



Confirmation of *COL4A6* variants in X-linked nonsyndromic hearing loss and its clinical implications

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Abstract

Hearing loss (HL) is one of the most common sensory defects, of which X-linked nonsyndromic hearing loss (NSHL) accounts for only 1–2%. While a *COL4A6* variant has been reported in a single Hungarian family with NSHL associated with inner ear malformation, causative role of *COL4A6* variants and their phenotypic consequences in NSHL remain elusive. Here we report two families in which we identified a male member with X-linked HL. Each has inherited a rare hemizygous *COL4A6* variant from their respective mothers, NM_001287758.1: c.3272 G > C (p.Gly1091Ala) and c.951 + 1 G > C. An in vitro minigene splicing assay revealed that c.951 + 1 G > T leads to skipping of exon 15, strongly suggesting a pathogenic role for this variant in the HL phenotype. The p.Gly1091Ala variant is classified as a variant of unknown significance based on the variant interpretation guidelines. This report provides evidence for variants in the *COL4A6* gene resulting in X-linked NSHL. It highlights the importance of in-depth genetic studies in all family members in addition to the proband, especially in multiplex families, to determine the precise etiology of HL.

Introduction

One out of every 1000 live births is affected with severe to profound congenital hearing loss (HL), and 50% of the sensorineural HL cases are hereditary in nature. Approximately 80% of nonsyndromic hearing loss (NSHL) cases are inherited in an autosomal recessive manner, while 20% are autosomal dominant and 1–2% are X-linked. Autosomal recessive mutations in *GJB2* are by far the most common genetic etiology and account for more than 30% of NSHL in children in Western Europe. From more than 120 deafness

genes/loci reported in literature to date, six loci (DFNX1-6) with five genes cloned (*PRPS1*, *POU3F4*, *SMPX*, *AIFM1*, and *COL4A6*), have been implicated in X-linked NSHL [1]. Many syndromic X-linked disorders are associated with HL, such as Norrie syndrome, X-linked deafness-dystonia-optic neuropathy, and Alport syndrome presenting with HL as a major clinical finding [1]. Interestingly, Alport syndrome, a heterogeneous condition characterized by non-immune glomerulopathy and progressive sensorineural HL can be inherited as an autosomal dominant, autosomal recessive, or X-linked condition. The X-linked forms accounts for ~70% of Alport syndrome cases and is associated with variants in the *COL4A5* gene [2–4]. A contiguous X-chromosome deletion involving *COL4A5* and *COL4A6* presents with X-linked Alport syndrome with HL and leiomyomatosis in the gastrointestinal, respiratory, and female reproductive tracts [5–7]. The autosomal forms of Alport syndrome are less common compared to the X-linked type and occur due to sequence variants in the *COL4A3* and *COL4A4* genes [8]. The *COL4A6* gene on Xq22.3 encodes a classical type IV collagen with a close homology to all the other five chains. Its protein structure most closely resembles that of *COL4A2* and *COL4A4* [9]. *COL4A6* is expressed in the *stria vascularis* and spiral ganglia cells in the mouse inner ear, and the alpha-6-chain

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of collagen type IV is a part of the basement membrane of the inner ear and is thought to have an important role in development of the cochlea. Being a member of the collagen family, COL4A6 forms a triple helical structure with two other alpha-5-chains, which is stabilized by the presence of glycine as every third residue. We hypothesize that the presence of a splice variant with skipping of an exon in Family A and a mutation of a glycine residue at the highly conserved residue, Gly1091Ala in Family B can result in destabilization of the collagen molecule with inner ear abnormalities and HL.

Here we present data on two families from North America segregating two separate variants in the *COL4A6* gene as a cause of their X-linked NSHL. Both probands had deaf parents and were identified from a larger subgroup of families within a North American Repository of DNA from individuals with HL [10]. DNA from all samples underwent initial *GJB2* and *GJB6* testing, mitochondrial testing, and subsequent testing for a deafness gene panel using NGS. The *COL4A6* variants identified by NGS were confirmed by Sanger sequencing in the proband and family members. Further in silico and splicing analyses reaffirm our hypothesis that the two identified variants in the *COL4A6* gene are a plausible basis for their HL.

Material

Patient ascertainment

Deaf probands from North America and their family members were ascertained for this study through several sources, including the Annual Survey of Deaf and Hard of Hearing Children and Youth, conducted at the Gallaudet Research Institute of Gallaudet University (GU), an institution of higher education for the deaf and hard of hearing. The Annual Survey collected educational, etiologic, and audiologic data, as well as demographic information such as race, parental mating type, and hearing status of siblings on a nationwide sample of nearly 50,000 deaf and hard of hearing students who receive special education services because of their HL [10]. Participants were also recruited through the GU Alumni Association [11]. All participants completed the informed consent process approved by the GU Institutional Review Board.

Audiological testing

Pure tone audiometry was performed according to current standards to determine air conduction thresholds at 0.125, 0.25, 0.5, 1, 2, 4, 6, and 8 kHz. Audiological data were available only in certain deaf members from Family B. The audiogram for individual B-II:3 revealed profound bilateral HL.

Clinical history

Proband A-III:1, ascertained through GU Alumni Association, was 24 years old and reported severe HL (Fig. 1A). There was no report of any skin findings, renal abnormalities, or ophthalmologic changes. Although we did not receive audiograms from the family, parents of A-III:1 are also reported to have HL. The father, individual A-II:1, has deaf parents and a deaf brother. The mother, A-II:2, reported HL from high fever during a varicella infection as a child.

Proband B-III:1 was ascertained from an audiology clinic in Mexico at 18 months of age. He had congenital deafness, no response on ABR testing, and did not cooperate for a behavioral hearing test. His father, paternal uncle, and mother (B-II:3, B-II:2, B-II:4) are reported to have severe to profound HL with a prelingual onset. The paternal grandmother, B-I:2, reported premature graying and was noted to have dystopia canthorum. No additional clinical or phenotypic details were available.

Cranial or temporal bone imaging studies are not available for Families A and B.

Methods

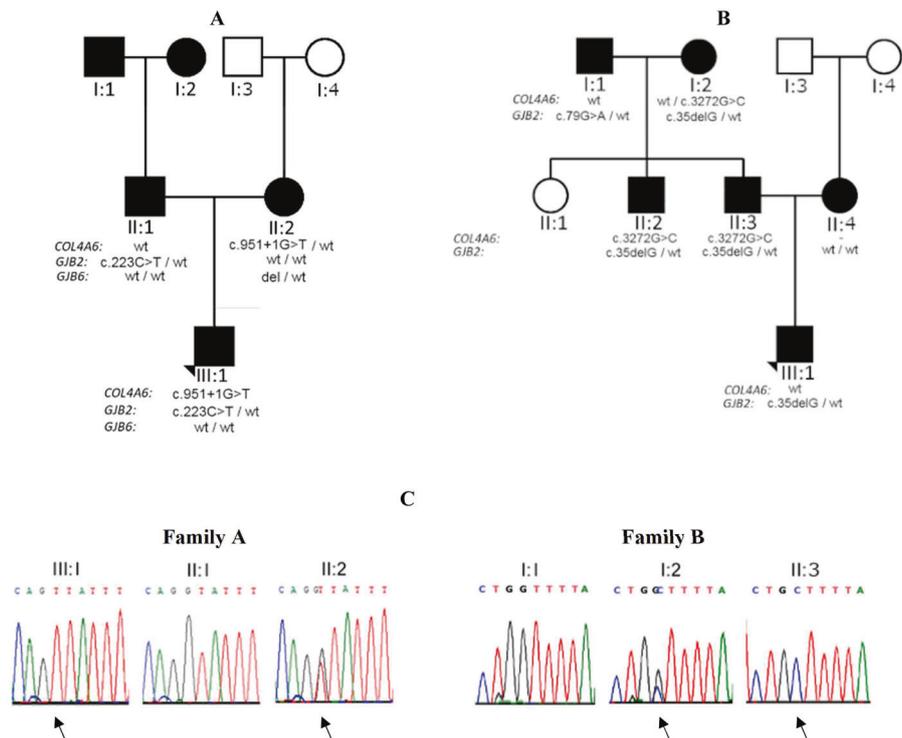
Molecular testing

DNA from Families A and B was extracted from peripheral blood samples using Pure Gene (Gentra Systems) protocols. DNA samples from these two families were screened for mutations in exons 1 and 2 of *GJB2* by Sanger sequencing as described in Pandya et al. [10]. The del (GJB6-D13S1830) deletion was tested using primers described by del Castillo et al. [12]. Mitochondrial testing was performed as described by Pandya et al. [13]. Additional NGS was performed on a panel of 187 HL genes as described in Yan et al. [14]. A microarray or whole-exome sequencing (WES) was not performed.

Minigene splicing assay and sequencing of in vitro transcript

In vitro minigene assay was performed as described by Booth et al. [15]. Briefly, wild-type exons 15 and 16 and ~360 base pairs of each flanking intron of *COL4A6* were polymerase chain reaction (PCR) amplified and ligated into the pET01 vector (MoBiTec, Goettingen, Germany). The c.951 + 1 G > T variant was introduced to the wild-type vector according to the manufacturer's protocol. Wild-type and mutant vectors were transfected into HEK293 cells. Total RNA was harvested 48 h post transfection and complementary DNA (cDNA) was transcribed according to the manufacture protocol. PCR using primers specific to the 5'

Fig. 1 Two families with *COL4A6* variants and sanger sequencing trace for *COL4A6*. **A, B** Families A and B with their multiplex pedigrees and segregation for variants in *GJB2*, *GJB6*, and *COL4A6*. **C** Electropherogram for Sanger sequencing of the hemizygous male (A-III:1 and B-II:3), heterozygous mother (A-II:2 and B-I:2), and unaffected father (A-II:1 and B-I:1).



and 3' native exons of the pET01 vector was performed and products were visualized on an agarose gel. Gel products were extracted and Sanger sequenced.

Pathogenicity determination tools

COL4A6 variants identified in the family members were interpreted according to the ACMG guidelines along with its recent modification for hereditary HL [16].

Predictions of pathogenicity for the variants of interest were made using Adjusted CADD scores [17], MutationT@ster2 [18], PolyPhen-2 [19], and SIFT [20].

Results

Variant discovery

Family A

Initial screening of *GJB2*, *GJB6*, and *MT-RNR1* in the proband A-III:1 identified the dominant variant in *GJB2* c.223 C>T (p.Arg75Trp) that was paternally inherited (Fig. 1A). A heterozygous *GJB6* [del (GJB6-D13S1854)] was noted in the mother A-II:2 but not in the proband. Additionally, the HL NGS gene panel identified a maternally inherited donor splice site variant c.951 + 1 G>T in *COL4A6* (Fig. 1C). The splice site variant in *COL4A6* c.951 + 1 G>T affects the canonical GT dinucleotide consensus sequence at the donor site of intron 14. The

splicing assay (Fig. 2) for the *COL4A6* c.951 + 1 G>T variant reveals skipping of exon 15 with introduction of a premature stop codon, further confirmed by Sanger sequencing the pET01_ *COL4A6* cDNA transcript (Fig. 2C). ACMG variant interpretation guidelines and our minigene study suggest the variant is likely pathogenic (Table 1) [21].

Family B

The cause for HL in the proband B-III:1 remained uncertain following the initial identification of a heterozygous *GJB2* c.35delG variant (Fig. 1B), which is involved in autosomal recessive NSHL. Subsequent testing with the HL gene panel in the trio also failed to identify an etiology in the proband. Subsequently, we identified a *COL4A6* variant c.3272 G>C (p.Gly1091Ala) in the father of the proband, B-II:3 that was also present in his mother B-I:2 and brother B-II:2 who both have HL (Fig. 1C). Pathogenicity tools predict that the variant is disease causing (Table 1). It generated a CADD score of 23 and disrupts the Gly-X-Y motif in *COL4A6*. ACMG variant interpretation guidelines suggest it to be a variant of unknown significance (Table 1) [21].

Discussion

Family A

The *GJB2* p.Arg75Trp variant in heterozygous state has been identified in at least 11 individuals with HL [22–27].

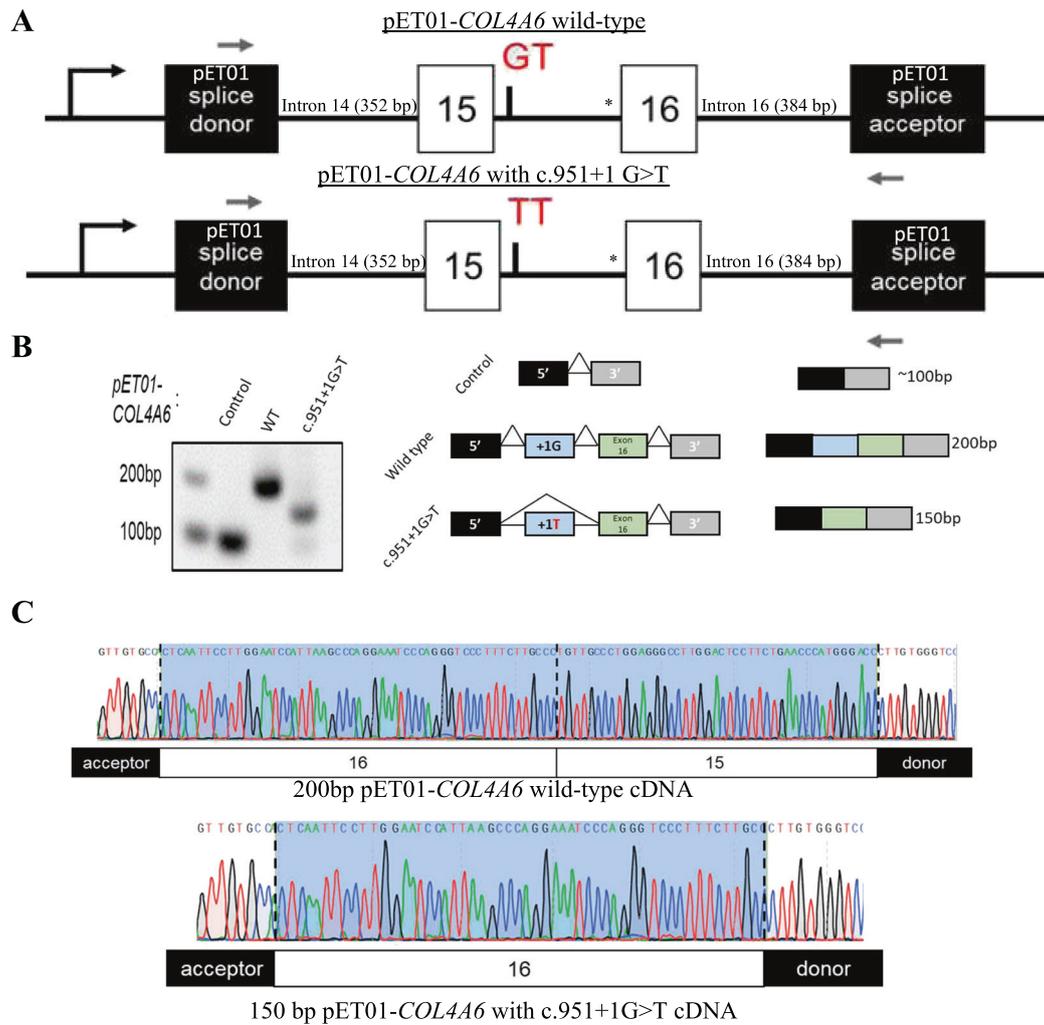


Fig. 2 Schematic of splicing assay for the COL4A6 c.951 + 1G > T variant identified in Family A. **A** Depiction of the altered splice acceptor site in intron 15 (TT) which is bypassed. **B** Verification of pET01-COL4A6 transfection. Wild-type and mutant cDNA harvested and transcribed from pET01-COL4A6 transfected HEK293 cells.

Agarose gel shows 200 bp wild-type product that includes exons 15 and 16, and a 150 bp product with exon 15 skipped in the mutant c.951 + 1G > T lane. **C** Sanger sequence confirmation of extracted product noted in agarose gel in (**B**).

Table 1 Pathogenicity tool scores and ACMG criteria applied to the COL4A6 variants identified in Families A and B.

COL4A6 variant	Tool	MutationT@ster	PolyPhen-2	SIFT
c.951 + 1 G > T	CADD	Score	Score	Score
c.3272 G > C	Interpretation	Score	Interpretation	Score
Family	ACMG criteria [21]	Interpretation	Evidence	dbSNP ID
Fam. A	c.951 + 1 G > T	Likely pathogenic	PM2, PS3	rs1235090929
Fam. B	c.3272 G > C	VUS	PM2, PP3	rs769241359

PM2 (rare in population database), PS3 (functional minigene evidence), PM2 (low population frequency), and PP3 (in silico evidence).

Most reported individuals had severe to profound HL that was either congenital or progressive in infancy, and some individuals also had palmoplantar keratoderma. This variant occurred de novo in three individuals and was identified in three affected parents, which supports that this variant is inherited in a dominant manner [22, 25–28]. Additionally the p.Arg75Trp variant has not been identified in large population studies. The functional assay demonstrated that the change to a Tryptophan (Trp) at position 75 has a dominant negative effect on the protein, disrupts the function of the gap junction [21, 28–30], and is important for the postnatal development of the organ of Corti and normal hearing [31, 32].

The novel splice site variant in *COL4A6* c.951 + 1 G > T is not reported in the ClinVar database and has a minor allele frequency of 0.00001 (1/177373, gnomAD). The inappropriately spliced mRNA with the loss of exon 15 results in a disrupting 15-amino-acid deletion and aberrant protein suggesting it as a clinically significant alteration.

In the proband, we hypothesize that both the *COL4A6* and *GJB2* pathogenic variants contribute to the HL and postulate the HL to be more severe than in either of his parents; however, we did not obtain audiometric data to confirm this hypothesis. The father's HL is attributed to the dominant *GJB2* variant, and although we do not have audiometric data for the mother, heterozygous carriers for the *COL4A6* gene have been reported to exhibit mild to moderate HL [33]. The contribution of the *GJB6* [del (GJB6-D13S1854)] deletion, an autosomal recessive variant, to HL in the mother is not likely to be significant. Trying to identify an etiology in the proband that also explains the HL in the parents in this family contributed to defining a more precise molecular etiology.

Family B

The *COL4A6* variant c.3272 G > C (p.Gly1091Ala) identified in the proband's father has an allele frequency of 0.001075 in the Latino population (gnomAD). Based on the segregation in the family with deaf members, pathogenicity tool prediction, and disruption of the Gly-X-Y motif in the *COL4A6* protein, we propose this is the cause of deafness in B-II:2 and his brother B-II:3 (variant call using SVI), although the etiology in the proband remains to be determined. It certainly helps clarify that the father will not pass this variant to his sons but that his daughters would be carriers.

Conclusions

This report adds highly relevant data to the previously reported genes implicated in nonsyndromic X-linked

sensorineural HL. To date, only one other family with a *COL4A6* sequence variant has been reported with HL in which the index proband and his mother were identified with a missense mutation in exon 23 of the *COL4A6* gene, c.1771 G > A p.Gly591Ser, which segregated in other affected male members in the family with HL [33]. The present report confirms the role of *COL4A6* variants in two additional families with X-linked NSHL.

Although the dominant *GJB2* variant explained HL in Family A, a family structure with two deaf parents persuaded us to look further. While WES data were not available in these cases and could have provided additional insight, the comprehensive NGS panel resulted in the identification of the novel *COL4A6* likely pathogenic variant in the proband. This finding has genetic counseling implications given the different inheritance pattern for each variant. Similarly, in Family B we are yet to identify an etiology for the HL in the proband, however, the dense family history of HL made us probe further in the older generations yielding the *COL4A6* variant in the proband's father as a likely explanation of his and his brothers HL. This information allows more precise genetic counseling for Family A due to the proband's offspring having a 50% risk of developing HL due to the *GJB2* variant, and all his daughters (and none of his sons) inheriting the *COL4A6* variant with variable expression that can be passed on to his grandsons. Thus, caution needs to be exercised when evaluating deaf individuals when both parents have HL. When determining the etiology of HL, clinicians should strive to utilize current technology to having a precise molecular etiology as it enables more accurate counseling for recurrence risk assessment and prognosis.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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References

1. Corvino V, Apisa P, Malesci R, Laria C, Auletta G, Franze A. X-linked sensorineural hearing loss: a literature review. *Curr Genomics*. 2018;19:327–38.
2. Knebelmann B, Breillat C, Forestier L, Arrondel C, Jacassier D, Giatras I, et al. Spectrum of mutations in the *COL4A5* collagen gene in X-linked Alport syndrome. *Am J Hum Genet*. 1996;59:1221.
3. Plant KE, Green PM, Vetrie D, Flinter FA. Detection of mutations in *COL4A5* in patients with Alport syndrome. *Hum Mutat*. 1999;13:124–32.

4. Wang F, Ding J, Yu Lx, Yang Jy. Detection of COL4A5 gene mutations in Alport syndrome by analyzing cDNA of skin fibroblasts. *J Beijing Med Univ.* 2002;3:219–224.
5. Mothes H, Heidet L, Arrondel C, Richter KK, Thiele M, Patzer L, et al. Alport syndrome associated with diffuse leiomyomatosis: COL4A5-COL4A6 deletion associated with a mild form of Alport nephropathy. *Nephrol Dial Transplant.* 2002;17:70–4.
6. Uliana V, Marcocci E, Mucciolo M, Meloni I, Izzi C, Manno C, et al. Alport syndrome and leiomyomatosis: the first deletion extending beyond COL4A6 intron 2. *Pediatr Nephrol.* 2011;26:717–24.
7. Sá MJN, Fieremans N, de Brouwer AP, Sousa R, e Costa FT, Brito MJ, et al. Deletion of the 5' exons of COL4A6 is not needed for the development of diffuse leiomyomatosis in patients with Alport syndrome. *J Med Genet.* 2013;50:745–53.
8. Kashan CE. Alport Syndrome. In: Adam MP, Ardinger HH, Pagon RA, et al., editors. *GeneReviews*® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2021.
9. Zhou J, Ding M, Zhao Z, Reeders ST. Complete primary structure of the sixth chain of human basement membrane collagen, alpha 6 (IV). Isolation of the cDNAs for alpha 6(IV) and comparison with five other type IV collagen chains. *J Biol Chem.* 1994;269:13193–9.
10. Pandya A, Arnos KS, Xia XJ, Welch KO, Blanton SH, Friedman TB, et al. Frequency and distribution of GJB2 (connexin 26) and GJB6 (connexin 30) mutations in a large North American repository of deaf probands. *Genet Med.* 2003;5:295–303.
11. Arnos KS, Welch KO, Tekin M, Norris VW, Blanton SH, Pandya A, et al. A comparative analysis of the genetic epidemiology of deafness in the United States in two sets of pedigrees collected more than a century apart. *Am J Hum Genet.* 2008;83:200–7.
12. del Castillo FJ, del Castillo I, Moreno-Pelayo MA, Brownstein Z, Marlin S, Adina Q, et al. Prevalence and evolutionary origins of the del(GJB6-D13S1830) mutation in the DFNB1 locus in hearing-impaired subjects: a multicenter study. *Am J Hum Genet.* 2003;73:1452–8.
13. Pandya A, Xia X, Erdenetungalag R, Amendola M, Landa B, Radnaabazar J, et al. Heterogenous point mutations in the mitochondrial tRNA Ser (UCN) precursor coexisting with the A1555G mutation in deaf students from Mongolia. *Am J Hum Genet.* 1999;65:1803–6.
14. Yan D, Tekin D, Bademci G, Foster J, Cengiz FB, Kannan-Sundhari A, et al. Spectrum of DNA variants for non-syndromic deafness in a large cohort from multiple continents. *Hum Genet.* 2016;135:953–61.
15. Booth KT, Azaiez H, Kahrizi K, Wang D, Zhang Y, Frees K, et al. Exonic mutations and exon skipping: lessons learned from DFNA5. *Hum Mutat.* 2018;39:433–40.
16. Oza AM, DiStefano MT, Hemphill SE, Cushman BJ, Grant AR, Siegert RK, et al. Expert specification of the ACMG/AMP variant interpretation guidelines for genetic hearing loss. *Hum Mutat.* 2018;39:1593–613.
17. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet.* 2014;46:310.
18. Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. *Nat Methods.* 2014;11:361.
19. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. *Nat Methods.* 2010;7:248.
20. Ng PC, Henikoff S. SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Res.* 2003;31:3812–4.
21. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med.* 2015;17:405–23.
22. Richard G, White TW, Smith LE, Bailey RA, Compton JG, Paul DL, et al. Functional defects of Cx26 resulting from a heterozygous missense mutation in a family with dominant deaf-mutism and palmoplantar keratoderma. *Hum Genet.* 1998;103:393–9.
23. Putcha GV, Bejjani BA, Bleoo S, Booker JK, Carey JC, Carson N, et al. A multicenter study of the frequency and distribution of GJB2 and GJB6 mutations in a large North American cohort. *Genet Med.* 2007;9:413.
24. Mani RS, Ganapathy A, Jalvi R, Srisailapathy CS, Malhotra V, Chadha S, et al. Functional consequences of novel connexin 26 mutations associated with hereditary hearing loss. *Eur J Hum Genet.* 2009;17:502.
25. Yuan Y, Huang D, Yu F, Zhu X, Kang D, Yuan H, et al. A de novo GJB2 (connexin 26) mutation, R75W, in a Chinese pedigree with hearing loss and palmoplantar keratoderma. *Am J Med Genet Part A.* 2009;149:689–92.
26. Lee JY, In S, Kim HJ, Jeong S, Choung YH, Kim YC. Hereditary palmoplantar keratoderma and deafness resulting from genetic mutation of Connexin 26. *J Korean Med Sci.* 2010;25:1539–42.
27. Weegerink NJ, Pennings RJ, Huygen PL, Hoefsloot LH, Cremers W, Kunst HP. Phenotypes of two Dutch DFNA3 families with mutations in GJB2. *Ann Otol Rhinol Laryngol.* 2011;120:191–7.
28. Pang X, Chai Y, Sun L, Chen D, Chen Y, Zhang Z, et al. Characterization of spectrum, de novo rate and genotype-phenotype correlation of dominant GJB2 mutations in Chinese hans. *PLoS ONE.* 2014;9:e100483.
29. Deng Y, Chen Y, Reuss L, Altenberg GA. Mutations of connexin 26 at position 75 and dominant deafness: essential role of arginine for the generation of functional gap-junctional channels. *Hear Res.* 2006;220:87–94.
30. Marziano NK, Casalotti SO, Portelli AE, Becker DL, Forge A. Mutations in the gene for connexin 26 (GJB2) that cause hearing loss have a dominant negative effect on connexin 30. *Hum Mol Genet.* 2003;12:805–12.
31. Inoshita A, Iizuka T, Okamura H, Minekawa A, Kojima K, Furukawa M, et al. Postnatal development of the organ of Corti in dominant-negative Gjb2 transgenic mice. *Neuroscience* 2008;156:1039–47.
32. Inoshita A, Karasawa K, Funakubo M, Miwa A, Ikeda K, Kamiya K. Dominant negative connexin26 mutation R75W causing severe hearing loss influences normal programmed cell death in postnatal organ of Corti. *BMC Genet.* 2014;15:1.
33. Rost S, Bach E, Neuner C, Nanda I, Dysek S, Bittner RE, et al. Novel form of X-linked nonsyndromic hearing loss with cochlear malformation caused by a mutation in the type IV collagen gene COL4A6. *Eur J Hum Genet.* 2014;22:208.