A novel LOXHD1 variant in a Chinese couple with hearing loss

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Abstract
Objective: To perform molecular diagnosis and genetic counseling in a young Chinese couple with congenital hearing loss.
Methods: Variant screening analysis was performed by PCR and direct Sanger sequencing or targeted next-generation sequencing of all known hearing loss genes. Novel variants were evaluated by PolyPhen2 and PROVEAN software tools to evaluate possible effects on protein function.
Results: We identified causative variants in the young couple: c.235delC (rs80338943)/c.299-300delAT (rs111033204) compound heterozygous variants of GJB2 in the husband and c.1828G>A (p.Glu610Lys, rs535637788)/c.2825-2827delAGA compound heterozygous variants of LOXHD1 in the wife. The LOXHD1 c.1828G>A variant has only previously been reported in a Mexican-American individual in the 1000 Genomes Project database. Using PolyPhen2 and PROVEAN, we speculated that the LOXHD1 variant c.1828G>A is potentially pathogenic.
Conclusion: We carried out molecular diagnosis in a young couple with congenital hearing loss, and identified different disease-causing genes in the two individuals. The LOXHD1 variant c.1828G>A present in the wife had not previously been reported in individuals with congenital hearing loss. We determined this to be a potential pathogenic variant, and a novel variant associated with hearing loss in a Chinese individual.

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Introduction

Hearing loss (HL) is the most frequent sensory deficit in humans, with a prevalence of around 1/1000 in newborns.\(^1,2\) Approximately 50% to 60% of hearing loss cases are caused by genetic factors.\(^3\) The genetic mode of HL inheritance can be autosomal recessive, autosomal dominant, mitochondrial, or X/Y-linked. To date, 121 genes have been reported to be associated with hearing loss (http://hereditaryhearingloss.org/): 45 are autosomal dominant genes,\(^7\) 71 are autosomal recessive, and 5 are X-linked. However, most of these genes have only been reported in one or a few families.\(^4\) Epidemiological studies showed that variants in \(GJB2\), \(SLC26A4\), and \(12S\) rRNA genes are highly correlated with hereditary HL.\(^2\) The most frequent genetic cause of HL is variants in \(GJB2\), and most of these cases occur with non-progressive HL. Variants in \(SLC26A4\), \(CDH23\), and \(MYO3A\) were also shown to be associated with naturally occurring progressive HL.\(^5,6\)

The genetic diagnosis of HL is very important because the findings can be used to aid treatment decisions, and provide prognostic information and genetic counseling for the patient’s family.\(^7\) Here, we describe a young couple with HL in whom the husband carried compound heterozygous variants of \(GJB2\), and the wife had an extremely rare form of deafness and compound heterozygous variants of \(LOXHD1\). We provided genetic counseling for this couple and followed them up during their pregnancy.

Patients and methods

Study population

We recruited a young Chinese couple (husband: 27 years old; wife: 25 years old) with congenital HL and 100 healthy controls (aged 25–30 years) from Gansu Provincial Maternal and Child Health Care Hospital. The couple had been married for 6 months and requested pre-pregnancy genetic counseling. The study was in accordance with the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of Gansu Provincial Maternal and Child Health Care Hospital. Written informed consent was obtained from all participants.

Sample collection and genomic DNA preparation

Blood samples (2–3 mL) were collected from the probands and their parents and control individuals. Genomic DNA was extracted using a Tiangen DNA extraction kit (Tiangen Biotech, Beijing, China) according to the manufacturer’s instructions and quantified spectrophotometrically.

Targeted next-generation sequencing (NGS) and Sanger sequencing

First, the coding exon and flanking sequences of \(GJB2\) were screened by PCR and direct sequencing using primers and conditions described in Table 1. If no \(GJB2\) variant was found, targeted capture of candidate disease genes (\(n = 165\), Table 2) was performed using a GenCap custom
### Table 1. Primers and PCR conditions for GJB2 and LOXHD1.

| Primer name  | Sequence (5’–3’) | Product size (bp) | Amplification reaction conditions |
|--------------|------------------|-------------------|----------------------------------|
| GJB2-F       | CATGCTTGCTTACCCAGACTCA | 873               | 95°C for 5 minutes, then 20 cycles of 94°C for 30 seconds, 62°C for 45 seconds, and 72°C for 1 minute. |
| GJB2-R       | TAGCGACTGAGCCCTTGCAGGC |                 |                                  |
| GJB2-S1      | TGGGTTTTGTATCTTCTGATG  |                  |                                  |
| GJB2-S2      | GCCTACCGGAGACATGAGAG  |                  |                                  |
| LOXHD1-E14F  | GTTAGTAGGGCTGGTCTCCC  | 355               | 95°C for 5 minutes, then 15 cycles of 94°C for 30 seconds, 58°C for 45 seconds, and 72°C for 1 minute. |
| LOXHD1-E14R  | AGTTGCTTAACCACGACGCTC |                  |                                  |
| LOXHD1-E19F  | CAACAAAATCCACGAGATTC | 594               |                                  |
| LOXHD1-E19R  | GAGGTTGTTGGAAGGATCTGAG |                |                                  |

GJB2-S1 and GJB2-S2 are the sequencing primers for GJB2.

### Table 2. Genes in the hearing loss panel.

| Nuclear genes associated with hereditary HL |
|--------------------------------------------|
| ACTG1 ADGRV1 ALX3 BSND CABP2 CCDC50 CDH23  |
| CEACAM16 CHD7 CIB2 CLDN14 CLPP CLRNI COCH  |
| COL1A1 COL1A2 COL1A1 COL2A1 COL4A3 COL4A4  |
| COL4A5 COL4A6 COL9A1 COL9A2 CRYM DFNBS9 DIABLO |
| DIAPH1 DIAPH3 DSPP ECM1 EDN3 EDNRB ELMOD3 |
| ESPN ESRRB EYA1 EYA4 FGF3 FGF8 FGFR1       |
| FGFR3 FLNA FOX11 FREM1 FXN GATA3 GIPC3     |
| GJB1 GJB2 GJB3 GJB6 GLYAT GPMC2 GRHL2      |
| GRXCR1 GSDME HARS HARS2 HGF HMX1 HOXA2     |
| HSD1B7B IL13 ILDR1 KARS KCNE1 KCN10 KCNQ1 |
| KCNQ4 KITLG KRT9 LAMA3 LARS2 LHFPL5 LOXHD1 |
| LRTOMT MARVELD2 MIR96 MITF MPZ MSR3 MYH14  |
| MYH9 MYO15A MYO1A MYO1E MYO3A MYO6 MYO7A    |
| NDP NDRG1 NEFL NELL2 NF2 OPA1 OTOA         |
| OTOF OTOG OTOG P2RX2 PABPN1 PAX3 PCDH15    |
| PCDH9 PDZD7 PMP22 PNPT1 POLR1C POLR1D POU3F4 |
| POU4F3 PROK2 PROKR2 PRPS1 PTPN11 PTTRP PTPRR |
| RDX RPGR SALL1 SALL4 SEC23A SEMA3E SERPINB6 |
| SIX1 SIX5 SLC17A8 SLC19A2 SLC2A4 SLC2A5 SMAD4 |
| SMPX SNAI2 SOX10 STRC TBC1D24 TCIRG1 TCOF1 |
| TECTA TIMM8A TJP2 TMC1 TMEM126A TMIE TMRPSS3 |
| TMPRSS4 TNC TPRN TRIOB8 TRMU TSPEAR TYR    |
| USH1C USH1G USH2A WFS1 WHRN               |

| Mitochondrial gene |
|--------------------|
| MT-RNR1 RNR-TL1 MT-COI RNR-TS1 MT-TK RNR-TE |
enrichment kit (MyGenostics, Beijing, China). Briefly, 1 μg of DNA library was mixed with BL buffer and a GenCap hypercholesterolemia probe (MyGenostics) and heated in a PCR cycler at 95°C for 7 minutes then 65°C for 2 minutes. A total of 23 μL HY buffer (pre-warmed to 65°C; MyGenostics) was added and the mixture was incubated at 65°C for 22 hours for hybridization. MyOne beads (50 μL; Thermo Fisher Scientific Inc., Rockford, IL, USA) were washed three times in 500 μL binding buffer (1×) and resuspended in 80 μL binding buffer (1×). Next, 64 μL binding buffer (2×) was added and the mixture was transferred into a tube containing 80 μL MyOne beads, and spun for 1 hour on a rotator. The beads were then washed once with WB1 buffer at room temperature for 15 minutes and three times with WB3 buffer at 65°C for 15 minutes. Elution buffer was used to elute the bound DNA, which was amplified as follows: 98°C for 30 seconds then 15 cycles of 98°C for 25 seconds, 65°C for 30 seconds, and 72°C for 30 seconds, followed by 72°C for 5 minutes. PCR products were purified using SPRI beads (Beckman Coulter Inc., Brea, CA, USA) following the manufacturer’s protocol. Enrichment libraries were sequenced on an Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA, USA) for 100-bp paired reads.

After sequencing, high-quality reads were retrieved by filtering out adaptors, low-quality reads, and short sequences (<40 bp). Data quality control standards were: 10× > 95%, depth = 200 ± 30. The SOAPaligner program (SOAP v2.21) was used to align clean read sequences to the human reference genome (UCSC Genome Browser hg19). After removing duplicates with Picard software (v1.119), single nucleotide polymorphisms (SNPs) were identified using SOAPsnp v1.03. Subsequently, reads were realigned to the reference genome using the Burrows–Wheeler alignment program (0.7.12-r1044), and insertions or deletions (InDels) were detected by the HaplotypeCaller of GATK software (https://software.broadinstitute.org/gatk/, GATK-3.5) and filtered by VariantFiltration of GATK software. We annotated the identified SNPs and InDels using the ExomeAssistant program. Short read alignment and candidate SNP and InDel validation were performed using MagicViewer.

We performed Sanger sequencing for all identified variants in the probands and their parents. PCR primers for Sanger sequencing were designed by Primer 3.0 software (http://bioinfo.ut.ee/primer3-0.4.0/). Primers and PCR conditions for GJB2 and LOXHD1 are shown in Table 1. DNA sequencing was performed on an ABI 3500DX Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Bioinformatics analysis
If a novel variant was found that was not reported in the Human Gene Variant Database (http://www.hgmd.cf.ac.uk/) or ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/), we used PolyPhen2 (http://genetics.bwh.harvard.edu/pph2) and PROVEAN (http://provean.jcvi.org/index.php) tools to predict its possible functional role. To exclude the possibility that the variant was a polymorphism, we also performed direct sequencing in 100 healthy controls.

Results
Variant analysis
PCR and direct Sanger sequencing identified c.235delC (rs80338943)/c.299-300delAT (rs111033204) compound heterozygous variants of GJB2 in the husband. c.235delC was inherited from his mother.
and c.299-300delAT was inherited from his father (Figure 1).

No GJB2 variants were identified in the wife, so targeted NGS was used to search for potential pathogenic variants. She was shown to carry c.1828G>A (p.Glu610Lys, rs535637788)/c.2825-2827delAGA compound heterozygous variants of LOXHD1, with c.2825-2827delAGA inherited from her mother and c.1828G>A from her father (Figure 2). Her hearing loss is an extremely rare form known as DFNB77 (OMIM: 613079). Variant c.2825-2827delAGA has previously been reported to be associated with DFNB77,7 but variant c.1828G>A (p.E610K) was only reported in a Mexican-American individual in the 1000 Genomes database. It has not been reported to be associated with DFNB77. PCR and direct Sanger sequencing did not identify this variant in any of our 100 healthy controls.

**Bioinformatics analysis score**

PolyPhen2 and PROVEAN tools were used to evaluate the possible functional role of variant c.1828G>A. PolyPhen2 gave a score of 1, suggesting that the site might be a damaging variation. The PROVEAN score was −3.203, and the site was considered “deleterious”.

**Pregnancy outcome**

Following molecular analysis, we provided genetic counseling to the young couple with HL. We explained that their children were unlikely to have HL because they both carried different genetic variants. During their pregnancy, they underwent regular prenatal

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**Figure 1.** Results of Sanger sequencing. Compound heterozygous variants were detected in the proband. c.235delC (rs80338943, left)/c.299-300delAT (rs111033204, right) of GJB2. The father carried the heterozygous variant c.299-300delAT, while the mother and fetus carried the heterozygous variant c.235delC.
checkups which detected the presence of the c.235delC \textit{GJB2} variant and the c.1828G>A \textit{LOXHD1} variant in the fetus (Figures 1 and 2). The baby was born in June 2018, and both ears passed the hearing screening test.

**Discussion**

We identified causative variants of HL in both individuals of a young Chinese couple. The variants of the husband were common and the c.2825-2827delAGA \textit{LOXHD1} variant of the wife was previously associated with DFNB77; however, the c.1828G>A \textit{LOXHD1} variant of the wife has only been reported in a Mexican-American individual in the 1000 Genomes database, and not in the HGMD database or elsewhere. PolyPhen2 and PROVEAN tools suggested that it is a likely pathogenic variant.

\textit{LOXHD1} is located on chromosome 18q12-q21 and contains at least 43 exons.\textsuperscript{8} It encodes lipoxygenase homology domain 1-containing protein 1 which has 15 PLAT domains\textsuperscript{4} that are involved in targeting proteins to the plasma membrane and mediating protein interactions.\textsuperscript{9–12} Mouse studies showed that the \textit{Loxhd1} product is localized to the stereocilia of sensory hair cells, and that \textit{Loxhd1} variants can induce deafness with defects in the stereocilia followed by hair cell degeneration.\textsuperscript{13} This indicates that \textit{LOXHD1} plays an important role in maintaining normal hair cell function.

Although DFNB77 has previously been associated with \textit{LOXHD1} variants, it is a highly heterogeneous disease both phenotypically and genetically. More than
23 probands with DFNB77 have been reported worldwide on PubMed, and 37 different disease-causing variants have been identified (Table 3).\(^5,7–21\) Most of these probands come from Asia, suggesting that it has a high incidence of DFNB77. They show different auditory characteristics and audiometric phenotypes, varying from mild to profound and from stable to progressive sensorineural HL.\(^5,7–21\) Animal
studies revealed that homozygous missense variants of Loxhd1 induced profound deafness while homozygous nonsense variants caused progressive HL. However, Wesdorp et al. found that the type of variant (nonsense or missense) did not associate with HL severity, and that the combination of a nonsense and missense variant could cause different audiometric phenotypes. Such research is limited, so correlations between LOXHD1 variants and phenotypic characteristics of HL remain unclear.

LOXHD1 variants have not only been linked to HL but are also associated with late-onset Fuchs corneal dystrophy (FCD), a genetic disorder of the corneal endothelium. A case–control study by Stehouwer et al. reported a significant association between FCD and hearing disorders, but this should be investigated in larger sample sizes. We believe that it is important to check for ophthalmology disorders in patients with HL caused by LOXHD1 variants; however, we found no FCD phenotype in the current proband with LOXHD1 variants.

In conclusion, we carried out molecular diagnosis in a young couple with congenital HL and identified different disease-causing variants in the two individuals. The husband had compound heterozygous variants of GJB2, while the wife had the extremely rare HL known as DFNB77 and compound heterozygous variants of LOXHD1. We followed up the pregnancy outcome of this couple, and report that both ears of their baby passed the hearing screening test. To the best of our knowledge, this is the third case reported in Chinese individuals and the first in the northwest of the country. PCR and direct Sanger sequencing cannot provide effective detection of diseases caused by such rare variants. However, with the development of molecular diagnostic technology, the cost of tests is decreasing and NGS will become a more effective way of providing accurate molecular diagnosis and genetic counseling for rare diseases.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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