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A dominant variant in *DMXL2* is linked to nonsyndromic hearing loss

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Purpose: To explore the genetic etiology of deafness in a dominant family with late-onset, progressive, nonsyndromic hearing loss.

Methods: Genome-wide linkage analysis was performed for 21 family members. Candidate pathogenic variants were identified by whole-exome sequencing of selected family members and confirmed by Sanger sequencing of all family members. Cochlear expression of *Dmxl2* was investigated by reverse-transcription polymerase chain reaction (RT-PCR) and immunostaining of the organ of Corti from mice.

Results: The causative gene was mapped to a 9.68-Mb candidate region on chromosome 15q21.2 (maximum logarithm of the odds score = 4.03) that contained no previously described deafness genes. Whole-exome sequencing identified heterozygous c.7250G>A (p.Arg2417His) in *DMXL2* as the only candidate pathogenic variant segregating the hearing loss. In mouse cochlea, expression of *DMXL2* was restricted to the hair cells and the spiral ganglion neurons.

Conclusion: Our data indicated that the p.Arg2417His variant in *DMXL2* is associated with dominant, nonsyndromic hearing loss and suggested an important role of *DMXL2* in inner ear function.

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Key Words: autosomal dominant; *DMXL2*; inner ear; nonsyndromic hearing loss; synaptic vesicle

INTRODUCTION

The genetic causes of hearing loss are attributable to pathogenic variants in more than 100 genes with diverse functions in the auditory system. In addition, the cause remains unresolved in more than 50 loci genetically mapped for nonsyndromic deafness (Hereditary Hearing Loss, http://hereditaryhearingloss.org). For many deafness genes, including *WFS1* (MIM 606201), *KARS* (MIM 601421), and *TBC1D24* (MIM 613577), various types of pathogenic variants may lead to distinct phenotypes ranging from nonsyndromic deafness to syndromic deafness or non-deafness-related syndromes.

DMXL2 encodes rabconnectin-3α, the α subunit of the rabconnectin protein complex that concentrates on synaptic vesicles at the synapse and plays an essential role in neurosecretion.[1–3] In the hypothalamus, it is expressed in exocytosis vesicles of tanycytes and hypothalamic neurosecretory neurons. Conditional deletion of a single allele of *Dmxl2* in mouse neurons led to delayed puberty and very low fertility.[4] In affected members of a consanguineous family, a homozygous 15-nucleotide deletion in *DMXL2* resulted in progressive endocrine and neurodevelopmental disorders, including incomplete puberty, central hypothyroidism, peripheral polyneuropathy, mental retardation, and abnormal glucose regulation.[4] In the inner ears of zebrafish, rabconnectin-3α is expressed in basal regions of hair cells where synaptic vesicles are enriched. Recessive nonsense pathogenic variants in the zebrafish rabconnectin-3α gene *rbc3a* resulted in abnormal auditory and vestibular dysfunctions due to defective acidification of synaptic vesicles.[5]

So far, no report has linked *DMXL2* to syndromic or nonsyndromic deafness in humans. In this study, we identified a heterozygous missense variant in *DMXL2* that was associated with dominant, nonsyndromic hearing loss in a large Chinese Han family.

MATERIALS AND METHODS

Subjects and clinical evaluation

We recruited a large Chinese Han family (family KH) from Zhejiang Province, China, with autosomal-dominant nonsyndromic hearing loss. This family spanned seven generations and had at least 16 members affected by hearing loss before the age of 20 years. Eleven affected and 11 unaffected members participated in the present study (**Figure 1a**). Informed consent

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was obtained from all participating subjects. This study was approved by the ethics committee of Xinhua Hospital, Shanghai Jiaotong University School of Medicine.

The hearing levels of all participating members were measured by pure tone audiometry. For affected members, a complete medical history and physical examination were performed to exclude the possibility of environmental causes or syndromic hearing loss. Additional auditory evaluations included otoscopic examination, otoacoustic emission, and temporal bone high-resolution computed-tomography scanning.

Whole-genome linkage analysis

Multipoint genome-wide linkage analysis was performed for 21 family members (marked with asterisks in **Figure 1a**) using the HumanOmniZhongHua-8 BeadChip (Illumina, San Diego, CA) containing 900,015 SNP markers. Genotypes of 6,301 Tag SNPs (distributed in every 0.5 cM of genomic region) were chosen for calculation of the logarithm of odds (LOD) scores using the Merlin v. 1.1.23 parametric linkage analysis package. The inheritance model was assumed to be dominant with full penetrance. The disease allele frequency was assumed to be 0.0001.

Whole-exome sequencing and verification of the pathogenic variants

Whole-exome sequencing was performed in three affected (II-1, IV-1, and IV-4) members and one unaffected (IV-6) members (marked with triangles in **Figure 1a**) as previously described.[6] Exons and flanking intronic regions of 20,794 genes (33.2 Mb, 97.2% of consensus coding sequence coding exons), microRNAs, and other noncoding RNAs were captured by the Illumina TruSeq Exome Enrichment Kit and sequenced on a HiSeq 2000 instrument (Illumina). Image analysis, error estimation, and base calling were performed using the Illumina Pipeline (version 1.3.4). Reads were aligned to NCBI37/hg19 assembly using the BWA Multi-Vision software package. SNPs and indels were identified using SOAPsnp software and the GATK Indel Genotyper, respectively. Candidate pathogenic variants were defined as nonsense, missense, splice-site, and indel variants with allele frequencies of 0.001 or less in public variant databases dbSNP and 1000 Genomes and in previous sequencing data of 1,000 Chinese Han adult controls with normal hearing (in-house whole-exome sequencing data using the same platform).

Candidate pathogenic variants were further genotyped for all 22 family members by Sanger sequencing. Possible pathogenic effects of the identified variant were evaluated by computational tools, including Mutation Taster (http://www.mutationtaster.org), PROVEAN, and SIFT (with cutoff scores set at −1.3 and 0.05, respectively; http://sift.jcvi.org).

Reverse-transcriptase PCR and immunostaining of *Dmxl2* in mouse cochlea

Reverse-transcriptase PCR (RT-PCR) was performed for total RNA extracted from P1 and P60 mouse cochlea using

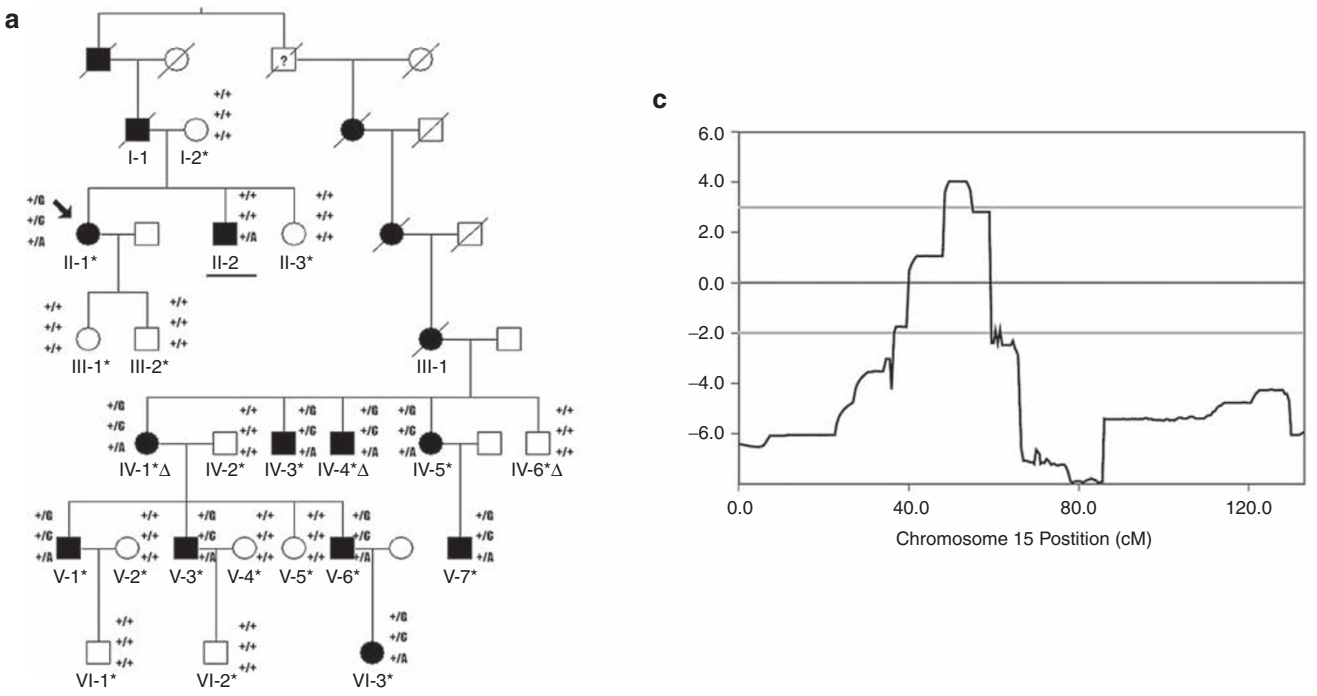

Figure 1 Pedigree, audiograms, and linkage analysis results of family KH. (**a**) Pedigree of family KH. Individuals selected for linkage analysis and whole-exome sequencing are marked with asterisks and triangles, respectively. Proband II-1 is indicated by an arrow. Genotypes of the three candidate variant (G: c.2155A>G in *AP4E1*, NM_001252127; C: c.940T>C in *CYP19A1*, NM_000103; A: c.7250G>A in *DMXL2*, NM_001174116) are shown for all family members. "+" indicates the reference allele. Individual II-2, who harbored a key recombination event, is underlined. (**b**) Audiograms of the affected individuals in family KH. (**c**) Logarithm of the odds (LOD) scores of genome-wide linkage analysis for chromosome 15. The maximum LOD score was recalculated to 4.33 after inclusion of the genotypes of the three candidate variants in II-2.

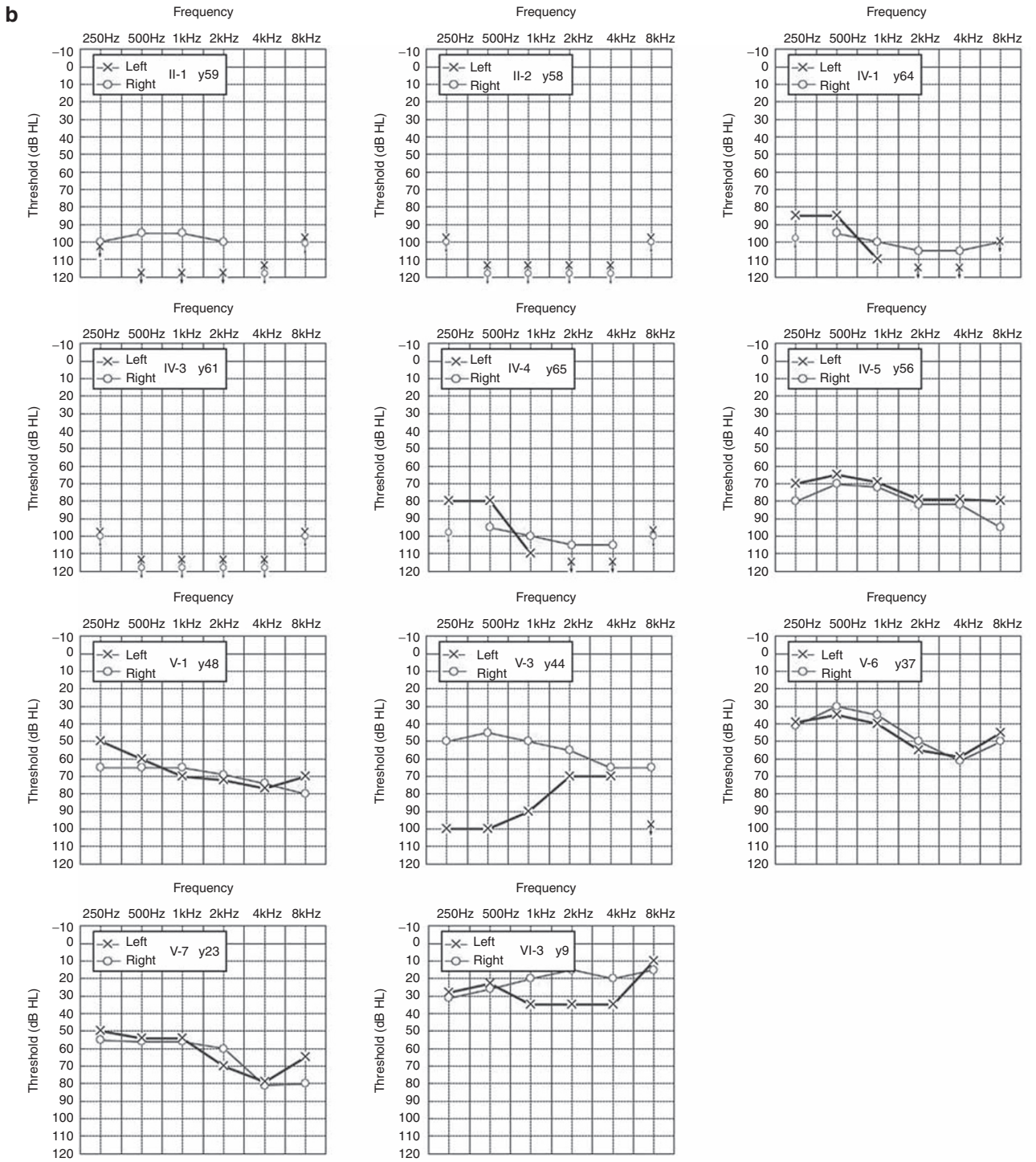

Figure 1 (Continued)

the SuperScript One-Step RT-PCR System (ThermoFisher Scientific, Waltham, MA). Forward and reverse primer pairs were designed in exons 41 and 43 and exons 42 and 44 of *Dxml2*, resulting in a 151-bp and a 279-bp PCR product, respectively. Immunofluorescence staining of DMXL2 was performed in cross-sections of the organ of Corti from mice as previously described.[7] Briefly, mouse cochleae were first decalcified in 0.12 M EDTA for 1 to 2 days. The cochlea was embedded in 30% sucrose for dehydration and then in OCT overnight, followed by cryosectioning into 8-µm-thick slices. Tissues and slides were incubated in primary antibody diluted in PBS containing 0.5% triton, 5% donkey serum, and 1% BSA at room temperature overnight and detected with species-specific Alexafluor-conjugated secondary antibodies (ThermoFisher) diluted to

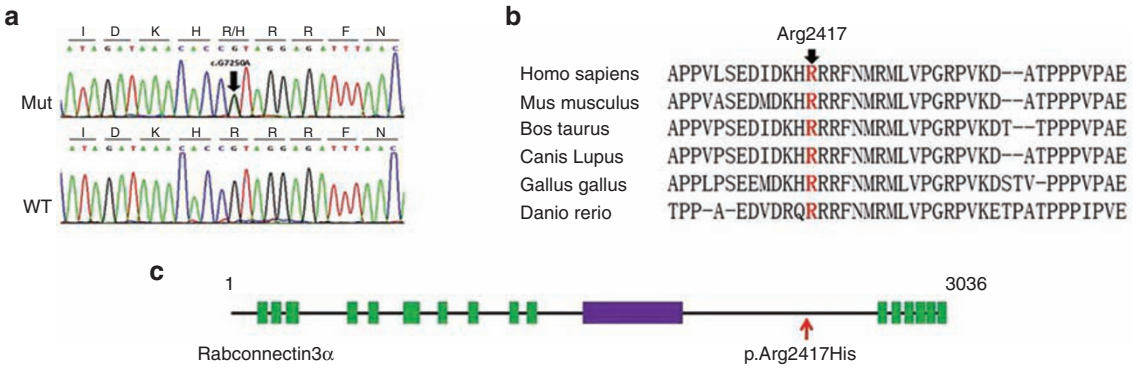

Figure 2 The p.Arg2417His variant in *DMXL2*. (**a**) Chromatograms of the variant and reference sequences. (**b**) Conservation of the Arg2417 residue of DMXL2 in *Homo sapiens*, *Mus musculus*, *Bos taurus*, *Canis lupus*, *Gallus gallus*, and *Danio rerio*. (**c**) Domain structure of the DMXL2 protein with the location of the p.Arg2417His variant marked by an arrow. Green boxes: WD domains; purple box: the Rav1p_C domain.

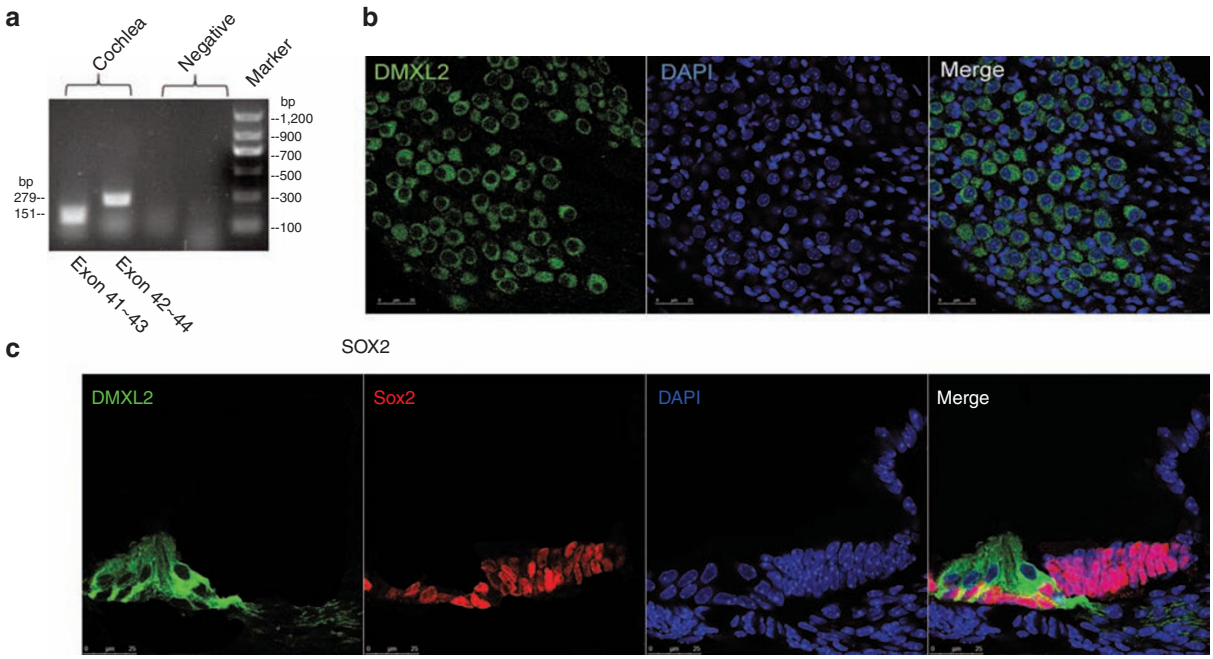

Figure 3 Expression of *Dmxl2* in mouse cochlea. (**a**) RT-PCR showing expression of *Dmxl2* (the 151-bp and the 279-bp PCR products) in total RNA extracted from P1 mouse cochlea. Mouse tail genomic DNA were used for negative controls. (**b**) Immunostaining of the P6 organ of Corti from mice showing the expression of DMXL2 (green) in the spiral ganglion neurons. (**c**) Expression of DMXL2 in the outer hair cells (OHC) and inner hair cells (IHC) as well as the neurofilaments of spiral ganglion neurons. Red: SOX2 staining marking the supporting cells. Blue: DAPI staining of the cell nuclei. Bars, 25 μm.

1:500. All images were screened using the Leica SP5 confocal microscope. Antibodies used in this study included rabbit anti-DMXL2 (ab122552, Abcam, Cambridge, MA) detecting three major isoforms of DMXL2, rabbit anti-MYOSIN VIIA (25–6790, Proteus, Ramona, CA), and goat anti-SOX2 (sc-17320, Santa Cruz, Dallas, TX). The RT-PCR and immunostaining experiments were performed in triplicate in C57BL/6 mice. No randomization was used and no blinding was used.

RESULTS

Phenotype characterization

Audiograms of the 11 affected members of family KH are shown in **Figure 1b**. The bilateral, mild to moderate hearing loss began during the second decade of life and gradually progressed to severe to profound hearing loss during the sixth decade. Click-evoked otoacoustic emission was absent for all affected members. No inner ear malformation was detected by temporal bone high-resolution computed-tomography scanning. The affected members reported no symptoms associated with vestibular dysfunction. The hearing loss was found to be nonsyndromic because no additional abnormality was observed in the affected individuals.

Positional mapping of the family

Multipoint genome-wide linkage analysis of 10 affected (affected individual II-2 was recruited later and was not included in the

linkage analysis) and 11 unaffected family members (marked with asterisks in **Figure 1a**) generated a 9.68-Mb positional candidate interval on chromosome 15q21.2 between markers rs16962243 and rs8037652 (**Figure 1c** and **Supplementary Table S1** online). A maximum LOD score of 4.03 was obtained for marker rs11071224. None of the positional candidate genes within the interval has been linked to syndromic or nonsyndromic deafness, implicating that the hearing loss was probably caused by a novel deafness gene.

Identification of the variant associated with hearing loss

Three affected members and one unaffected members (marked with triangles in **Figure 1a**) were selected for whole-exome sequencing. For all four individuals, the median depth was more than 100× and the percentage of targeted regions covered by 10 or more reads was more than 89.0%. Within the positional candidate interval on chromosome 15q21.2, 92.3% of the targeted region was covered by 10 or more reads and the remaining 7.7% of the poorly covered region was screened via Sanger sequencing. A total of three candidate pathogenic variants, all within the positional candidate interval on chromosome 15q21.2, were detected in the three affected individuals but not in the unaffected individual (**Supplementary Table S1** online). Sanger sequencing in the other family members—particularly in individual II-2 (underlined in **Figure 1a**), who was recruited later and was not included in the linkage analysis—showed that the only variant segregating with the hearing loss was c.7250G>A (p.Arg2417His) in exon 29 of *DMXL2* (NM_001174116, recalculated maximum LOD score = 4.33; **Figure 1a**). This p.Arg2417His variant was predicted to change a highly conserved Arg2417 residue between the Rav1p_C domain and the C-terminal WD domains of DMXL2 (**Figure 2**) and was predicted to be deleterious by the Mutation Taster (prediction score = 0.999), PROVEAN (prediction score = −2.86), and SIFT (prediction score = 0.027) computational tools. It has a minor allele frequency of 0.00003 (4 in 121,174 alleles) in the Exome Aggregation Consortium (ExAC) database and was not present in 1,000 ethnically matched adult controls with normal hearing (the average and minimum NGS depths were 52.4× and 42.5×, respectively, at the variant site).

Cochlear expression of murine *Dmxl2*

To further elucidate the role of *DMXL2* in hearing, we studied the expression of *Dmxl2* in mouse cochlea. RT-PCR showed the expression of *Dmxl2* in total RNA extracted from the P1 and P60 mouse cochlea (**Figure 3a** and **Supplementary Figure S1** online). In cryosections of P1, P6, and P60 of the organ of Corti from mice, restricted immunofluorescence staining of *Dmxl2* could be detected in the inner and outer hair cells as well as in the spiral ganglion neurons (**Figure 3b**,**c** and data not shown). Interestingly, the immunostaining observed in hair cells was markedly enriched at the basal region of the hair cells and the neurofilament extremity of spiral ganglion neurons projected into the hair cells, suggesting that DMXL2 may function on the presynaptic and postsynaptic sides of the hair cell innervations.

DISCUSSION

In this study, we provided genetic evidence to support that the p.Arg2417His variant in *DMXL2* is associated with dominant, nonsyndromic hearing loss, including: (i) linkage analysis identified a 9.68 -Mb positional candidate interval on chromosome 15q21.2 (maximum LOD score = 4.33) containing *DMXL2*; (ii) whole-exome sequencing identified heterozygous p.Arg2417His in *DMXL2* as the only candidate pathogenic variant segregating with the hearing loss in family KH; (iii) the p.Arg2417His variant changed a highly conserved amino acid of DMXL2, has an extremely low allele frequency (0.00003 in the ExAC database, not present in 1,000 ethnically matched normal hearing controls), and was predicted to be deleterious by Mutation Taster, PROVEAN, and SIFT computational tools; and (iv) the mRNA and protein expression of *Dmxl2* in mice is consistent with its role in hearing. However, the whole-exome sequencing approach used in the current study has several limitations: (i) noncoding regions were not covered; (ii) approximately 10% of targeted coding regions are below the 10× read depth; and (iii) copy-number variants cannot be reliably detected. Therefore, it remains possible that the causative variant could not be identified by whole-exome sequencing and that the p.Arg2417His variant in *DMXL2* may be in linkage disequilibrium only with the true causal variant.

So far, only a homozygous 15-bp in-frame deletion (c.5824_5838del/p.1942_1946del) in *DMXL2* has been reported in a consanguineous family, which led to a newly classified syndrome including gonadotropic axis deficiency, central hypothyroidism, peripheral demyelinating sensorimotor polyneuropathy, intellectual disability, and profound hypoglycemia progressing to nonautoimmune insulin-dependent diabetes mellitus.[4] Hearing loss, however, was not present in the affected individuals in this family. Quantitative RT-PCR of the blood lymphocytes showed that the homozygous p.1942_1946del mutation resulted in a 75% reduction of the *DMXL2* mRNA level. In family KH, the affected individuals had no gonadotropic and neurodevelopmental abnormalities other than late-onset, progressive hearing loss. The distinct phenotypes suggest that the heterozygous p.Arg2417His variant is likely associated with nonsyndromic hearing loss through a gain-of-function or dominant-negative mechanism specifically affecting the inner ear function.

Our study is, to our knowledge, the first to reveal *DMXL2* as a deafness-associated gene in humans. Consistently, it has been shown that rbc3α, the zebrafish DMXL2 ortholog, was localized to the basal region of zebrafish hair cells and that mutant alleles of *rbc3α* isolated from a large-scale screen led to auditory and vestibular defects in zebrafish.[5] In hair cells of the mutant zebrafish, the synaptic vesicles had elevated pH and the cytosolic V1A subunit of the V-ATPase was no longer enriched in synaptic regions, suggesting that Rbc3α modulates synaptic transmission in hair cells by promoting V-ATPase activity and acidification of the synaptic vesicles. In our study, immunostaining of DMXL2 in the organ of Corti from mice also showed

extensive expression in the basal region of hair cells where synaptic vesicles are present (**Figure 3c**). Interestingly, strong expression of *Dmxl2* was also detected in the neurofilament extremity of spiral ganglion neurons projected into the hair cells (**Figure 3b**,**c**), suggesting that DMXL2 may function on the postsynaptic sides of the hair cell innervations as well. Our results were consistent with previously reported data for mouse inner ear gene expression that showed that *Dxml2* was enriched in hair cells by 3.86-fold (false discovery rate <0.05) and was expressed in spiral and vestibular ganglia.[8,9]

In conclusion, our study identified *DMXL2* as a novel candidate gene for human deafness, which may provide new insights into the molecular mechanism of hearing and hearing disorders. Functional studies of *DMXL2* are needed to further explore its role in the synaptic machinery of hair cells and the postsynaptic nerve pathways.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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DISCLOSURE

The authors declare no conflict of interest.

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