Original Research *Feature article*

A novel variant in *DMXL2* gene is associated with autosomal dominant non-syndromic hearing impairment (DFNA71) in a Cameroonian family

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Impact statement

Prior to the present study, only one report from China has showed an association between a variant in *DMXL2* and HI in humans. Our study implicates for the first time the *DMXL2* gene in NSHI in Africans, and thus also confirms the contribution of *DMXL2* to HI in humans. The genetics of HI in Africans is not well elucidated beyond the study of pathogenic or likely pathogenic variants in connexin genes that were shown to be infrequent in most African populations. The present study therefore enriches the list of HI genes in Africans.

Abstract

Approximately half of congenital hearing impairment cases are inherited, with non-syndromic hearing impairment (NSHI) being the most frequent clinical entity of genetic hearing impairment cases. A family from Cameroon with NSHI was investigated by performing exome sequencing using DNA samples obtained from three family members, followed by direct Sanger sequencing in additional family members and controls participants. We identified an autosomal dominantly inherited novel missense variant [NM_001174116.2: c.918G>T; p.(Q306H)] in *DMXL2* gene (MIM:612186) that co-segregates with mild to profound non-syndromic sensorineural hearing impairment . The p.(Q306H) variant which substitutes a highly conserved glutamine residue is predicted deleterious by various bioinformatics tools and is absent from several genome databases. This variant was also neither found in 121 apparently healthy controls without a family history of hearing impairment , nor 112 sporadic NSHI cases from Cameroon. There is one previous report of a large Han Chinese NSHI family that segregates a missense variant in *DMXL2*. The present study provides additional evidence that *DMXL2* is involved in hearing impairment etiology, and we suggest *DMXL2* should be considered in diagnostic hearing impairment panels.

Keywords: Non-syndromic hearing impairment, autosomal dominant inheritance, *DMXL2*, Africa

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Introduction

Hearing impairment (HI) is, globally, a significant public health concern, with higher burden in lower income countries.[1] Approximately 3.6% of the general population and 14.8% of adults ≥50 years of age in sub-Saharan Africa live with disabling HI.[2] Identifying the underlying cause of HI is critical, as it allows for targeted therapeutic decision making.[3] About 30 to 50% of congenital cases of HI in sub-Saharan Africa are inherited, with non-syndromic HI (NSHI) representing approximately 86.1% to 92.5% of all genetic HI cases.[2,4] NSHI is mainly inherited in an autosomal recessive (AR) manner (~80% of cases), while autosomal dominant (AD) inheritance is less frequent but not negligible, as it accounts for ~18% of NSHI cases.[5]

The genetics NSHI in Africans is unclear. Pathogenic variants in *GJB2* which constitute the major etiology of genetic HI in Asian and European populations were found to be rare in Africans.[6] HI is highly genetically heterogeneous, as about 121 genes and 170 loci have been described to date (Hereditary Hearing Loss Homepage). However, targeted exome sequencing has also demonstrated a lower identification rate of pathogenic and likely pathogenic (PLP) variants in > 100 HI genes, amongst sporadic HI cases in populations of African ancestry, i.e. Black South

Africans, African Americans, and Nigerians, compared to Asians and Europeans.[7,8] However, the HI PLP detection rate was highly improved with the targeted selection of multiplex families segregating NSHI, from Cameroon.[9] Moreover, the prevalence of PLP variants in AR NSHI genes, based on gnomAD, and selected from Deafness Variation and ClinVar databases,[10] was evaluated at 5.2 per 100,000 persons for Africans or African Americans, which is lower than the 96.9 per 100,000 persons prevalence for Ashkenazi Jews.[11,12] Because of this low contribution of known variants in AR NSHI genes in African populations, next-generation sequencing techniques have a high potential of identifying novel PLP variants, and novel HI genes in populations of African descent as was demonstrated for other populations.[13–15]

In the present study, we have used whole exome sequencing (WES) and identified a missense variant in *DMXL2* (DFNA71; MIM:617605) in a multiplex family from the Bamileke tribe from Cameroon, segregating AD NSHI. We strengthen the evidence that *DMXL2* contributes to HI, as there is only a single report of a missense *DMXL2* variant co-segregating with NSHI in a large Han Chinese family.[16]

Materials and methods

Participants' recruitment

Patients' recruitment procedure has previously been described.[17] Briefly, the proband of a Cameroonian family segregating HI (Family 23, Figure 1(a)) was selected via a school for HI individuals, and the other family members were recruited thereafter. Medical records of all our participants were analyzed by a general practitioner, an ENT specialist, and a medical geneticist, and detailed personal and family histories were obtained through a rigorous clinical interview. For all HI participants, in addition to a systemic general examination, an otological assessment including pure tone audiometry (PTA) was performed. To classify hearing levels, we used recommendations from the Bureau International d'Audiophonologie (BIAP), Belgium.[18] Isolation of genomic DNA from peripheral blood was done within the division of Human Genetics at the University of Cape Town in South Africa, using the chemagic extraction protocol. All hearing-impaired participants were shown to not carry PLP variants in *GJB2* gene or the "*GJB6*-D13S1830" deletion, and the results were previously published.[18]

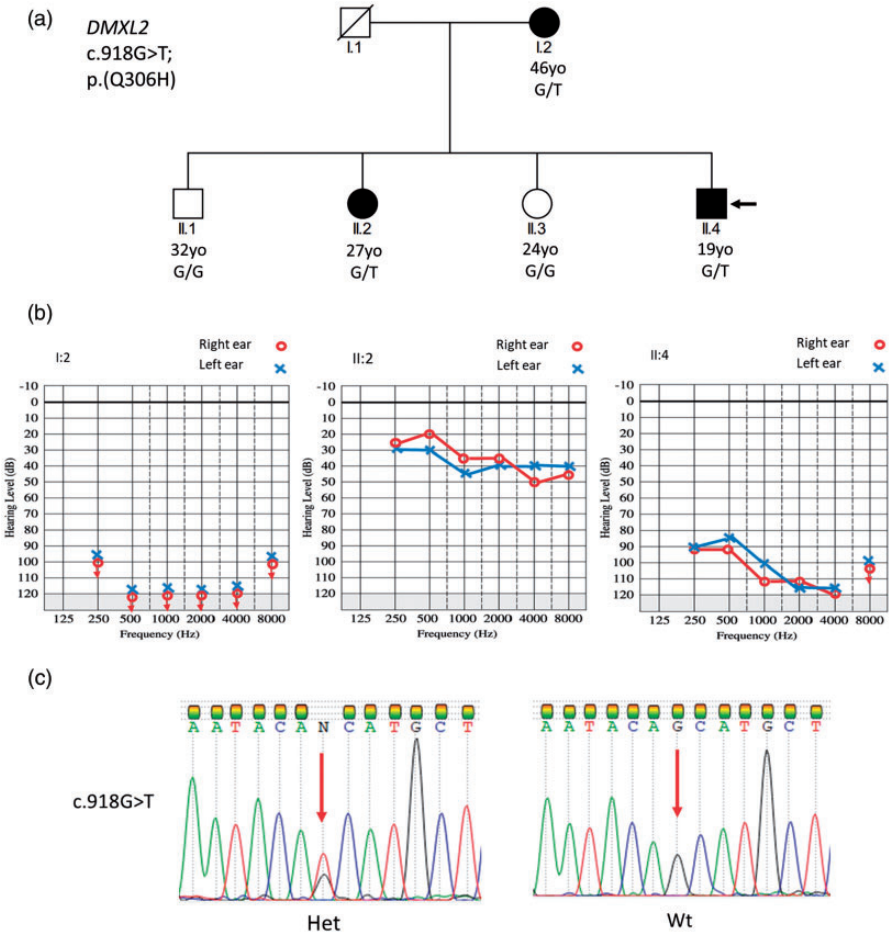

Figure 1. Pedigree, audiometry results, and sequencing chromatograms of family 23. (a) The family tree and co-segregation of the DMXL2 variant NM_015263.5: c.918G>T are compatible with AD mode of inheritance of the HI within the family. The proband is designated by the black arrow. (b) Pure tone audiometry (air conduction) of the three affected individuals, showing a bilateral profound hearing loss for the mother (I.2), a bilateral mild HI for child II.2, and bilateral profound HI for child II.4. (c) Sequencing chromatograms displaying the wild type and variant alleles. The red arrow indicates the nucleotide where the variant occurs. Het: heterozygous for the alternate allele; Wt: homozygous for the wild type; yo: years old.

Additionally, a total of 112 Cameroonian patients with isolated NSHI cases of presumed genetic etiology (described in Table S1) were included in order to explore the frequency of the novel *DMXL2* variant in this group. Moreover, a total of 121 apparently healthy controls individuals from Cameroon, without a family history of HI, were chosen from randomly selected blood donors at Yaoundé Central Hospital and tested for the identified variant.

Whole exome sequencing and data analyses

WES was executed for DNA samples from two affected (I.2, and II.4) and one unaffected (II.3) (Figure 1(a)) members from Family 23 at Omega Bioservices (Norcross, GA, USA) company. Illumina instructions were followed to prepared samples, and an Illumina sequencer was used for the sequencing using 150 bp pair-end reads. Low-quality reads were removed, and Burrows–Wheeler Aligner-MEM software (BWAv0.7.15) was used to align the filtered reads to the human reference genome hg19.[19,20] After sorting and marking duplicate reads, the genome analysis toolkit (GATK) v4.0.6.0 was used to call single nucleotide variants (SNVs) and insertions/deletions (InDel) implementing base quality score recalibration.[21] Plink v1.9 was used to check the sex of each individual.[22] Via identity-by-descent sharing (plink v1.9) and the KING algorithm,[22,23] familial relationships were verified.

Annotation and filtering strategy

ANNOVAR was used for filtering and variant annotation,[24] following methods described previously.[20] In brief for the analysis, (1) The AD mode of inheritance was considered; (2) Exonic and splice site variants were selected; (3) We prioritized variants with an expected effect on pre-mRNA splicing or protein function (missense, start-loss, frameshift, splice site, nonsense, etc.) with a minor allele frequency (MAF) of <0.0005 in all populations of the gnomAD database; and (4) Bioinformatics prediction scores were retrieved from dbNSFP and dbscSNV to assess the deleterious effect of missense and splicing variants respectively, including polymorphism phenotyping v2 (PolyPhen-2), SIFT, MutationTaster, deleterious annotation of genetic variants using neural networks (DANN), combined annotation dependent depletion (CADD), and Genomic Evolutionary Rate Profiling (GERP++) scores.[25–30] Previously reported association between our candidate variants/genes and HI was obtained from Human Phenotype Ontology (HPO) ClinVar, Online Mendelian Inheritance in Man (OMIM), and Hereditary Hearing Loss Homepage (HHL) databases, and genes expressed in the inner ear or genes known to be implicated in human/animal HI were retained.[31] The presence of known pathogenic HI variants from the ClinVar database was also assessed without any MAF cut-off.

Sanger sequencing

The candidate variant obtained from filtering was validated using direct sequencing and its co-segregation with HI phenotype amongst the other family members that were available (I.2, II.1, II.2, II.3, and II.4; Figure 1(a)) was also evaluated. Additionally, a total of 112 individuals with sporadic NSHI of putative genetic origin, and 121 apparently healthy control individuals from Cameroon, without a family history of HI, were screened for the candidate variant. Primers to target our variant of interest in exon 8 (forward 5′-TCCAAAGCAGTTCATTTGTGTCT-3′; reverse 5′-CTGTGAACATCATAAGAACCGGG-3′) of *DXML2* gene were assessed through NCBI BLAST. PCR and Sanger sequencing reactions were both performed using the aforementioned primers within the Division of Human Genetics at the University of Cape Town in South Africa. FinchTV v1.4.0 software was used to manually check sequencing chromatograms, and the latter were aligned to the *DMXL2* reference sequence (ENSG00000104093; obtained through Ensembl server) using UGENE v34.0.

Conservation of amino acids and protein modeling analyses

To assess how conserved the amino acid affected by the p.(Q306H) missense variant is, a multiple sequence alignment (MSA) of human DMXL2 with comparable proteins was performed. A BLASTp search (using the PSI-BLAST algorithm)[32] of the mutant protein against the non-redundant protein sequence database was realized, and the first isoform of the hits from each taxonomic group was retrieved for MSA. The MSA was performed with CLUSTAL O v1.2.4[33] using custom scripts and the sequence trimmed to retain residues around the position of interest without gaps. Jalview v2.10.5[34] was then used to visualize the alignment. Additionally, the secondary structure of DMXL2 wild type and mutant proteins was predicted by using PSIPRED v4.0.[35]

The crystal structure of human DMXL2 protein was not available in the protein data bank (PDB).[36] As such, comparative modeling methods were used to build the three-dimensional (3D) structure of human DMXL2. We obtained the amino acid sequence of DMXL2 through the NCBI database, and executed a pBlast search against PDB. We first built the homology model of the wild type using the PDB structure 2YMU (a highly repetitive propeller structure) as a template, and subsequently constructed the structure of the mutant by mutating the targeted residue using MODELLER.[37] PYMOL viewer was used to visualize the protein structure.

Results

Study subjects phenotypes

In this family, there was no history of ototoxic treatment, severe ear infections, head trauma, neonatal asphyxia, or was any other environmental factor identified as a possible cause of HI for any of the three HI members (I.2, II.2, and II.4; Figure 1(a)). None of them had a history of ophthalmological or neurological symptoms, and the physical examination did not reveal any vestibular, neurologic, or other systemic abnormalities. The segregation of NSHI for family

23 was compatible with an AD mode of inheritance, and both parents were unrelated (Figure 1(a)). Before our study, no formal otological evaluation was done for any of the HI individuals. The audiometry testing performed at the time of this study revealed that HI is bilateral and sensorineural for the three affected individuals. The mother (I.2) experienced prelingual and progressive HI in the past, and during the present recruitment, PTA revealed profound HI (Figure 1(b)). In one of the two affected children (II.4), HI was congenital, and the otological assessment at the time of the recruitment revealed a profound HI phenotype (Figure 1(b)). The other affected child (II.2) did not report any HI, until audiometric assessment for this study revealed a mild HI (Figure 1(b)).

Whole exome sequencing and identification of the candidate variant

WES was performed using DNA samples from three individuals; the average sequencing depth of the targeted region was 226X (I.2), 209X (II.3), 236X (II.4), and the fraction of the targeted region covered >10× was 96.27%. The mean base quality score across the samples was 36, implying high base call accuracy (>99.9%), while the mean ratio of reads that were mapped out of the target region (off-target reads) to the ratio of reads mapped unto the target region (on-target reads) was 2.7% (0.027), indicating high capture quality of the kit. Our filtering strategy described in the methods section identified a mono-allelic missense variant [NM_015263.5:c.918G>T; p.(Q306H)] in *DMXL2*. This variant which leads to the substitution of glutamine by a histidine residue [NM_015263.5:p.(Q306H)] and co-segregates with HI following an AD mode of inheritance is expected to be damaging by MutationTaster (disease-causing, score: 0.937), Polyphen2 HDIV (possibly damaging, score: 0.895), CADD (score: 20.9), and DANN (score: 0.941). The variant is absent from gnomAD, trans-omics for precision medicine (TOPMed), Greater Middle East (GME) variome project, UK10K, and dbSNP databases, and 121 apparently healthy control individuals from Cameroon.

Sanger sequencing confirmed the occurrence of the variant in the heterozygous state in the affected mother (I.2) and children (II.2 and II.4), while it was absent in unaffected children (II.1 and II.3) (Figure 1(c)). This candidate variant was not identified in 112 isolated NSHI cases from Cameroon. The variant pathogenicity was categorized as of uncertain significance (PP1, PP3, and PM2) as referred to the American college of Medical genetics (ACMG) recommendations for variants interpretation.[38,39]

Analysis of NM_015263.5(DMXL2):p.(Q306H) variant

The 3036 amino acids protein (NP_056078.2) was retrieved from NCBI GenPept as a FASTA file and hereafter referred to as DMXL2.

Amino acids evolutionary conservation

Performing an NCBI PSI-BLAST search of DMXL2 through the non-redundant protein database revealed the position of the variant (p.Q306H) to be conserved amongst all selected non-Homo sapiens species (Figure 2). This reflects a strong evolutionary conservation, typical of a significant functional role.

DMXL2 secondary structure prediction

Helices and strands are secondary structural features that form motifs, which in turn constitute functional domains of proteins. Alterations of the secondary structural elements could thus affect the function of the protein. PSIPRED v4.0 server expects our candidate variant (NM_015263.5:p.Q306H) to have a significant impact on DMXL2 secondary structure. The variant was predicted to occur within a helix and its occurrence diminishes the helical propensity at several residues (^{304}IH^{305} and K^{316}). In addition, the short helix at ^{346}RHI^{350} is abrogated (Red boxes in Figure S1). The variant further alters the β-strand propensity at several residues as indicated by the black boxes in Figure S1.

Protein modeling

3D modeling of DMXL2 showed that the residue p.Q306 in DMXL2 exists in a β-sheet (Figure 3(a)). The p.Q306 in wild-type displays hydrogen bonding with T339 and L318 (Figure 3(b)). The p.(Q306H) variant substituted the polar side chain with non-polar aromatic ring. As a consequence, a hydrophobic interaction is established with nearby residues as shown in Figure 3(c). As a result of these various contacts and different nature of amino acids, a slight shortening of the β-strand was observed in the altered protein. (Figure 3(e)).

Discussion

To our knowledge, this study is only the second report on the implication of the *DMXL2* gene in AD NSHI. This study provides evidence that a missense variant [p.(Q306H)] in *DMXL2* is associated with NSHI in an African family from Cameroon and co-segregates with NSHI in an AD manner. This novel p.(Q306H) variant affects a conserved glutamine amino acid residue of DMXL2, is expected deleterious by various bioinformatics tools, and is predicted to significantly alter DMXL2 function and structure. The variant is likely to be private, as it was not detected in 112 sporadic NSHI cases, 121 apparently healthy control individuals without HI from Cameroon, and was absent from gnomAD, TOPMed, UK10K, GME, and dbNSP databases.

A missense variant in *DMXL2* was shown to be associated with HI for the first time in 2017 by Chen *et al.*;[16] they reported on a large seven-generations Han Chinese family with AD HI (DFNA71), that segregated a mono-allelic missense variant p.(Arg2417His) in *DMXL2*. Chen *et al.*[16] also found *Dmxl2* gene to be expressed in the outer and inner hair cells of the mouse cochlea, and its ganglion neurons, suggesting an implication in the synaptic mechanism of hair cells. Moreover, orthologs of rabconnectin-3α were shown to be expressed in the cochlear of zebrafish and to be critical for hair cells innervation. Pathogenic nonsense variants in the rabconnectin-3α gene of zebrafish led to vestibular dysfunctions and HI caused by abnormal acidification of synaptic vesicles.[16,40]

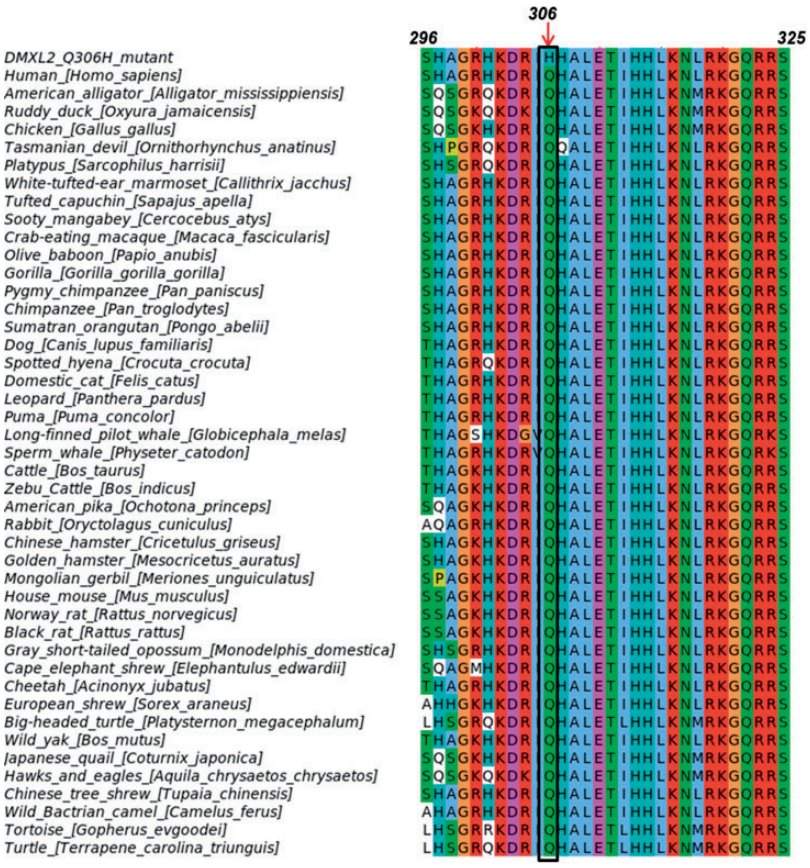

Figure 2. Conservation of the mutational position DMXL2:p.(Q306H) (designated by the red arrow) across species.

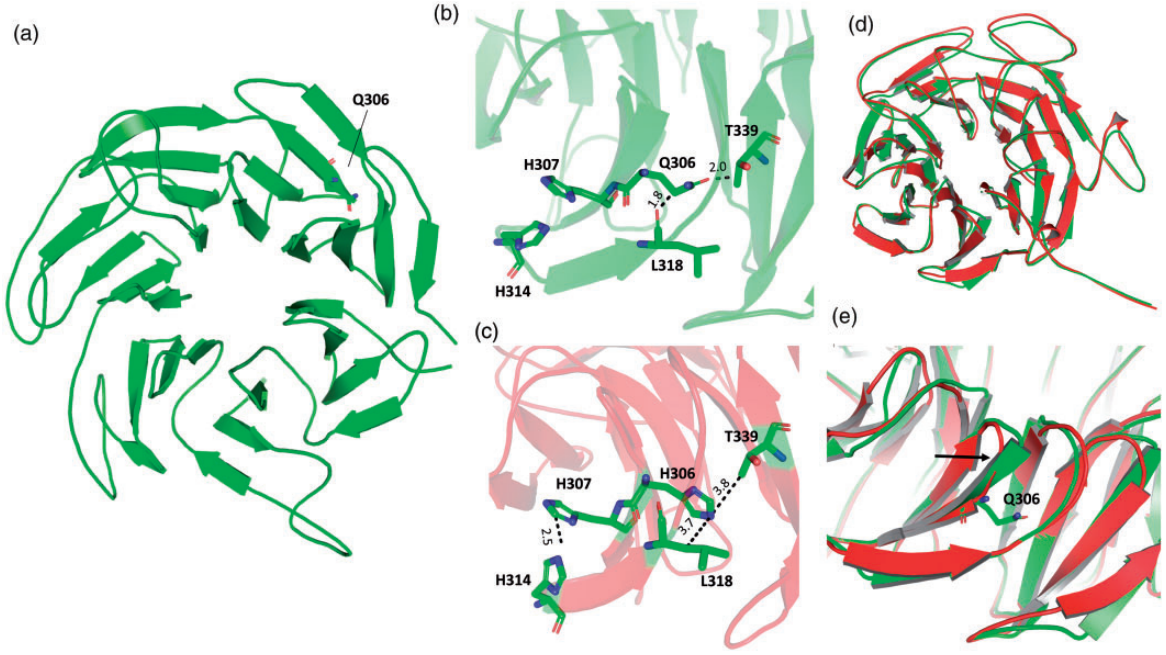

Figure 3. Model of the 3D structures of wild-type DMXL2 and its Q306H mutant. (a) The overall structure of DMXL2 (b) Close-up view of the interactions at position 306 of wild and Q306H (c). The wild and mutant are represented by green and red colors, respectively. The superposed structure of wild and mutant type (d) shows the shortening of the β-strand in the mutant protein as indicated by an arrow in the close-up view (e).

The phenotype of patients included in the present study has some similarities with Han Chinese patients reported by Chen *et al.*,[16] as they equally presented with bilateral and progressive sensorineural NSHI that was inherited in an AD manner, without clinical signs of vestibular dysfunction or any syndromic abnormality. Chen *et al.*[16] described in their study a late onset of the HI (occurring during the second decade of life), while two of the three patients reported here, present a prelingual onset of HI, with at least one patient presenting with congenital HI. Additionally, one of the three affected Cameroonian family members (II.2: 27 years old) presented with mild HI, while the two others (I.2: 46 years old, and II.4: 19 years old) presented profound HI, suggesting variable expressivity, a well-known feature of many AD traits.[41] It is also possible that II.2 could still expressed a more severe HI phenotype later in life, similar to what was reported for patients from China.[16] A regular assessment of hearing for individual II.2 is critical. Our results thus suggest that variants in *DMXL2* might lead to a NSHI phenotype that can occur in infancy and present with variable expressivity. Studies of more individuals with HI due to *DMXL2* variants are needed to refine the phenotype.

Variants in *DMXL2* have been associated with diseases other than HI. A biallelic 15-bp in-frame deletion in *DMXL2* (p.1942_1946delSDGNG) was identified in a Senegalese family and led to polyendocrine-polyneuropathy syndrome (PEPNS; MIM:616113).[42] PEPNS is a progressive neurodevelopmental and endocrine disorder occurring in early childhood and is characterized by hypoglycemia, growth retardation, gonadotropic axis deficiency, peripheral demyelinating polyneuropathy, hypothyroidism, progressive non-autoimmune insulin-dependent diabetes mellitus, intellectual disability, pyramidal signs, and cerebellar ataxia.[42] Early infantile epileptic encephalopathy-81 (EIEE81; MIM:618663) which is also called Ohtahara syndrome was recently shown to be associated with variants in *DMXL2*.[43] EIEE81 is a developmental brain disorder that involves severe psychomotor development, intractable seizures, dysmorphic features (such as hypotonic facies and epicanthal folds), mild peripheral polyneuropathy, and sensorineural HI.[43] None of our patients or those previously described in the Han Chinese family NSHI presented with any neurological or endocrine phenotypes. Variants in *DMXL2* are thus associated with two clinical forms of sensorineural HI: (1) the non-syndromic form as that described in the present study and by Chen *et al.*,[16] and (2) the form that occurs as part of a syndrome with neurological abnormalities, as described in the EIEE81.[43]

DMXL2 (on chromosome 15q21.2) encodes DmX-like protein 2 [DMXL2, also called rabconnectin-3α (RC3)],[43] the α subunit of the rabconnectin protein complex that condenses on synaptic vesicles and is involved in neurotransmitters secretion.[16,44] DMXL2 is a vesicular protein made up of 3036aa (Mr 339,753 Da) and is involved in the Ca2+-dependent translocation of neurotransmitters across synaptic membranes.[43,45] The expression of RC3 was found in many tissues, which include ear hair cells, and the brain.[16,44] The exact neurophysiological role of RC3 in the brain remains elusive;[43] however, studies on the rat brain suggest that RC3 may serve as a scaffold protein for Rab3A and its effector proteins.[46] Rab3A, which was shown to co-immunoprecipitate with RC3 in the rat brain, plays a role in the exocytosis of neurotransmitters across synaptic membranes by facilitating the translocation and docking of synaptic vesicles to presynaptic membranes and also preventing the fusion of the vesicles to the plasma membrane, a Ca2+-dependent process.[46] In mammalians, RC3 is also expressed in the brain and at synaptic terminals where it binds RAB3A interacting proteins as a dimer with Rabconnectin-3b (*DMXL1*), and is thought to be important for autophagy and brain development.[43] *Dmxl2* homozygous knockout mice were shown to be embryonic lethal, while heterozygous *Dmxl2* ($Dmxl2^{+/-}$) mice display corpus callosum dysplasia and macrocephaly, giving more evidence of the implication of this gene in the development of the brain.[43,47] The implication of RC3 in the hearing process was revealed by Einhorn *et al.*[40] when they showed that the zebrafish RC3 ortholog (Rbc3α) was expressed in the inner ear of zebrafish, and was concentrated at the basal region of hair cells. Also, mutant alleles of *rbc3α* selected from a cohort of zebrafishes were associated with HI and vestibular dysfunctions.[40] *rbc3α* mutants also lack light adaptation responses in melanocytes and spontaneous eye movements, suggesting a possible visual defect.[40] No clinical sign of vestibular or visual abnormalities was described in family 23, or in the Chinese family described by Chen *et al.*;[16] however, given the visual impairment observed in the *rbc3α* mutants, studies on the possible expression and function of DMXL2 in the retina are recommended. Last, Einhorn *et al.*[40] showed that the pH of the synaptic vesicles in the hair cells of the *rbc3α* mutant zebrafish was elevated, and the cytosolic unit of the V-ATPase was no longer concentrated in synaptic junctions.[40] Interestingly, deacidification of the synaptic vesicles was shown to reduce the loading of neurotransmitters in vesicles and thus negatively impact neurotransmission.[48] Based on these results, Einhorn *et al.*[40] proposed that Rbc3α regulates the synaptic activity in hair cells by allowing the concentration of the V-ATPase holoenzyme on synaptic vesicles, modulating thus the acidification of the vesicle and its neurotransmitters concentration.

To further support the linkage of HI in "Family 23" with *DMXL2* gene, genetic linkage analysis using the logarithm of the odds (LOD) score method could be useful. Also, functional analyses using cell lines and/or animal models are needed to further confirm the deleterious effect of the p.(Q306H) variant on DMXL2 function and structure. In addition to examining the exome, identifying variations in parts of the genome not covered by WES might be of importance. Indeed, variants in noncoding DNA sequences including deep intronic variants were shown by previous studies to be associated with HI.[49] Although 10× depth of coverage may not be sufficient to detect small proportions of heterogeneous genetic variations in population-based studies involving large numbers of samples (coverage of

at least 20× is generally ideal), family-based studies retain their power to detect such variants at 10× depth of coverage given that they generally look for segregation of sometimes private and/or rare variants within a family.[50]

This study confirms the contribution of *DMXL2* to NSHI in humans and demonstrates for the first time such association in Africans. Our study thus enriches the list of HI-causing genes in humans and should consequently contribute to increasing the solving rate of genetic HI cases in clinical settings. The present study adds more evidence to the efficacy of WES in detecting causative variants in NSHI cases from sub-Saharan. Additional studies are necessary to assess the contribution of *DMXL2* gene to NSHI in other populations.

Conclusions

The present study reports a novel mono-allelic missense variant in *DMLX2* [p.(Q306H)] that co-segregates with NSHI in a Cameroonian family with an AD mode of inheritance. This study provides additional support for the use of WES to identify variants that contribute to HI in sub-Saharan Africa. These data will complement and improve clinical diagnosis, and our knowledge of HI pathophysiology, globally.

AUTHORS' CONTRIBUTIONS

AW and SML conceived the project; EWT performed the recruitment and molecular experiments; IS, TB, LMN, AA performed the bioinformatics analysis; KKE, EWT, AN and S M performed the *in silico* analysis of the identified variant; EWT issue the first version of the paper and all the authors reviewed and edited it; AW supervised the entire project; all authors have read and agreed to the final version of the paper.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

All research procedures in the present study were authorized by the Columbia University's institutional review board (IRB-AAAS2343), the Institutional Research Ethics Committee for Human Health of the Gynaeco-Obstetric and Paediatric Hospital of Yaoundé, Cameroon (No. 723/CIERSH/DM/2018), and the Human Research Ethics Committee of the University of Cape Town's Faculty of Health Sciences (HREC 484/2019). All participants were informed and provided written consent, including permission to publish data, in respect of the Declaration of Helsinki.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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