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Old gene, new phenotype: Splicing altering variants in *CEACAM16* cause recessive non-syndromic hearing impairment

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Abstract

Background—Hearing loss is a genetically and phenotypically heterogeneous disorder.

Objectives—The purpose of this study was to determine the genetic cause underlying the post-lingual progressive hearing loss in two Iranian families.

Methods—We used OtoSCOPE, a next generation sequencing platform targeting >150 genes causally linked to deafness, to screen two deaf probands. Data analysis was completed using a custom bioinformatics pipeline and variants were functionally assessed using mini-gene splicing assays.

Results—We identified two homozygous splice-altering variants (c.37G>T and c.662-1G>C) in the *CEACAM16* gene, segregating with the deafness in each family. The mini-gene splicing results revealed the c.37G>T results in complete skipping of exon 2 and loss of the AUG start site. The c.662-1G>C activates a cryptic splice site inside exon 5 resulting in a shift in the mRNA reading frame.

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Conflict of interest statement

The authors declare no conflict of interest.

Contribution Statement

KTB, KK and HA: conception and study design; designed and performed experiments; gathered and analyzed data; wrote initial manuscript draft, critically read and revised manuscript.

HN: study design; gathered clinical data and samples; critically read and revised manuscript.

RJS: conception and study design; critically read and revised manuscript.

All authors have approved the finalized manuscript.

Conclusions—These results suggest that loss-of-function mutations in *CEACAM16* result in post-lingual progressive hearing impairment and further support the role of *CEACAM16* in auditory function.

Keywords

Deafness; *CEACAM16*; non-syndromic hearing loss; splice-site

Introduction

The genetic, mutational and phenotypic landscape of deafness is vast. Currently, more than 150 genes (<http://hereditaryhearingloss.org/>) harboring in aggregate more than 7000 pathogenic variants (<http://deafnessvariationdatabase.org/>) are implicated in hearing loss. This genetic and mutational heterogeneity gives rise to a broad phenotypic spectrum. Roughly 1 in 5 genes associated with hearing loss exhibit pleiotropy: alterations in these genes can give rise to autosomal recessive (AR) or autosomal dominant (AD), non-syndromic or even syndromic forms of hearing loss. Unraveling this genotype-phenotype continuum is essential in the era of personalized medicine.

Normal cochlear function depends on the harmonious synchrony of many molecular factors. The tectorial membrane (TM), for example, is a complex extracellular matrix of collagens and glycoproteins that covers the surface of the cochlear sensory epithelium and attaches to the tallest row of stereocilia of outer hair cell (OHC) hair bundles (as reviewed by Richardson et al[1]). Its physical properties are dependent on its both porous structure, which is defined by its macromolecular matrix, and the interstitial fluid within this matrix. Changes in these properties alter fluid flow dynamics, basilar membrane motion and wave tuning, and lead to hearing loss, as evidenced by the broad spectrum of pathogenic variation in genes that encode TM proteins.

One gene essential to normal TM function is *CEACAM16* (OMIM# 614614). It is expressed almost exclusively in the inner ear[2], where it is transcribed and translated in the supporting cells and secreted into the matrix of the TM[3]. The encoded 425-amino acid (aa) peptide belongs to the large and diverse carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family. Unlike other members of this family, *CEACAM16* has two Ig-like V-type domains flanking two Ig-like C2-type domains and no transmembrane domain/anchor[4, 5]. *CEACAM16* interacts with other TM proteins like *TECTA* and *TECTB*[3, 4] and its targeted deletion in mice results in patho-morphological defects of the TM and hearing loss[2, 3]. To date, *CEACAM16* has been described only as a cause of ADNSHL (two families and one de novo case[4, 6, 7].) Here, we show that variation in *CEACAM16* also causes mild-to-moderate progressive ARNSHL, adding *CEACAM16* to the growing list of genes that cause both ADNSHL and ARNSHL in humans.

Materials and Methods

Subjects

Two Iranian families with progressive mild-to-moderate hearing loss were ascertained for this study. Affected individuals underwent a clinical evaluation and pure tone audiometry. After obtaining written informed consent to participate in this study, blood samples were obtained from all family members. All procedures were approved by the human research Institutional Review Boards at the Iran University of Medical Sciences and the Welfare Science and Rehabilitation University, Tehran (Iran), and the University of Iowa, Iowa City, Iowa (USA).

Targeted Genomic Enrichment and Massively Parallel Sequencing

Targeted genomic enrichment with massively parallel sequencing (TGE+MPS) using the OtoSCOPE® platform (version 7) was performed on the proband from each family to screen ~150 known genes implicated in NSHL and common syndromic forms of deafness for pathogenic and likely pathogenic variants as described[8–11]. Enriched libraries were sequenced on the Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA) using 100bp paired-end reads at the Genomics Division of the Iowa Institute of Human Genetics.

Bioinformatic analysis

Data analysis was performed using a custom bioinformatic and annotation pipeline, as described[8, 12, 13]. Briefly, sequencing reads were mapped to the NCBI Build 37 reference genome using BWA[14] followed by variant calling using Genomic Analysis Tool Kit (GATK)[15]. Variants were first filtered for quality (depth >10 and quality score >30) followed by minor allele frequency (MAF) (<2% in: 1000 Genomes Project database; the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project Exome Variant Server (EVS); the Exome Aggregation Consortium (ExAC); Genome Aggregation Database (gnomAD)). Variants were then prioritized based on conservation (GERP and PhyloP), predicted deleteriousness (SIFT, PolyPhen2, MutationTaster, LRT and the Combined Annotation Dependent Depletion (CADD)) and variant-type (missense, nonsense, indel or splice-site). Potential effects on splicing were analyzed using Human Splicing Finder 3.0 (HSF) (<http://www.umd.be/HSF3/>). All samples were analyzed for CNVs using a sliding-window method to assess read-depth ratios[16]. Variant nomenclature follows the recommended HGVS naming convention[17]. Variants reported here have been submitted to the Deafness Variation Database (<http://deafnessvariationdatabase.org/>) for integration and curation.

Segregation analysis Sanger Sequencing

Segregation analysis of candidate variants was completed by Sanger sequencing on an ABI 3730 Sequencer (Perkin Elmer, Waltham, MA). All sequencing chromatograms were compared to published cDNA sequence for *CEACAM16* (NM_001039213.3); nucleotide changes were detected using Sequencer v5 (Gene Code Corporation, Ann Arbor, MI).

In vitro splicing analysis

In vitro splicing mini-gene assays were carried out as described[9]. Wild-type (WT) *CEACAM16* (NM_001039213.3) exon 2 or exon 5 was PCR amplified with gene-specific primers and ligated into the pre-constructed pET01 Exontrap vector (MoBiTec, Goettingen, Germany). Using the manufacture's protocols, variants were introduced into the wild-type sequences using QuikChange Lightning Site-Directed Mutagenesis (Agilent, Santa Clara, CA). Colonies were selected, grown and plasmid DNA was harvested using the Zyppy Plasmid Midiprep Kit (ZYMO Research, Irvine, CA). After sequence confirmation, WT and mutant mini-genes were transfected in triplicate into COS7 and HEK293 cells, and total RNA was extracted 36-hours post-transfection using the Quick-RNA MiniPrep Plus kit (ZYMO Research, Irvine, CA). Using a primer specific to the 3' native exon of the pET01 vector, cDNA was synthesized using RNA SuperScript™ III Reverse Transcriptase (ThermoFisher Scientific, Waltham, MA). After PCR amplification, products were visualized on a 1.5% agarose gel, extracted and then sequenced.

Results

Families

We ascertained two families of Iranian origin, L-890076 and L-8800015, with progressive mild-to-moderate ARSNHL (Figure 1A,B). In all affected individuals, the hearing loss was post-lingual, starting in the second decade. With the exception of hearing loss, physical examination of affected individuals was unremarkable (Table 1).

Variant Identification

Probands from both families were screened using OtoSCOPE® v7. In proband IV.2 of family L-8900076, after variant filtering as described above, 12 variants remained. Further filtering for recessive inheritance left a single homozygous canonical-splice site variant (c.662-1G>C) in exon 5 of *CEACAM16*, which segregated with the deafness phenotype in affected siblings and in the extended pedigree (Figure 1A). In the second family, L-8800015, proband II.2 had only a single variant after filtering, a homozygous transversion (c.37G>T) in the last coding nucleotide of exon 2 in *CEACAM16*. Segregation analysis showed that this variant also segregated with the hearing-loss phenotype (Figure 1B). No copy number variants were detected in either proband.

Computational and In vitro splicing analysis

Both variants, c.662-1G>C and c.37G>T, were computationally predicted to alter the WT splicing sequencing motif and impact splicing. Visualization of the splicing products for WT and mutant exon 5 revealed: a 234-base pair (bp) band for the empty vector corresponding to the 5' and 3' native exons; a 513-bp product with WT sequence showing inclusion of exon 5 of *CEACAM16*; and two bands for the mutant at 410 bp and 234 bp (Figure 2A). For the WT and mutant exon 2, bands were seen at 405 bp and 234 bp, respectively (Figure 2B). Sequencing all bands confirmed break points and splicing events.

Discussion

To date, the only phenotype associated with pathogenic variants in *CEACAM16* is ADNSHL at the DFNA4B. Herein, we have implicated *CEACAM16* as the causative gene underlying the deafness phenotype in two Iranian families. Both variants we identified occur in a canonical splice-site, are computationally predicted to alter RNA splicing, and are absent from both the gnomAD or ExAC databases (Table 2). Individuals homozygous for either of these variants have progressive hearing loss, whereas heterozygous carriers have normal hearing (Figure 1).

Human *CEACAM16* is a 7-exon gene although only exons 2–7 are protein coding (Figure 1C). While the precise function of *CEACAM16* is unclear, it is believed to stabilize and organize the protein network of the TM by interacting with TECTA and having an epistatic role on TECTB[2–4, 18, 19]. Gain-of-function mutations result in ADNSHL and while loss of *Ceacam16* results in murine hearing loss, until this report, a human counterpart was unknown.

In family L-890076, we identified and confirmed segregation of a novel homozygous transversion (c.662-1G>C) in the donor site of exon 5 of *CEACAM16* (Figure 1A, 1C). The c.662-1 guanine is highly conserved and is computationally predicted to alter proper splicing. To confirm this prediction, we tested the splicing output from mini-genes containing WT or mutant exon 5 DNA sequence. In the WT construct, we confirmed the expected inclusion of the cDNA sequence of exon 5 (Figure 2A). In the mutant, we identified two splicing events. The first is the activation of a cryptic acceptor site (AG) at position c.763–764 the consequence of which is loss of first 103 nucleotides of exon 5 (c.662_764del) and a shift in the mRNA reading frame resulting in premature protein truncation (p.Phe221Cysfs*16) which is predicted to undergo nonsense mediated decay (NMD) resulting in a null allele[20] (Figure 1C). The second splicing event is the skipping of the entire exon (Figure 2A) and consequently the loss of the c.622_940 in the mRNA, resulting in an in-frame deletion (p.Phe221_Ala313delinsSer). Of these two events, the former is more robust (Figure 2A). Exon 5 encodes an Ig-like C2-type 2 domain. If NMD did not occur, the p.Phe221Cysfs*16 peptide would lack most of the Ig-like C2-type 2 domain and all of the C-terminus Ig-like V-type domain (Figure 1C), and if exon 5 is skipped completely, the resultant peptide would lack the entire Ig-like C2-type 2 domain, implying that loss of part or all of exon 5 results in hearing loss.

In family L-8800015, we identified a novel, highly conserved, homozygous variant (c.37G>C) in two siblings with deafness (Figure 1B). The variant occurs in the last nucleotide of exon 2 (Figure 1C). Given the variant is located in the U1 small nuclear ribonucleoprotein (snRNP) binding site[21–23], we assessed its effects on splicing. The results revealed a 405-bp and a 234-bp band for the WT and mutant exon 2 mini-genes respectively. Sequencing of the mutant 234-bp band confirmed complete skipping of exon 2, the first coding exon of the gene (Figure 2B). As a consequence, the native ATG-methionine start site is missed. Scanning exon 3, there is an alternative ATG (c.50–52) the use of which alters the reading frame and produces a stop codon after 156 nucleotides. This allele most likely gets targeted

for NMD. Besides housing the translation start site, exon 2 also encodes the signal sequence required to translocate CEACAM16 extracellularly (Figure 1C).

We also noticed an interesting finding in the WT exon 2 mini-gene. The amplified WT band was 405 nucleotides although we expected a 367-bp product (Figure 2B). There is currently only one annotated RefSeq transcript for *CEACAM16* (NM_001039213.3). In this transcript, exon 2 spans chr19:45204631–45204763 with the annotated AG acceptor site at chr19:45204629–45204630. We did not detect this annotated acceptor in any of our experiments. Rather, we detected the inclusion of 38-bp upstream of annotated intron 1 (chr19:45204597–45204630) (Figure 2B), with the AG of the novel acceptor site at chr19:45204591–45204592. We further investigated this inclusion event computationally using Human Splicing Finder (HSF). The annotated acceptor site has an HSF score of 86.81 with no MaxEnt score, whereas the acceptor site we identified has an HSF score of 87.6 and a MaxEnt score of 7.3. It is unclear how the inclusion of these additional 38 bps would affect the *CEACAM16* transcript but this alternative splicing could be a regulatory mechanism for its temporal and spatial expression [22, 24]. Further experiments are needed to elucidate this finding.

The phenotype in our patients is similar to that seen in the *Ceacam16* null mouse, which also has progressive hearing impairment [2, 3]. To date three pathogenic variants (p.Thr140Pro, p.Gly169Arg and p.Leu365Arg) in *CEACAM16* have been linked to ADNSHL (Table 2). The phenotype associated with each genotype is similar: post lingual progressive with the onset typically late first decade or early second, initially affecting the high frequencies. The phenotype described here in families L-8900076 and L-8800015 (Figure 1A and B) is identical to that seen in DFNA4B individuals. Interestingly, similarity between ADNSHL and ARNSHL phenotypes caused by the same gene is rarely seen with deafness. An in-depth clinical evaluation revealed affected individuals have normal vestibular reflexes, vision, skin, cognitive function and developed normal speech (Table 1). *CEACAM16* is expressed in the testes and *Ceacam16* null mice are fertile. Although a semen analysis was not completed, based on the murine data we do not expect fertility issues for affected individuals.

Finally, that these variants alter mRNA splicing is potentially noteworthy given the advances in therapies targeting RNA splicing, such as antisense oligonucleotides [25–30]. The variants described here both reside in a canonical splice-site making spliceosome-mediated RNA *trans*-splicing (SMaRT) [31] an option to bypass the altered splicing and produce a functional *CEACAM16* mRNA molecule and peptide.

In summary, we have refined the mutational spectrum of *CEACAM16* as a cause of hearing loss by including splice-altering variants. This finding expands the phenotypic spectrum of *CEACAM16* to include ARNSHL and adds *CEACAM16* to the list of deafness genes that exhibit allelic and phenotypic heterogeneity. Expanding our understanding of the mutational spectrum of disease genes is an important step in providing the correct clinical molecular interpretation and diagnosis for patients with hearing loss.

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Figure 1.

Pedigrees, Audiograms and *CEACAM16* gene and protein schematic. Filled symbols denote individuals with hearing loss; bold represents the *CEACAM16* mutant alleles segregating in each family. Audiograms were obtained with air conduction with the frequency range of 250 Hz to 8,000 Hz. For family L-8800015, serial audiograms for individual II.2 are shown. C) Human *CEACAM16* has 7 exons. Non-coding, signal peptide, Ig-like V-type domains, C2-type domains and linker region are denoted by grey, yellow, green, blue, red and black, respectively. Novel variants identified in this study (top) and on bottom known DFNA4B variants.

Figure 2.

Mini-gene splicing assays. Gel electrophoresis of Wild-type, empty pET01 vector and the c. 662-1G>C (A) and c.37G>T (B). A) The c.662-1G>C variant causes the activation of a cryptic acceptor site. Sequence chromatograms show the read through at each exon junction and sequence alignment show the deletion because of the activation of the cryptic acceptor site. B) The c.37G>T variant causes complete exon skipping confirmed by sequencing. A novel acceptor site identified upstream of exon 2 in the wild-type. Sequencing was used to map the upstream acceptor site. Asterisk indicates RefSeq annotated acceptor site for exon 2.

Table 1

Clinical Summary

Family	Individual	Current Age	Age of onset	Speech	Optical	Skin	Cognition	Balance
L-8900076	III.1	59	13	NL	NL	NL	NL	NL
	IV.2	34	10	NL	NL	NL	NL	NL
	IV.3	27	12	NL	NL	NL	NL	NL
	IV.4	37	10	NL	NL	NL	NL	NL
L-8800015	II.2	31	12	NL	NL	NL	NL	NL
	II.3	25	13	NL	NL	NL	NL	NL

Age is in years. For Speech, language developed normally. NL, Normal.

Table 2

Variant Summary

Inheritance	Phenotype	Genomic	cDNA	Protein	EVS	MAF		Conservation			Deleteriousness				Reference
						IKG	ExAC	gnomAD	GERP	PhyloP	MT	PP2	SIFT	LRT	
AR	Post-lingual Progressive	chr19:45204763;G>T	c.37G>T	-	0	0	0	0	C	C	D	B	T	U	This Study
		chr19:45208859;G>C	c.662-1G>C	-	0	0	0	0	C	C	-	-	-	-	This Study
AD	DFNA4B	chr19:45207323;A>C	c.418A>C	p.Thr140Pro	0	0	0	0	C	C	P	PD	T	U	[4]
		chr19:45207410;G>A	c.505G>A	p.Gly169Arg	0	0	0	0	C	C	P	PD	D	U	[6]
De novo		chr19:45211286;T>G	c.1094T>G	p.Leu365Arg	0	0	0	0	C	C	D	PD	D	-	[7]

Nucleotide numbering starting at the +1 position of transcript NM_001039213.2. Minor Allele Frequency (MAF) databases include: the Exome Variant Server (EVS), 1000 Genomes (1KG), Exome Aggregation Consortium (ExAC) and genome Aggregation Database (gnomAD). Deleteriousness was assessed using 5 prediction algorithms: MutationTaster (MT), PolyPhen-2 (PP2), Scale Invariant Feature Transform (SIFT), Likelihood Ratio Test (LRT) and Combined Annotation Dependent Depletion (CADD). C, conserved; P, Polymorphism; PD, Possibly Damaging; T, Tolerated; D, Damaging, Disease Causing or Deleterious; B, Benign; U, Unknown; “-”, score not given.