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Research Article

Identification of Novel *TMC1* Compound Heterozygous Mutations Related to Autosomal Recessive Hearing Loss by Targeted Capture Sequencing

Abstract

Mutations in the transmembrane channel-like gene1 (*TMC1*) are known to cause autosomal dominant and recessive forms of nonsyndromic hearing loss DFNA36 and DFNB7/11, respectively. Here, we characterized a 5-year old girl with severe sensorineural hearing loss. By combining targeted capture of 140 known deafness genes, next-generation sequencing and bioinformatic analysis, we identified two compound heterozygous mutations *TMC1* c.1333C>T (p.R445C) and c.1765A>G (p.M589V) as the disease-causing mutations. Our results indicated that *TMC1* c.1333C>T (p.R445C) and c.1765A>G (p.M589V) lead to hearing loss in this family and highlight the power of combining deafness gene capture and next generation sequencing for identification of defective genes in sporadic cases with hearing loss. This approach will facilitate identification of the mutation spectrum of all known deaf genes.

Background

Hearing loss is a common sensory defect in humans. Nonsyndromic hereditary forms, in which hearing loss is the only clinical sign, are known to be genetically heterogeneous [1]. To date, more than 200 genetic loci and 140 responsible genes for hereditary hearing loss have been identified (Hereditary Hearing Loss Homepage, <http://hereditaryhearingloss.org/>). The extremely genetic heterogeneity of hearing impairment is a major challenge for traditional genetic testing and counseling. Targeted DNA capture and massively parallel sequencing are ideal tools to address this challenge.

Next generation sequencing, is a revolutionary technology that allows large amounts of genomic sequence information to be obtained rapidly and at a low cost [2]. Because of its large capacity to survey the whole exome and genome in an unbiased manner, NGS (next generation sequencing) is well suited to identifying the causative mutations of hereditary hearing loss. NGS has commonly been used to identify disease genes within even a limited number of patient samples [3-7]. Novel genes for non-syndromic [8,9] and syndromic [10] hearing loss have also been identified using the targeted NGS approach. Targeted deaf gene enrichment usually increases this proportion by at least 1000 fold [11,12]. In this study, we applied targeted capture of 140 known deafness genes, next-generation sequencing and bioinformatic analysis to analyze the genetic pathogenesis of one sporadic patient with severe sensorineural

TMC1 is predicted to encode membrane proteins with six transmembrane domains that may be involved in the functional maturation of cochlear hair cells [13]. Although the specific functions of the protein encoded by *TMC1* are unknown, bioinformatic analysis [14] and data obtained from *in vitro* heterologous systems

[13], suggest that *TMC1* functions as a membrane channel or transporter [15]. *TMC1* mutations cause nonsyndromic autosomal dominant and recessive hearing loss at the DFNA36 and DFNB7/11 loci, respectively. More than 30 recessive mutations in *TMC1* are associated with autosomal recessive nonsyndromic hearing loss (ARNSHL) at the DFNB7/11 locus in 39 families worldwide [14,16-24].

In this study, we identified one small ARNSHL pedigree with severe sensorineural hearing loss caused by the c.1333C>T (p.R445C) and c.1765A>G (p.M589V) mutation of *TMC1* through targeted deaf gene capture, next generation sequencing and bioinformatic analysis. This is the first study to identify *TMC1* c.1333C>T (p.R445C) and c.1765A>G (p.M589V) as the ARNSHL-associated mutation in this family.

Materials and Methods

Clinical data

Family C01701 is a two-generation Chinese family with autosomal recessive nonsyndromic hearing loss. To identify candidate mutations, DNA samples were obtained from 3 members of family C01701. Mutations of *GJB2* and *SLC26A4* had been excluded previously. Fully informed written consent was attained from two guardians. The study was approved by the Chinese PLA General Hospital ethics of research committees. Clinical information was gathered through multiple interviews with the family members. Medical history was obtained using a questionnaire regarding the following aspects of this condition: subjective degree of hearing loss, age at onset, evolution, symmetry of the hearing impairment, use of hearing aids, presence of tinnitus, medication, noise exposure, pathological changes in the

ear and other relevant clinical manifestations. Otoscopy, physical examination and pure tone audiometric examination (at frequencies from 250 to 8000 Hz) were performed to identify the phenotype. Immittance testing was applied to evaluate middle-ear pressure, ear canal volume and tympanic membrane mobility. Unaffected phenotype status was defined by a threshold lower than age- and gender-matched 50th percentile values for all frequencies measured. Physical examination of all members revealed no signs of systemic illness or dysmorphic features. CT scans of the temporal bone in the patient were performed. The diagnosis of profound sensorineural hearing impairment was made according to the WHO criteria based on audiometric examination. Tandem gait and Romberg tests were performed to evaluate balance.

Deafness gene capture and Illumina library preparation

Each DNA sample is quantified by agarose gel electrophoresis and Nanodrop (Thermo). Libraries were prepared using Illumina standard protocol. Briefly, 3 microgram of genomic DNA was fragmented by nebulization, the fragmented DNA is repaired, Illumina adapters are then ligated to the fragments, and the sample is size selected aiming for a 350–400 base pair product. The size selected product is PCR amplified (Each sample is tagged with a unique index during this procedure), and the final product is validated using the Agilent Bioanalyzer.

The amplified DNA was captured with a deafness related Gene Panel using biotinylated oligo-probes (MyGenostics GenCap Enrichment technologies). The probes were designed to tile along 140 deafness related genes. The capture experiment was conducted according to manufacturer's protocol. The enrichment libraries were sequenced on Illumina HiSeq 2000 sequencer for paired read 100bp.

Bioinformatics analysis

For sequencing analysis, high-quality reads were retrieved from raw reads by filtering out the low quality reads and adaptor sequences using the Solexa QA package and the cutadapt program (<http://code.google.com/p/cutadapt/>), respectively. SOAPaligner program was then used to align the clean read sequences to the human reference genome (hg19).

After the PCR duplicates were removed by the Picard software, the SNPs was firstly identified using the SOAPsnp program (<http://soap.genomics.org.cn/soapsnp.html>). Subsequently, we realigned the reads to the reference genome using BWA and identified the insertions or deletions (InDels) using the GATK program (http://www.broadinstitute.org/gsa/wiki/index.php/Home_Page). The identified SNPs and InDels were annotated using the Exome-assistant program (<http://122.228.158.106/exomeassistant>). Magic Viewer was used to view the short read alignment and validate the candidate SNPs and InDels. Nonsynonymous variants were evaluated by four algorithms, Ployphe, SIFT, PANTHER and Pmut, as described previously to determine pathogenicity.

Mutational analysis of *TMC1*

The segregation of the *TMC1* c.1333C>T (p.R445C) and c.1765A>G (p.M589V) mutations was tested in 3 family members

(I:1, I:2 and II:1), including the one whose gDNAs has been subjected to 140 deafness-associated gene NGS analysis, using PCR followed by bidirectional Sanger sequencing of the amplified fragments (ABI 3100, Applied Biosystems, USA). Nucleotide alteration(s) was identified by sequence alignment with the *TMC1* GenBank sequence using the Genetool software.

Multiple sequence alignment

Multiple sequence alignment was performed according to a Homologene program with default settings and the sequences NP_619636.2 (*H. sapiens*), XP_001093188.2 (*M. musculus*), XP_528322.2 (*P. troglodytes*), XP_002689695.2 (*B. taurus*), XP_005616877.1 (*C. lupus*), NP_083229.1 (*M. musculus*), NP_001101991.1 (*R. norvegicus*), NP_001006580.1 (*G. gallus*) and XP_695621.4 (*D. rerio*).

(http://www.ncbi.nlm.nih.gov/homologene?cmd=Retrieve&dopt=MultipleAlignment&list_uids=23670)

Results

Family and clinical evaluations

Family C01701 is a two-generation Chinese family with severe sensorineural hearing loss (Figure 1A) and includes 1 affected patients: II:1 (female, 5 years old). Hearing impairment was severe, prelingual and stable (Figure 1B).

Vestibular analysis was performed in the patient II:1 who did not complain about dizziness, vertigo, or imbalance. All position tests produced no nystagmus without vertigo sensation. II:1 did not have obvious delayed gross motor development. The physical examinations revealed no signs of systemic illness. High-resolution computed tomography of the temporal bone in II:1 was normal,

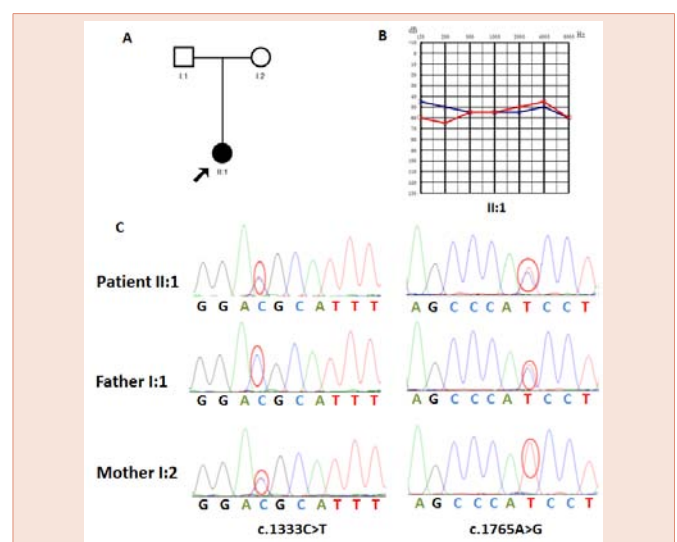


Figure 1: A. Pedigree of Family C01701 with ARNSHL Affected subject is denoted in black. Arrow indicates the proband; B. Audiogram of affected subject showed severe to profound sensorineural hearing loss. C. Electro-pherograms analysis of *TMC1* in family C01701 showing the compound heterozygous mutations (c.1333C>T and c.1765A>G) co-segregated with the phenotype.

excluding inner-ear malformations. This phenotype was consistent with that reported for DFNB7/11.

Targeted deafness gene capture and massively paralleled sequencing

We sequenced all the coding exons plus ~100 bp of the flanking intronic sequence of 140 deafness genes in one affected (II:1) member of family C01701. Under the autosomal recessive or autosomal dominant de novo mode, two variants leading to amino acid change were detected in *TMC1*: c.1333C>T (p.R445C) and c.1765A>G (p.M589V). Of these, one variant, c.1333C>T (p.R445C), was reported previously as a pathogenic mutation [25], whereas c.1765A>G (p.M589V) was novel and had not been found in the dbSNP databases.

Mutation detection and analysis

Using Sanger sequencing, three participating family members (1 affected and 2 unaffected) in family C01701 were genotyped to identify the mutations. Compound heterozygous c.1333C>T and c.1765A>G mutations of *TMC1* were identified in the affected family member of the second generation (II:1). *TMC1* p.R445C was found heterozygous in mother with normal hearing (I:2), whereas p.M589V was found heterozygous in father with normal hearing (I:1) (Figure 1C), which is consistent with autosomal recessive inheritance.

We used TMHMM2.0 to predict the TMC1 protein with six membrane-spanning regions and cytoplasmic N and C terminal regions. R445C located in the TM4 domain and p.M589V is located in the cytoplasm between the TM4 and TM5 domains of TMC1. This predicted model of TMC1 membrane topology is similar to those for ion channels and transporters.

Two amino acid substitutions occurred in revolutionarily conserved regions across different species (Figure 2). It was predicted that both *TMC1*c.1333C>T and c.1765A>G mutations would be damaging by SIFT and Polyphen2.

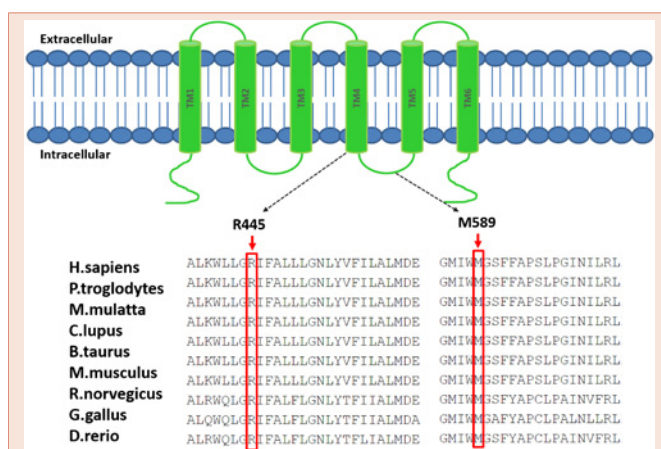


Figure 2: Conservation analysis. Protein alignment showing conservation of residues TMC1 R445 and M589 across nine species. These missense mutations occurred at an evolutionarily conserved amino acid (in red).

Discussion

In this study, we identified *TMC1* c.1333C>T (p.R445C) and c.1765A>G (p.M589V) as the disease-causing mutation in a two-generation Chinese family (C01701) with ARNSHL through multiple deaf genes capture, next-generation sequencing and bioinformatic analysis.

Massively parallel sequencing is a revolutionary technology that enables us to obtain large amounts of genomic sequence information in a rapid and low-cost manner [2]. With targeted gene capture, the proportion of DNA fragments containing or near targeted regions is greatly increased. Because of its ability to enrich deafness genes, NGS can be used to identify causative mutations of hereditary hearing loss. The use of NGS has frequently resulted in the identification of disease genes within a limited number of patient samples [4-7,26,27].

Mouse models with *Tmc1* defects [14, 15] support a role for *Tmc1* in the inner and outer hair cells, either in proper trafficking of other membrane proteins in these cells or in regulating the differentiation of immature hair cells into fully functional auditory receptors [13]. This model proposed that mechanical forces brought about by bending of stereocilia and tension on the tip links directly activate ion channels. If it is true that *Tmc1* is an ion channel localized mainly in the IHC, then it might be involved in the most basic auditory process of hair-cell transduction.

The predicted structure of TMC1 is similar to that of the α -subunit of voltage-dependent K^+ channels, which has six α -helical TM segments and intracellular N and C termini [28]. It was predicted that TMC1 might be an ion channel or transporter which mediated K^+ homeostasis in the inner ear [29]. The first four TM domains of the K^+ channel α -subunit act as voltage sensors for activation gating [30], whereas the intervening segment between TM5 and TM6 appears to confer channel selectivity [28]. One novel conserved *TMC1* sequence variant in this study c.1765A>G (p.M589V) lies within a large cytoplasmic loop between TM4 and TM5 that includes the TMC domain and a potential pore-forming loop [31].

Conclusions

Overall, we report here the clinical and genetic characteristics of one small Chinese family with ARNSHL. Identification of novel mutations has an important impact on clinical patient management, genetic counseling, molecular diagnosis, and development of advanced therapeutic strategies.

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