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Defect in the gene encoding the EAR/EPTP domain-containing protein TSPEAR causes DFNB98 profound deafness

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We report a consanguineous Iranian family affected by congenital profound sensorineural deafness segregating in an autosomal recessive mode. Auditory tests implicated at least a cochlear defect in these patients. We mapped the deafness, autosomal recessive (DFNB) locus involved by linkage analysis to a 4.8 Mb region at chromosome 21q22.3-qter. Exclusion of the DFNB8/10 gene *TMPRSS3*, located in this chromosomal interval, led us to identify a new deafness locus, DFNB98. Whole exome sequencing allowed us to identify a homozygous frame-shifting mutation (c.1726G>T+c.1728delC) in the gene *TSPEAR* (*thrombospondin-type laminin G domain and EAR repeats*). This truncating mutation (p.V576L*fs*X37) impeded the secretion of the encoded protein by cells transfected with the mutated gene. Alternative splicing of *TSPEAR* transcripts predict two protein isoforms, 522 and 669 amino acids in length, both of which would be affected by the mutation. These isoforms are composed of a thrombospondin-type laminin G (TSP) domain followed by seven tandemly organized epilepsy-associated repeats (EARs), probably forming a β-propeller domain. *Tspear* is expressed in a variety of murine tissues. Only the larger *Tspear* transcript was found in the cochlea, and the protein was detected by immunofluorescence at the surface of the hair bundles of sensory cells. The mammalian EAR protein family includes six known members. Defects in four of them, i.e. Lgi1, Lgi2, Vlgr1 and, we show here, *TSPEAR*, cause disorders with auditory features: epilepsy, which can include auditory features in humans; audiogenic seizures in animals; and/or hearing impairments in humans and mice. These observations demonstrate that EAR-containing proteins are essential for the development and function of the auditory system.

INTRODUCTION

The mammalian auditory sensory organ, the cochlea, houses about 20 different cell types (1). Partly because there are few cochlear cells, conventional molecular biology techniques have not been extensively used to study the molecular physiology of this sensory organ. However, the effectiveness of the genetic approach to identify molecules essential to a given function is not limited by small numbers of the cells or molecules involved, so we have proposed this approach for studying the cochlea (2). Indeed, analysis of human deafness genes and studies of the corresponding mouse models have provided major insights into proteins involved in the formation and functioning of the cochlea (3,4). Many of the proteins encoded by these genes are involved in ion homeostasis, in the development or functioning of the sensory cells (hair cells) or in the structure of the tectorial membrane that transfers the mechanical acoustic stimulation to the hair cell's sensory antenna, called the hair bundle (4). The genetic approach has also been effective for deciphering protein networks underlying morphological and functional characteristics of the cochlea.

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The early-onset forms of inherited isolated (non-syndromic) deafness studied so far are monogenic disorders. They are classified according to their mode of transmission. DFNA and deafness, autosomal recessive (DFNB) refer to deafness forms inherited by autosomal dominant and autosomal recessive modes of transmission, respectively, and DFNX and DFNY, to an X and Y chromosome-linked mode of transmission, respectively. The DFNB forms are mainly responsible for prelingual forms of severe to profound hearing impairment, whereas DFNA forms usually appear later and are often mild or moderate. To date, around 135 loci underlying non-syndromic deafness have been reported: 67 are associated with DFNB forms, 50 with DFNA forms, 5 with DFNX forms, 1 with a DFNY form and 2 with mitochondrial forms. Sixty-eight genes have now been identified, of which 43 are responsible for DFNB forms.

Our approach consists of studying large consanguineous families that live in geographic isolation to search for genes underlying DFNB forms (5). We have collected about 140 such families in Iran affected by severe to profound deafness. In these families, the involvement of the *GJB2* gene encoding connexin 26, causative of the most prevalent DFNB form, DFNB1, has been excluded. Here, we report a novel DFNB locus that we identified in one family. We then used a complete exome sequencing strategy to identify the causative gene, *TSPEAR* (*thrombospondin-type laminin G domain and EAR repeats*), which encodes a member of the EAR (Epilepsy-Associated Repeat) protein family.

RESULTS

A new DFNB locus underlying prelingual, profound deafness at chromosome 21q22.3-qter

In the six-generation consanguineous family 923, from an isolated village located in the central region of Iran, three individuals (VI-2, VI-4, VI-5), born from a marriage between two normally hearing third cousins (V-2, V-3), were affected by bilateral profound sensorineural deafness. The three other siblings (VI-1, VI-3, VI-6) were unaffected (Fig. 1). The ages of generation VI individuals were between 20 and 34 years old. In accordance with the parents' interviews indicating a prelingual onset of the deafness, language acquisition was severely impaired in all affected children. A complete medical history was obtained for each affected individual to exclude the possibility of infectious or environmental causes of hearing impairment. No other clinical signs were associated with the hearing impairment.

Pure-tone audiometry in affected subjects showed flat audiograms characteristic of profound hearing impairment, whereas the other members of the family had normal-hearing thresholds (Fig. 2). We recorded the auditory brainstem response (ABR) to a pure-tone sound stimulus to characterize further the hearing impairment. No ABR could be recorded even in response to a 100 dB sound stimulation in any of the three affected children. Ipsilateral stapedial reflexes were absent. The cochlea contains two types of sensory cells, the inner hair cells, which are the genuine sensory cells, and the outer hair cells, which amplify the incoming sound stimulation of the sensory epithelium (organ of Corti). Transient-evoked otoacoustic emissions (TEOAEs), which are routinely used for probing the activity of outer hair cells, could not be detected in the three affected siblings. The absence of ABR to a very loud (100 dB) sound stimulus, however, indicates additional defects of the cochlea or the auditory nerve. Whether the central auditory pathway was also impaired in the patients could not be evaluated due to the complete absence of ABR.

We performed a genome-wide linkage analysis in family 923 using 700 k single-nucleotide polymorphism (SNP) arrays (Illumina OmniExpress-12), followed by homozygosity mapping. SNP analysis and haplotype reconstruction defined a single critical region of 4.8 Mb, at chromosome 21q22.3-qter, carrying the DFNB locus. This region is flanked by rs1126134 SNP on the proximal side (q22.3), and includes rs15047, the most telomeric SNP on the distal side (qter) (Fig. 1). A maximum logarithm of odds score of 3.0 was found using polyAC markers within this region. The DFNB8/10 gene, *TMPRSS3*, has been mapped to this interval (6), so we tested whether *TMPRSS3* was involved in family 923. We sequenced the 13 exons and flanking intronic sequences in one affected child (VI-2) and his normal-hearing brother (VI-6), and did not detect a causative mutation in the affected child. Several DFNB forms of deafness have been shown to be allelic to dominant forms of deafness (DFNA), but no DFNA locus has been reported in this chromosomal region. Also, several deafness genes have been found to underlie both isolated and syndromic forms of deafness, but no locus for a syndromic form of deafness has been reported in this region. This led us to conclude that this chromosomal region carries a previously undescribed DFNB locus, DFNB98 (http://www.genenames.org).

A frame-shifting mutation in *TSPEAR*

The DFNB98 candidate region at chromosome 21q22.3-qter was large, so we used a whole-exome sequencing strategy to identify the causative gene. Total exomes from the three affected siblings and from a normally hearing sibling homozygote for the candidate region (VI-6) were sequenced (see Materials and Methods). We focused on exonic and flanking intronic variants within the candidate region, i.e. between nucleotide positions 43 341 362 and 48 084 747. The region contains 74 annotated protein-encoding genes and no miRNA-encoding sequences (February 2009 human reference sequence UCSC hg19/GRCh37). Alignment of the sequence reads in this region showed that 864 of the 876 targeted exons (98.6%) had >10 high-quality reads for all the DNA samples. We also sequenced the remaining 12 exons by the Sanger method.

In the candidate region, 91 variants, all single nucleotide substitutions except a 1 bp deletion, passed our quality control thresholds. These variants are predicted to result in 51 synonymous and 39 non-synonymous changes, and 1 frameshift. All these variants except two were present both in the affected siblings and the normally hearing sibling; they are also present in HapMap (the International HapMap Project, URL: http://hapmap.ncbi.nlm.nih.gov/), 1000 genomes (URL: http://www.1000genomes.org/) and the complete exome sequences of a large collection of individuals from European, American and African American populations, recorded in the Exome Variant Server, NHLBI Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.wa

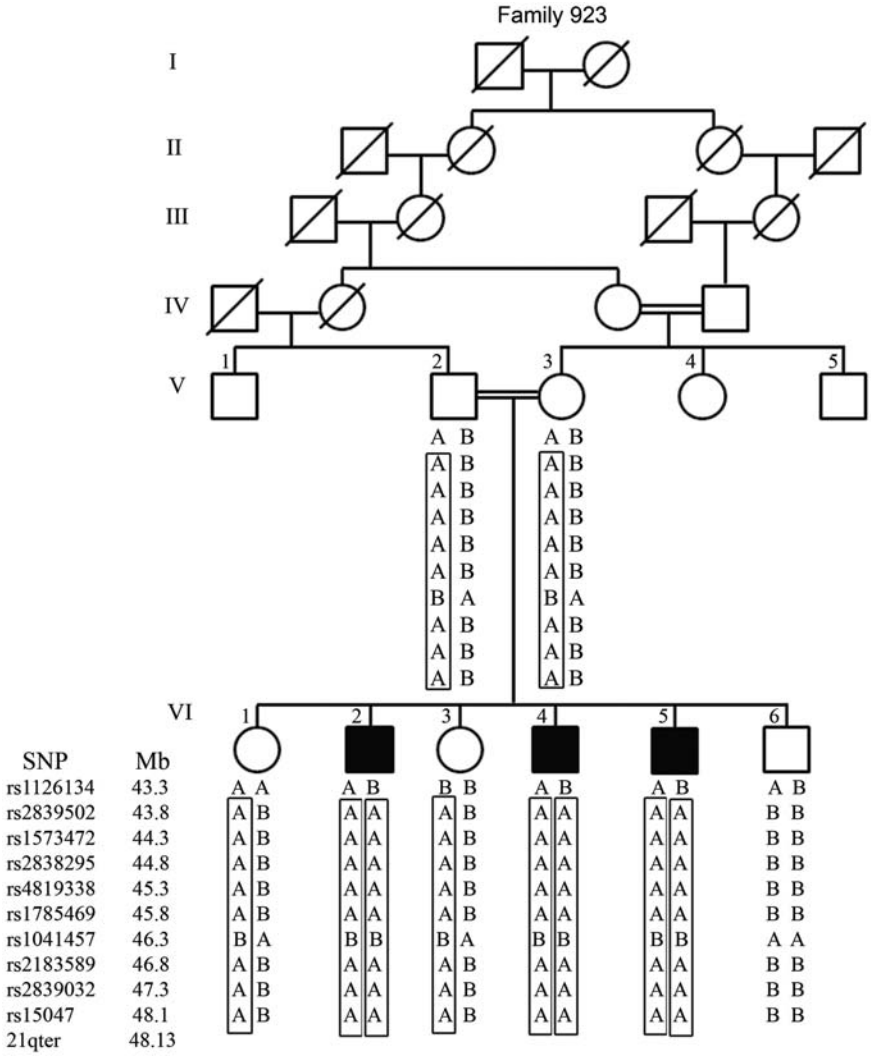

Figure 1. Segregation analysis with SNP markers at chromosome 21q22.3-qter. Deaf individuals are indicated by filled symbols, and unaffected individuals by open symbols. The haplotype associated with the DFNB98 allele is boxed. The physical distances between the SNP markers and the centromere are indicated on the left. The DFNB98 interval spans 4.8 Mb between rs1126134, on the proximal side, and rs15047, the most telomeric SNP. Mb, megabases (from human genome reference sequence build hg19).

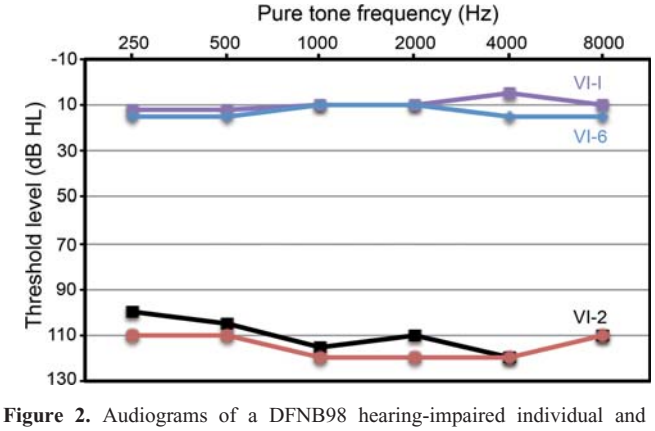

Figure 2. Audiograms of a DFNB98 hearing-impaired individual and of normal-hearing siblings. Pure-tone hearing thresholds for the right (red) and left (black) ears in patient VI-2 show bilateral, profound hearing impairment. Audiograms of the right ear of a heterozygote (VI-I) and a homozygote normal (VI-6) individual are shown in purple and blue, respectively. dB HL, decibel hearing level.

shington.edu/EVS/). Thus, they were not considered further. The two adjacent variants not previously detected were located in the *TSPEAR* gene. This gene covers 213 720 bp and contains 12 exons (Fig. 3). It encodes two alternatively spliced transcripts (Fig. 3). The larger transcript (3967 bp in length) encodes a predicted 669 amino acid protein. Both predicted Tspear isoforms contain a signal peptide and a thrombospondin-type laminin G (TSP) domain, followed by seven EAR/EPTP (Epitemptin) repeats organized in tandem. They do not contain a transmembrane domain, and are thus predicted to be extracellular proteins. They differ by the presence/absence of the TSP sequence encoded by the 3′ end of exon 3, exons 4 and 5, and by the inter TSP-EAR sequence encoded by the 5′ end of exon 6. *TSPEAR* orthologues are present in vertebrates and in the chordate *Ciona intestinalis* (see Supplementary Material, Fig. S1). The two neighbouring point mutations found in family 923 are a nucleotide substitution and a single base pair deletion, c.1726G>T+c.1728delC, in exon 10; the deletion is predicted to create a frameshift in

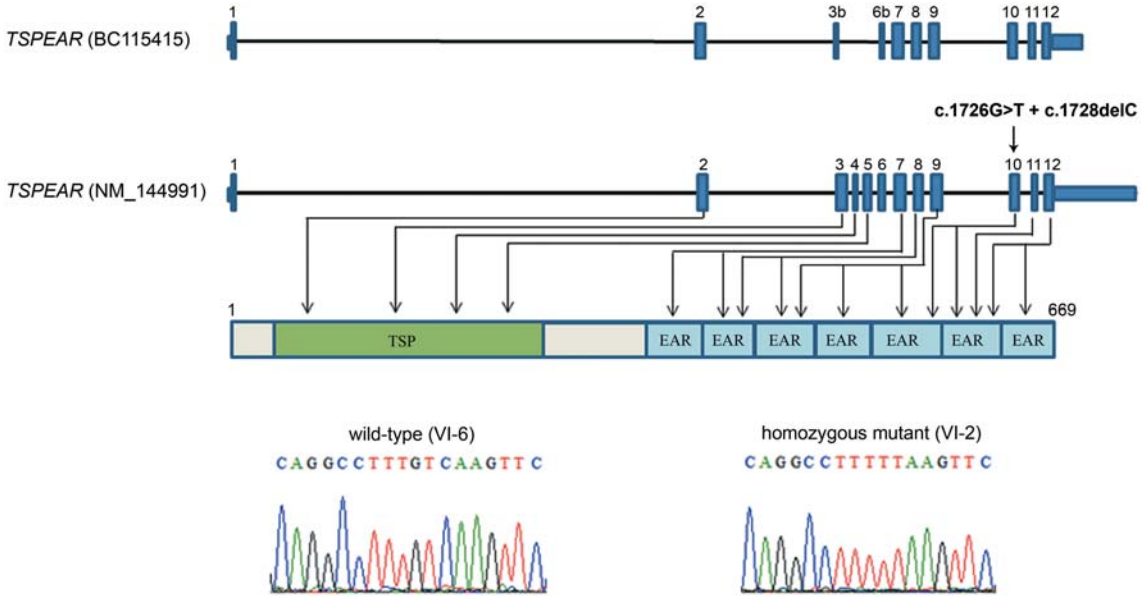

Figure 3. Structure of the *TSPEAR* gene and transcripts, and modular organization of the encoded protein. Upper panels: the two alternatively spliced *TSPEAR* transcripts are represented along with the exon/intron structure of the gene. The position of the mutation found in family 923 is shown on the long *TSPEAR* transcript. Bottom panels: DNA sequence chromatograms showing the c.1726G>T+c.1728delC mutation that is present in homozygous state in the deaf individual VI-2, and the corresponding wild-type sequence (individual VI-6). TSP, thrombospondin-type laminin G domain; EAR, epilepsy-associated repeat.

both the short and the long transcripts (p.V576L*fs*X37). Sequencing of exon 10 in all siblings of generation VI and their parents confirmed that these sequence variants were present in the homozygous state in the three deaf siblings (VI-2, VI-4, VI-5), absent from sibling VI-6 and present in the heterozygous state in the other two normally hearing siblings (VI-1, VI-3) and in both parents (V-2, V-3) (Fig. 3, and data not shown). These variants were absent from 152 unrelated normally hearing, Iranian individuals and from 200 normally hearing individuals living in France. Finally, mutations in *TSPEAR* were not detected in 55 other large Iranian families affected by deafness in which the deafness-causative genes have not yet been identified.

Tspear expression in the murine cochlea

Two murine *Tspear* transcripts have been reported (GenBank accession numbers AJ487520 and AK082868). The two predicted protein sequences, however, do not have a signal peptide. We analysed *Tspear* transcripts from the first postnatal day (P1) mouse cochlea, using rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) (see Materials and Methods). A single transcript was isolated, which differs in its 5′ end from the two previously reported transcripts by the presence of a 329 bp sequence derived from an additional exon (exon 1) that is located ∼140 kb upstream from exon 2. This sequence encodes a predicted signal peptide. The murine transcript (GenBank accession number JQ815565) includes the 12 exons of the gene, and is predicted to encode the larger Tspear isoform, 670 amino acids long, which shows 82% sequence identity with the human Tspear.

We then investigated the expression of *Tspear* in various murine tissues at different stages by reverse transcriptase (RT)–PCR, using primers from exon 8 and exon 12 sequences. In the microdissected organ of Corti, *Tspear* transcripts were detected at P1 and P7 (Fig. 4). Single-cell RT–PCR analysis of inner hair cells and outer hair cells from P7 mice, however, did not provide evidence of *Tspear* expression by these cells (0/13 'Tspear-positive' outer hair cells, and 4/19 'Tspear-positive' inner hair cells versus 0/10 positive extracellular fluid control samples; Fisher's exact test, $P = 0.27$; see Materials and Methods). *Tspear* transcripts were also detected in P7 cochlear ganglion and stria vascularis, in P7 vestibular end organs, in P1, P7 and adult inferior colliculus and the remaining brainstem, where auditory nuclei are located, and in P1, P7 and adult cerebellum, brain hemispheres and retina. At adult stage, *Tspear* transcripts were also detected in the liver, lung, kidney, intestine and testis, but not in the heart or skeletal muscles.

The c.1726G>T+c.1728delC mutation in *TSPEAR* prevents secretion of the protein

The c.1726G>T+c.1728delC mutations in *TSPEAR* are predicted to result in termination of translation in the sixth EAR repeat. This is reminiscent of the truncating mutations *Lgi1* and *Lgi2*, which prevent the secretion of the corresponding proteins (7,8). Therefore, we tested whether Tspear is a secreted protein, and whether the mutation found in family 923 impairs the secretion of this protein. V-5-tagged wild-type or mutant murine Tspear proteins were produced in transfected HEK293 cells. The culture media and cell lysates were analysed by western blotting with an anti-V5 monoclonal antibody. Both the wild-type and the mutant proteins were detected in the cell lysates, but only the wild-type form could be detected in the culture media, thus indicating

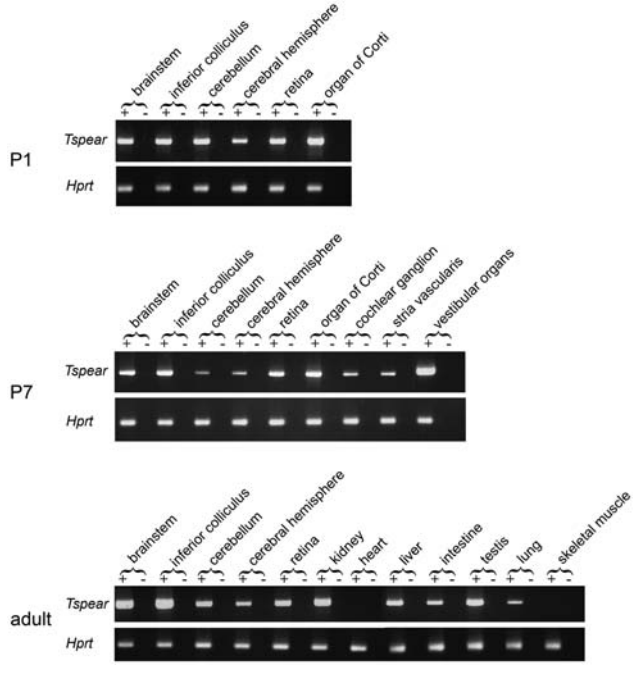

Figure 4. RT–PCR analysis of *Tspear* expression in murine tissues. RT–PCR amplification products obtained using primers located in exon 6 and exon 10 of *Tspear* are shown. A 660 bp product is observed in all tested tissues at P1, P7 and adult stage, except in the adult heart and skeletal muscle. PCR amplification of the *Hprt* ubiquitous transcript is shown as positive control. ± indicates the presence/absence of reverse transcriptase in the cDNA synthesis reaction mixture.

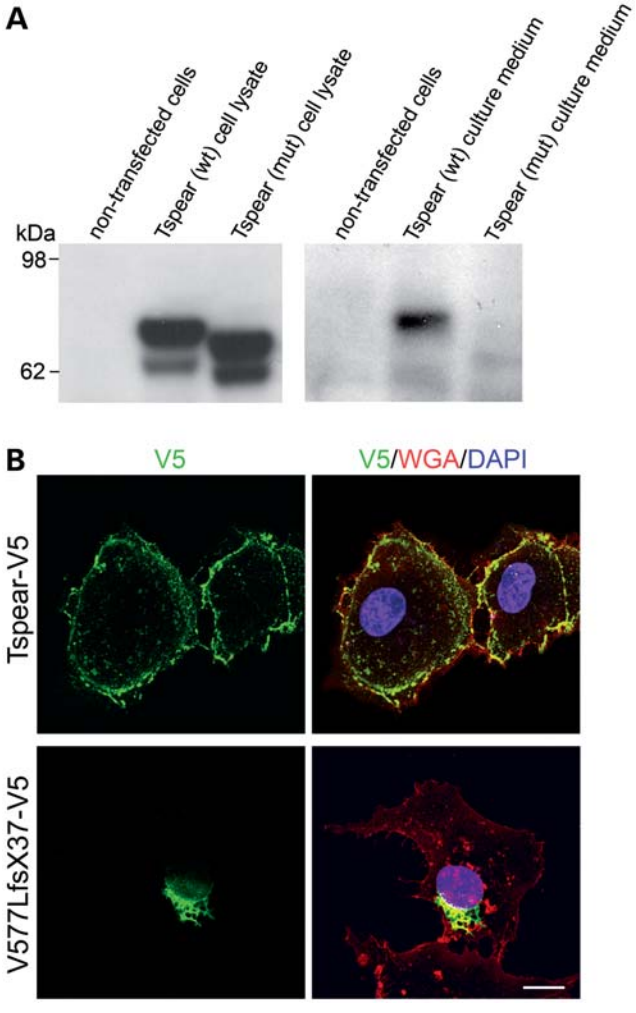

Figure 5. Detection of wild-type and mutant Tspear proteins in transfected cells. (**A**) Western blot analysis of the wild-type and mutant Tspear proteins in the cell lysate and culture medium of transfected HEK293 cells. Cell lysates and culture media from non-transfected cells and from transfected cells producing V5-tagged wild-type or mutant Tspear were analysed using an anti-V5 antibody. The wild-type (wt) and mutant (mut) Tspear proteins are detected in the cell lysates, but only the wild-type Tspear is detected in the culture medium, indicating that the p.V577L*fs*X37 truncating mutation prevents the secretion of the protein. The positions of the 62 kDa and 98 kDa molecular mass markers are indicated on the left. (**B**) Immunostaining of transfected COS-7 cells producing V5-tagged wild-type or mutant (p.V577L*fs*X37) Tspear proteins, using the anti-V5 antibody without cell membrane permeabilization. A cell surface staining is detected for the wild-type Tspear, but not for the mutant Tspear. Tetramethyl rhodamine iso-Thiocyanate-conjugated wheat germ agglutinin (WGA) was used to label the cell surface. Cell nuclei are labelled in blue (4′,6-diamidino-2-phenylindole staining). Scale bar: 10 μm.

defective secretion of the mutant protein (Fig. 5A). COS-7 cells were transfected with the same constructs and immunocytofluorescence analysis showed that the mutated Tspear was restricted to the endoplasmic reticulum next to the nucleus, indicating abnormal intracellular trafficking of this truncated form. In contrast, the wild-type Tspear was detected at the cell surface, which suggests the presence of an ubiquitous Tspear receptor/ligand in the plasma membrane (Fig. 5B).

Finally, to help identify the primary cell target(s) of the DFNB98 hearing defect, we studied the distribution of Tspear in the mouse cochlea by immunofluorescence labelling and confocal microscopy. The anti-Tspear polyclonal antibody used specifically detected Tspear in transfected cells (Fig. 6A). In the organ of Corti, Tspear labelling was restricted to the sensory hair cells (Fig. 6B), specifically at the base of the hair bundles of inner and outer hair cells. This labelling was observed in the absence of cell permeabilization, consistent with Tspear being a secreted protein.

DISCUSSION

We identified *TSPEAR* as the causative gene for a new form of prelingual, recessive deafness, DFNB98. Tspear belongs to a small family of proteins characterized by the presence of tandem EAR repeats. The mammalian family of EAR proteins consists of six members: the four members of the Lgi (leucine-rich, glioma-inactivated) subfamily (Lgi1-4) (9), Vlgr1 (Very large G protein-coupled receptor 1) and Tspear (Fig. 7). All of these proteins contain seven copies of EAR. Based on their similarity with WD-repeat domains, these repeats are predicted to form 7-fold β-sheet repeats, which together result in a β-propeller domain (10,11).

Our transfection experiments revealed that Tspear is an extracellular protein. Likewise, all the other mammalian EAR proteins are predicted to be secreted, and indeed, this has been demonstrated for most of them (9,10). All Lgi proteins are released into the extracellular medium of transfected cells (8,12–15). Vlgr1 has seven transmembrane domains and

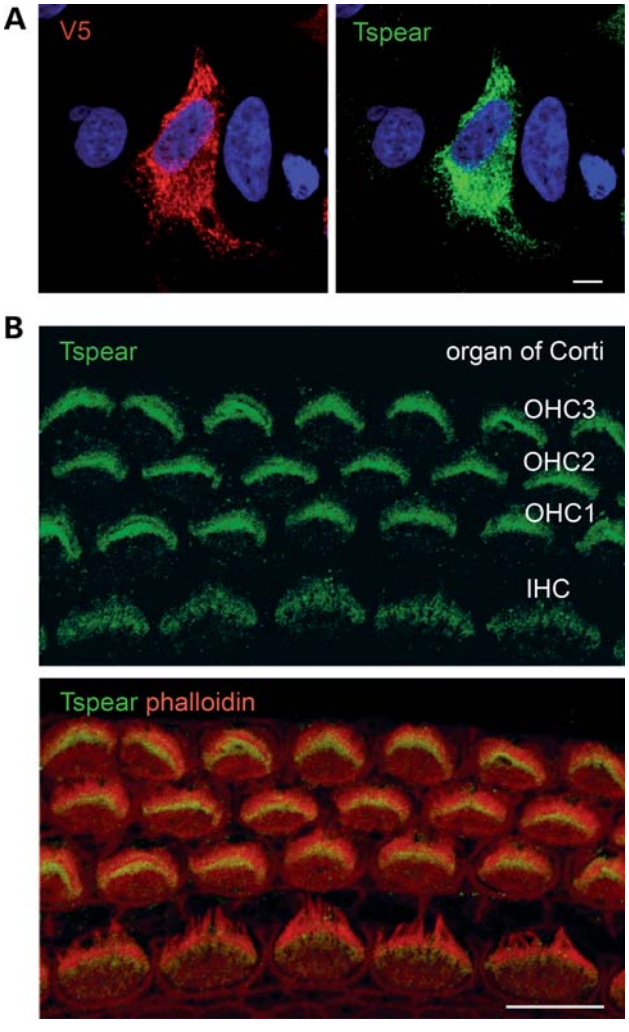

Figure 6. Immunofluorescence analysis of Tspear in the mouse cochlea. (**A**) HeLa cells were transfected with V5-tagged wild-type (Tspear-V5) murine Tspear clone. After cell membrane permeabilization, the cells were immunostained with anti-Tspear and anti-V5 antibodies. (**B**) Whole-mount immunostaining of a P1 mouse organ of Corti, using an anti-Tspear antibody (confocal microscopy). Tspear is detected at the basal region of the stereocilia of inner hair cells (IHCs) and outer hair cells (OHCs). Phalloidin was used to label F-actin. Scale bar: 5 μm.

is predicted to be a G protein-coupled receptor protein with a very large extracellular region, but several alternatively spliced transcripts of *VLGR1* indicate the existence of secreted Vlgr1 isoforms (16,17). In addition, the ectodomain of the transmembrane form, close to the first transmembrane domain, contains a G protein-coupled receptor proteolytic site.

The EAR repeat was first identified in Lgi1/Epitempin and Vlgr1, which are both defective in monogenic forms of epilepsy, hence the name. *LGI1* underlies autosomal dominant lateral temporal epilepsy or autosomal dominant partial epilepsy with auditory features, auditory hallucinations and illusions, as well as the sudden inability to understand speech in humans (7,18). *Lgi1* knock-out mice suffer from spontaneous epileptic seizures, and do not survive beyond the third post-natal week (13,19,20). *Vlgr1* underlies a form of generalized epilepsy induced by sound (audiogenic seizures, classified as reflex epilepsy) in the Frings mouse strain (21) and the BUB/BnJ inbred mouse strain. This has been confirmed by work with knockout ($Vlgr1^{-/-}$) mice (17) and Vlgr1del7TM mice that lack the exons encoding the transmembrane domains and the cytoplasmic region of Vlgr1 (16). Subsequently, a nonsense mutation in *VLGR1* was reported in a family affected by febrile and afebrile seizures (22). However, *VLGR1* was subsequently demonstrated to be one of the genes responsible for type II Usher syndrome, which associates severe, congenital hearing impairment and delayed onset retinitis pigmentosa (23). However, neither audiogenic seizures nor other forms of epilepsy have been reported in these patients nor in their heterozygous parents who carry truncating mutations in *VLGR1*. A *Vlgr1* defect was then shown to cause hearing impairment in the inbred mouse strain BUB/BnJ, Frings mice (24), and in engineered $Vlgr1^{-/-}$ mouse mutants (25,26). A protein-truncating mutation in the Lgi2 gene in the *Lagotto romagnolo* dog results in benign familial juvenile epilepsy (8). Moreover, *LGI2* is a candidate gene for a partial epilepsy characterized by pericentric discharges in humans (27). A *Lgi4* defect in the mouse results in a failure of peripheral nerve development involving hypomyelination and delayed axonal sorting (14,15). In humans, an association between *LGI4* and benign familial infantile convulsions has been reported (28).

Lgi family members are expressed in a variety of tissues. We report that *Tspear* is similarly expressed in various murine tissues, including the cochlea, vestibule, retina, various regions of the central nervous system and many non-neural tissues. We found that the cochlear outer hair cells in P7 mice do not express the gene, although these cells do not function properly in the affected patients as shown by the absence of TEOAEs. However, the Tspear protein was detected at the surface of the hair bundles of immature inner and outer hair cells. These observations suggest that the protein is produced and secreted by non-sensory cells and binds to a receptor on the hair bundle surface. Although all Lgi proteins interact with a subfamily of ADAM (a disintegrin and metalloproteinase) receptors that lack any metalloproteinase activity (8,12,14,15,29), transfection experiments in COS-7 cells suggest the existence of another, probably ubiquitous, membrane receptor for Tspear. LGI1 and LGI2 directly interact with ADAM22 and ADAM23; they cannot bind to the COS-7 cell surface unless the cells are cotransfected with the cDNA encoding ADAM22 or ADAM23 (8,12,13). This indicates that COS-7 cells do not express these ADAM receptors. Therefore, the Tspear receptor on the COS-7 cell surface may be ubiquitous. In addition, *Tspear* transcripts were detected in the cochlear ganglion, which contains the primary auditory neurons that form synapses with the inner hair cells, and in the brainstem including in the inferior colliculus, which contains the various auditory nuclei. Animals are particularly susceptible to audiogenic seizures as shown by Ivan Pavlov in his conditioning experiments (30,31). The possible expression of *Tspear* in the auditory brainstem and subcortical regions is compatible with the involvement of this gene in audiogenic seizures. Several chromosomal loci implicated in the susceptibility to audiogenic seizures have been reported in the mouse (32–34), but none of them corresponds to the location of *Tspear* on chromosome 10qC1. It is

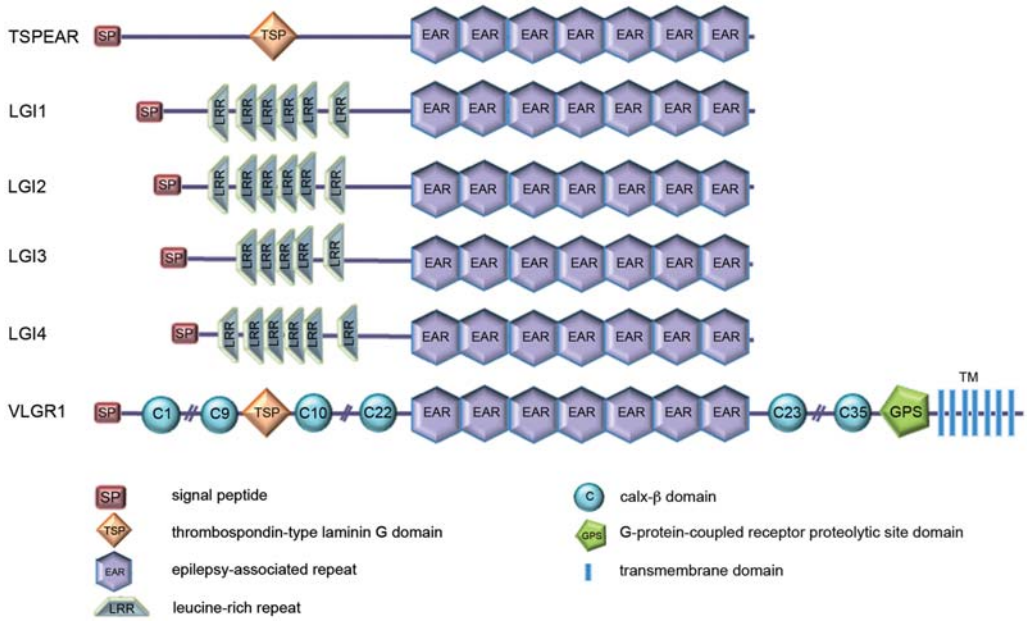

Figure 7. Family of EAR-domain-containing proteins.

not yet clear whether *Tspear* is involved in audiogenic seizures, in addition to hearing impairment, as observed for *Vlgr1* and *Gipc3* (35). This point could be addressed by studying *Tspear* conditional knockout mice carrying a deletion of *Tspear* only in central auditory nuclei.

Four of the six EAR family members in mammals—Lgi1, Lgi2, Vlgr1 and Tspear—are involved in one or several of the following abnormal phenotypes: epilepsy including occasional auditory features in humans, audiogenic seizures in the mouse and hearing impairment in humans and/or mice. All of them are present in various neural and non-neural tissues (NCBI database of the transcriptome, UniGene, URL: www.ncbi.nlm.nih.gov/unigene). As they all have indispensable roles only in the peripheral and central auditory system, it is tempting to speculate that they are involved in similar molecular pathways. Lgi1 has been shown to form a molecular complex with the AMPA receptor, PSD95, ADAM22 and stargazin, and to enhance AMPA receptor-mediated synaptic transmission (12,13). Because AMPA receptors are essential for rapid information processing in the auditory system (36), Tspear may behave in a similar way in the auditory system.

MATERIALS AND METHODS

Informed consent was obtained from all the subjects included in the study.

Audiological examination

Clinical examination was carried out on the three affected subjects (age 20 to 34 years) from family 923. Pure-tone audiometry was carried using air- or bone-transmitted sounds. Binaural mean pure-tone average of thresholds for air conduction (in dB HL) was calculated for pure-tone frequencies 0.5, 1 and 2 kHz ($PTA_{0.5,1,2\ kHz}$). Hearing impairment was additionally assessed by means of ABR and TEOAEs. Otoscopic examination and tympanometry with acoustic reflex testing were also carried out to rule out any conductive hearing impairment.

Genotyping

Genomic DNA was extracted from peripheral blood samples in all members of family 923 by standard procedures. DNA samples (500 ng) were hybridized on 700k Illumina OmniExpress-12 SNP arrays (Illumina) according to the manufacturer's protocol (http://www.illumina.com). Critical chromosomal regions were defined as segments where SNPs are identical on both alleles in all affected relatives, and discordant (heterozygosity or homozygosity for another allelic form) among unaffected relatives.

Whole-exome sequencing

Genomic DNAs from the three affected siblings and one unaffected sibling of family 923 (generation V1) (Fig. 1) were captured with their biotinylated oligonucleotide probes (Human All Exon v2—50 Mb, Agilent) using the Agilent in-solution enrichment method (SureSelect Human All Exon Kits Version 3, Agilent), followed by paired-end massively parallel sequencing on Illumina HiSEQ 2000. Sequence capture, enrichment and elution were performed according to the manufacturer's instruction and protocols (SureSelect, Agilent). Briefly, 3 μg of each genomic DNA was fragmented by sonication and purified to yield fragments of 150–200 bp. Paired-end adaptor oligonucleotides from Illumina were ligated on repaired, A-tailed fragments, then purified and enriched by six PCR cycles. Five hundred nanograms of these purified libraries were then hybridized to the SureSelect oligo probe capture library for 24 h. After hybridization,

washing and elution, the eluted fraction was PCR-amplified with 10 to 12 PCR cycles, purified and quantified by QPCR to obtain enough DNA template for downstream applications. Each eluted-enriched DNA sample was then sequenced on an Illumina HiSEQ 2000 as 75 bp, paired-end reads. Image analysis and base calling were performed using Illumina Real Time Analysis Pipeline version 1.14 with default parameters. The exome design covers 51 Mb of human genome corresponding to the exons and flanking intronic regions of 20 766 genes (~220 000 exons) and also 700 miRNAs in the human reference sequence UCSC (hg19/GRCh37, February 2009 release).

Sanger sequencing

To exclude *TMPRSS3* as a causative gene, PCR primers were designed to amplify and sequence all exons and flanking intronic regions of the gene using Sanger's technique (primer sequences available on request). To confirm the mutation in *TSPEAR* exon 10 and study its segregation in family 923, we used the following primer set: TSPEAR-F, 5′- CTG TGCCAAGATCCCACGTCCT-3′, and TSPEAR-R, 5′-CCA CAGGAAGGTCCCCAGGCCA-3′. PCR products were sequenced on an ABI 3130 capillary sequencer (Applied Biosystems), and sequence traces were analysed with Lasergene 8 software (DNASTAR).

RT–PCR analysis of *Tspear* expression

Total RNAs from the brainstem, inferior colliculus, cerebellum, brain hemispheres, retina, organ of Corti of P1 and P7 mice, and from the kidney, lung, testis, skeletal muscle and heart of adult mice were prepared using NucleoSpin® RNA II (Macherey-Nagel). The cDNAs were then synthesized from 1 μg of total RNA by RT using the SuperScript II Reverse Transcriptase kit (Invitrogen). The murine *Tspear* transcripts were PCR amplified using forward primer 5′-TGGTAGCCAACCACCGAGAAGGGG-3′ and reverse primer 5′-GCTCCTTGGCGCTGGAGTAGATGAG-3′, located in exons 6 and 10, respectively. PCR amplification of the Hprt ubiquitous transcript using forward primer 5′-GCTG GTGAAAAGGACCTCT-3′ and reverse primer 5′-CACAGG ACTAGAACACCTGC-3′ was used as a positive control. RNA samples without reverse transcriptase in the RT reaction mixture were used as negative controls.

Single-cell RT–PCR

Outer and inner hair cells were collected from excised organs of Corti of P7 mice. Single-cell RT–PCR was carried out as previously described (26). For each cell, we used surrounding extracellular fluid as a negative control. A multiplex, nested PCR was carried out using specific primer sets for the *Tspear* transcripts (*Tspear* multiplex forward 5′-CAA TGGCACTTCCACCC-3′ and reverse 5′-TGGGGATGTC CTGGAAC-3′; *Tspear* nested forward 5′-CACCTGTAC ATCTGGCTGG-3′ and reverse 5′-GGTGATGTTCAGCT CGTAGATA-3′) and for the transcripts of two control genes, specifically, *Myo7A* (encoding myosin VIIa), which is expressed in outer and inner hair cells, and *Slc26a5* (encoding prestin), which is expressed only in outer hair cells, as reported previously (26). The multiplex PCR consisted of 23 amplification cycles (40 s at 95°C, 45 s at 58°C and 50 s at 72°C). Amplified cDNA fragments ranged from 300 to 500 bp. The nested PCR was then carried out in a 25 μl mix containing 2 μl of the multiplex PCR mix and only one set of inner primers at a time, through 40 amplification cycles (40 s at 95°C, 45 s at 58°C and 50 s at 72°C).

Twenty outer hair cells and 20 inner hair cells were collected. Only hair cells that had the correct *Myo7A* and *Slc26a5* expression profiles were taken into account for analysis (13 outer hair cells and 19 inner hair cells). The number of 'Tspear-positive' cells was then counted and compared statistically to the number of extracellular fluid control samples ($n = 10$) that abnormally scored positive for the presence of the *Tspear* transcript, using the Fisher's exact test to check for significance ($P < 0.05$).

Cloning of a full-length *Tspear* cDNA

A murine *Tspear* cDNA was obtained by RACE-PCR performed on a double-strand cDNA library prepared from organs of Corti of postnatal day 1 (P1) C57/BL/6 mice, using Smart™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced.

Immunofluorescence analysis of the cochlea

For whole-mount immunolabelling, cochleas were dissected from temporal bones and fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. After three rinses in PBS (10 min each), cochlear sensory areas were microdissected and fixed again by immersion in 4% paraformaldehyde in PBS for 30 min at room temperature. The tissues were rinsed twice in PBS, and blocked in PBS containing 20% normal goat serum for 1 h at room temperature. The tissues were then incubated in PBS containing 1% bovine serum albumin and a rabbit anti-Tspear polyclonal antibody (AP9275c, Abgent) directed against a synthetic peptide (299–329) from the centre region of human Tspear (Q8WU66), overnight at 4°C. After three washes in PBS, the tissues were incubated with secondary antibodies (Alexa-Fluor-488-conjugated goat anti-rabbit $F(ab')_2$ IgG fragment, Molecular probes) diluted in PBS containing 1% bovine serum albumin for 1 h at room temperature. After three washes in PBS, the organs of Corti were mounted in Fluorsave (Callbiochem) and analysed on a laser scanning confocal microscope (LSM-700, Zeiss). Tetramethyl rhodamine iso-thiocyanate (TRITC)-conjugated phalloidin (Sigma) was used to label F-actin.

Cell culture, transfection and western blot analysis

A murine *Tspear* full-length cDNA (JQ815565) from the organ of Corti was cloned in the pcDNA3.1D/V5-His vector (Invitrogen) in frame with the C-terminal V5-tag. The mutant *Tspear* clone including the frame-shifting mutation (p.V577L*fs*X37) corresponding to that found in family 923

was prepared from the wild-type clone using QuikChange™ Site-Directed Mutagenesis kit (Stratagene). The recombinant construct was checked by sequencing. HEK293 cells were grown in the Dulbecco's modified Eagle's medium-GlutaMAX medium (Gibco) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 g/ml streptomycin and transiently transfected using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's instructions. Thirty-six hours after transfection, the medium was replaced by a serum-free medium (Optimem I- GlutaMAX, Gibco), and cells were incubated for an additional 12 h. The medium was then collected, supplemented with a protein inhibitor cocktail (Roche) and concentrated 30-fold using an Amicon Ultra-4 device (MWCO: 10 000, Millipore). HEK293 cells were lysed in 1 ml lysis buffer containing 10 mM Tris–HCl, pH 8, 150 mM NaCl, 1% NP-40, 10% glycerol, 1% Triton X-100 and protease inhibitors, for 1 h at 4°C. The cell lysate was clarified by centrifugation at 13 000 rpm at 4°C for 10 min. The cell lysates and culture media were analysed by western blot using the anti-V5 antibody (Invitrogen). Proteins were visualized using the enhanced chemiluminescence kit (Super-Signal West Pico Chemiluminescent Substrate, Thermo Scientifique).

Immunocytofluorescence analysis

HeLa and COS-7 cells were transiently transfected with the wild-type *Tspear*-V5 or mutant *Tspear*-V5 cDNA using Lipofectamine2000 (Invitrogen), and grown for 48 h before immunocytofluorescence analysis. Cell samples were fixed using 4% paraformaldehyde in PBS, and immunostained using either an anti-Tspear antibody (AP9275c, Abgent) or an anti-V5 antibody (Invitrogen). For membrane permeabilization condition, cells were incubated with 0.1% Triton X-100 for 10 min before incubation with the primary antibody. Alexa-Fluor-488-conjugated goat anti-rabbit $F(ab')_2$ IgG fragment, Alexa-Fluor-488-conjugated goat anti-mouse $F(ab')_2$ (Molecular probes) and Cy3-conjugated goat anti-mouse IgG (Amersham) were used as secondary antibodies. TRITC-conjugated wheat germ agglutinin (Sigma) and 4′,6-diamidino-2-phenylindole (Sigma) were used to label cell surface and cell nucleus, respectively.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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