Insertion of β -satellite repeats identifies a transmembrane protease causing both congenital and childhood onset autosomal recessive deafness

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Approximately 50% of childhood deafness is caused by mutations in specific genes. Autosomal recessive loci account for approximately 80% of nonsyndromic genetic deafness¹. Here we report the identification of a new transmembrane serine protease (TMPRSS3; also known as ECHOS1) expressed in many tissues, including fetal cochlea, which is mutated in the families used to describe both the DFNB10 and DFNB8 loci. An 8-bp deletion and insertion of 18 monomeric (~68-bp) β -satellite repeat units, normally present in tandem arrays of up to several hundred kilobases on the short arms of acrocentric chromosomes, causes congenital deafness (DFNB10). A mutation in a splice-

acceptor site, resulting in a 4-bp insertion in the mRNA and a frameshift, was detected in childhood onset deafness (DFNB8). This is the first description of β -satellite insertion into an active gene resulting in a pathogenic state, and the first description of a protease involved in hearing loss.

Two families with autosomal recessive sensorineural nonsyndromic deafness were previously independently reported with linkage to distal chromosome 21q. A locus in a Pakistani family was mapped telomeric to *D21S1225* in 21q22.3 (DFNB8; refs. 2,3), whereas the locus in a Palestinian family⁴ mapped to an approximately 1-Mb region between markers 1016E7.CA60 and 1151C12.GT45 (DFNB10; ref. 5). We previously reported the exclusion of nine genes in the DFNB10 critical region⁵⁻⁸ and the exclusion of three candidate genes (telomeric to the DFNB10 critical region) in the DFNB8 critical region³.

Further analysis of the DFNB10 critical region⁹ indicated the presence of four additional genes (*ZNF295*, *UMODL1*, *TMPRSS3* and *TSGA2*; Fig. 1a), including a new gene, *TMPRSS3* (for transmembrane protease, serine 3), with 13 exons spanning 24 kb (Fig. 1b; for exon-intron junctions, see Table A, http://genetics.nature.com/supplementary_info/). We detected four alternative transcripts, *TMPRSS3a* (2,438 bp), *TMPRSS3b* (2,524

Fig. 1 The DFNB10 critical region and *TMPRSS3*. **a**, A map of the DFNB10 critical region in chromosome 21q22.3. The positions of the microsatellite markers defining the DFNB10 and DFNB8 critical region in 21q22.3 are shown. The BAC contig between markers 1016E7.CA60 and 1151C12.GT45 is shown, with six known genes (*ABCG1*, *TFE3*, *TFE2*, *TFE1*, *PDE9A*, *NDUVF3*) and seven novel genes (*ZNF295*, *UMODL1*, *TMPRSS3*, *UBASH3A*, *TSGA2*, *SLC37A1*, *WDR4*) mapping to the critical region. **b**, *TMPRSS3* contains 13 exons (boxes) spanning 24 kb. **c**, There are four different transcripts, *TMPRSS3a-d* (coding regions in boxes, noncoding regions indicated by narrow box). **d**, A schematic of the *TMPRSS3* protein showing the transmembrane (TM), LDLRA, SRCR and protease domains and their position in the 454-aa peptide. **e**, Expression analysis of the *TMPRSS3* transcripts. RT-PCR specific to the *TMPRSS3a-d* transcripts were performed on cDNA from 27 human tissues and fetal cochlea as indicated.

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bp), *TMPRSS3c* (2,105 bp) and *TMPRSS3d* (1,359 bp), encoding putative polypeptides of 454, 327, 327 (same as *TMPRSS3b*) and 344 amino acids, respectively. The *TMPRSS3a* transcript contains all 13 exons with the initiating methionine codon in exon 2. The *TMPRSS3b* and *TMPRSS3c* transcripts start in introns 2 and 3, respectively, with putative initiating methionine codons in exon 5. The 3' end of *TMPRSS3d* is derived from 5 ESTs (for example AI978874) and continues into intron 9 (Fig. 1c).

Northern-blot analysis showed weak expression of the 2.4- and 1.4-kb *TMPRSS3a* and *TMPRSS3d* transcripts (data not shown). Semi-quantitative RT-PCR revealed that all four transcripts show distinct patterns of expression, but *TMPRSS3a* is the most abundantly and widely expressed transcript, including in fetal cochlea (Fig. 1e).

The putative peptide encoded by *TMPRSS3a* contains transmembrane (TM), low-density-lipoprotein receptor A (LDLRA), scavenger-receptor cysteine-rich (SRCR) and serine protease domains (Fig. 1d) similar to other proteases, including that encoded by the homologous gene *TMPRSS2*, which also maps on

Table 1 • Nucleotide changes detected during mutation analyses in *TMPRSS3*

	Change	Exon/intron	Amino acid	Sample	
1	157G→A	exon 3	V53I	DFNB8	polymorphic
2	IVS4-6 G→A	intron 4		DFNB8	pathogenic
3	453G→A	exon 6	V151V	cDNA 1:1	polymorphic
4	Ins(β-sat)+ del	exon 11		DFNB10	pathogenic
5	1367G→A	exon 13*		DFNB10	polymorphic
6	1571G→A	exon 13		DFNB8/cDNA 1:6	polymorphic
7	1942insG	exon 13		cDNA 2:5	polymorphic

The nucleotide changes follow the nomenclature rules of Dunnen and Antonakis²⁸. *Exon 13 variants are all in the 3'-untranslated region

21q, centromeric to the *DFNB10* critical region¹⁰. The serine protease domain (residues 217–444) is compatible with the S1 family of the PA clan of serine-type peptidases (serine or cysteine nucleophile; catalytic residues in the order His, Asp, Ser (or Cys) in sequence; all endopeptidases) for which the prototype is chymotrypsin¹¹, and shows between 45 and 38% identity with other transmembrane serine proteases (Fig. 1f).

As no recognizable leader sequence precedes the predicted hydrophobic TM domain, *TMPRSS3* is likely to be a type II integral membrane protein with a cytosolic amino terminus and an extracellular protease domain, similar to other transmembrane proteases¹². The LDLRA domain, which contains six disulfide-bound cysteines (C72, C79, C85, C92, C98 and C107), was originally found in the low density lipoprotein receptor as the binding site for low-density lipoprotein and calcium¹³, and has subsequently been described in numerous extracellular and membrane proteins. SRCR domains linked to serine protease domains have been reported in secreted or membrane-bound molecules with diverse biological roles in development and immunity¹⁴. The LDLRA and SRCR domains of *TMPRSS3* may be involved in binding with extracellular molecules or the cell surface.

The putative peptides encoded by the *TMPRSS3b* and *TMPRSS3c* transcripts contain only half of the SRCR domain, whereas the

Fig. 2 Identification of a β -satellite insertion in exon 11 of *TMPRSS3*. **a**, The Palestinian DFNB10 pedigree is shown with homozygous affected and heterozygous individuals marked by filled and half-filled symbols, respectively. **b**, PCR amplification of exon 11 of *TMPRSS3* from selected individuals (labeled in **a** and indicated by larger symbols) shows inheritance of the 1702-bp band with deafness. N, hearing control; -ve, no DNA control; M, marker. The position of the normal 476-bp, mutant 1702 and heteroduplex (HD) bands seen in heterozygotes are indicated (arrow pointing to the left). **c**, A chromatogram from a normal individual is shown aligned with chromatograms showing the 5' (DFNB10 5') and 3' (DFNB10 3') sequences of the insertion. β -satellite sequences specific to the chromatograms from affected individuals are underlined in red and the 8 nt deleted at the point of insertion are boxed in red in the normal chromatogram. This would result in a frameshift mutation from G393 within the protease domain of *TMPRSS3* and termination at 404 amino acids after the addition of 11 unrelated amino acids.

Fig. 3 Homology of the β -satellite monomers and FISH analysis. **a**, An alignment of the 18 β -satellite monomers inserted into exon 11 of *TPMRSS3* shows the high homology of the monomeric subunits. **b**, Two-color FISH analysis of chromosomes 21 from homozygous affected individuals from the DFNB10 family was performed using a BAC containing *TPMRSS3* (KB169B4, red) and a β -satellite probe generated by PCR from the insertion (green). The overlapping green and red probes produce yellow. **c**, Homology between two sections of exon 11 of *TPMRSS3* (shaded gray) and the β -satellite repeats results in the recombination and subsequent insertion of 18 β -satellite repeats at the position marked by an arrow. **d**, The homology of the second block, 74 nucleotides of exon 11 of *TPMRSS3* just before the point of insertion, with a sequence-spanning repeats 10 and 11 of the insertion.

putative product of *TPMRSS3d* contains only half of the protease domain (Fig. 1c). The *TPMRSS3b* and *TPMRSS3c* transcripts may be artifacts of RACE experiments or may produce soluble forms of the protease as has been observed for hepsin¹⁵.

Direct sequencing of all 13 exons of *TPMRSS3* (PCR amplified from DNA samples from the Palestinian DFNB10 and Pakistani DFNB8 families^{2,4,5} and controls) detected two sequence variants in the Palestinian/DFNB10 family (changes 4 and 5; Table 1). Amplification of exon 11 of *TPMRSS3* from a DFNB10 sample resulted in a 1.7-kb product instead of the 476-bp product that was amplified from normal controls. Southern-blot and PCR-assay analyses of exon 11 of *TPMRSS3* showed that the size of the insertion does not vary between family members or generations (data not shown), unlike fragile sites and repeat-expansion disorders¹⁶. Eight affected individuals were homozygous for the 1.7-kb product and 13 obligate carriers had one copy. The insertion segregated with the phenotype (Fig. 2a,b). Sequence analysis revealed a complex rearrangement with the deletion of 8 bp and the insertion of 18 complete β -satellite repeat monomers with 10 and 2 bp derived from β -satellite repeats at the 5' and 3' ends of the rearrangement, respectively (Fig. 2c). The sequences of the 18 β -satellite monomers, although highly conserved, are also highly variable (52–93% divergence between repeats; Fig. 3a). Monomeric, approximately 68-bp repetitive units of β -satellites (or *Sau3A* repeats) have been detected on the short arms of all human acrocentric chromosomes and chromosomes 1, 9 (centromeric), 19p and Y (refs. 17–19). At least four families of β -satellites have been defined¹⁹ and the β -satellite insertion into *TPMRSS3* is likely to be derived from either p21β2 or p21β7 repeats.

Using two-color FISH, we detected *TPMRSS3* signal only on chromosome 21q22.3, and β -satellites at the described localizations in normal chromosomes (data not shown). Colocalization was observed at 21q22.3 on chromosomes 21 derived from individuals heterozygous and homozygous for the insertion (Fig. 3b).

The mobile nature of repetitive sequences on the short arms of acrocentric chromosomes is well documented²⁰. Circular

extrachromosomal molecules present in many eukaryotic cells, small polydisperse circular DNAs (spcDNA), may contain β -satellites²¹ or other repeats present on the short arms of human acrocentric chromosomes (for review, see ref. 22), and are probably produced by unequal homologous recombination between or within repetitive sequences. The insertion into *TPMRSS3* in the DFNB10 family may have arisen by recombination of spcDNA containing β -satellites with a region of minimal homology spanning exon 11 of *TPMRSS3* (Fig. 3c,d). Although chromosomal rearrangements involving β -satellites have been described^{23,24}, this is the first description of β -satellite insertion into an active gene resulting in a pathogenic state.

Three *TPMRSS3* sequence variants were observed in homozygosity in the Pakistani/DFNB8 family² (changes 1, 2 and 6; Table 1). A G→A substitution at IVS4-6, whose inheritance was consistent with deafness in branches 3 and 4 of the DFNB8 family, creates an alternative splice-acceptor site (Fig. 4a,b) and was observed in 1 of 160 Muslim Indians and none of 30 European chromosomes. Haplotype analysis on the heterozygous, hearing individual with IVS4-6G→A, and his/her spouse and child, with 4 intragenic *TPMRSS3* SNPs (changes 1, 3, 4 and 6; Table 1) was consistent with a single mutational origin of IVS4-6G→A.

We next assessed the effect of IVS4-6G→A on splicing *in vitro* (Fig. 4b). RT-PCR and sequencing revealed a 4-bp insertion between exons 4 and 5 (Fig. 4c,d), consistent with the use of the putative splice acceptor site created by IVS4-6G→A. We screened 179 cloned RT-PCR products from an affected individual with an oligonucleotide that mimics the normal splice junction and one that includes the insertion. We found no hybridization with the 'normal' probe. Thus IVS4-6G→A can be considered to be a pathogenic mutation. Mutations in splice-acceptor sites that allow some normal splicing often result in

Fig. 4 Identification of a splice-site mutation in *TMPRSS3* in the Pakistani DFNB8 family. **a**, Chromatograms of normal and affected individuals from the DFNB8 family showing the G→A substitution at IVS4-6 underlined in red. The normal intron 4 splice-acceptor site (black arrow) and the putative acceptor site created by IVS4-6G→A (red arrow) are indicated. **b**, A genomic fragment containing exons 4 and 5 of *TMPRSS3* was inserted into an exon trapping (splicing) vector. **c,d**, RT-PCR from RNA of transfected COS7 cells identified a 4-bp insertion (underlined in **d**) between exons 4 and 5 (**b–d**) consistent with the use of the putative splice acceptor site created by IVS4-6G→A. The 4-bp insertion would result in a frameshift from C107, and termination at 132 aa after the addition of 25 unrelated amino acids.

milder phenotypes compared with null mutations in the same gene²⁵. *In vivo*, the IVS4-6G→A mutation in the DFNB8 family may allow limited normal splicing, and thus some normal *TMPRSS3* protein, accounting for the phenotypic difference between the DFNB8 and DFNB10 families.

Many human disorders such as hereditary pancreatitis (PRSS1, MIM 167800) and coagulation factor deficiencies (factors VII, IX, X and XII) are due to deficiency of serine proteases. This, however, is the first description of a protease involved in hearing loss. The phenotypic differences between the two families studied here may imply that *TMPRSS3* polymorphisms resulting in or associated with reduced expression or slightly abnormal function might be involved in age-related hearing loss, the most common form of hearing loss. The sensorineural hearing loss in both families with *TMPRSS3* mutations implies defects in the inner ear. *TMPRSS3* expression was demonstrated in fetal cochlea and many other tissues. *TMPRSS3* may be involved in the development and maintenance of the inner ear or the contents of the perilymph and endolymph. The levels of total protein in endolymph are extremely low, but endolymph proteins show a prominence of glycosylated acidic proteins and turnover of these proteins may require specific proteases²⁶.

Methods

Sample collection. The DFNB10 family (BT117) is a large, inbred, Palestinian family from a small town in Israel⁴. Pure-tone audiometric tests showed profound hearing loss in all affected individuals, without any hearing remnants, at a level of 75–80 decibels. Sensorineural deafness was confirmed in one-week-old twin girls by a brain stem-evoked potential (BERA) test. Blood samples were obtained from 36 hearing and 16 deaf individuals after obtaining informed consent and in accordance with institutional guidelines for human subjects.

The DFNB8 family is a large, consanguineous kindred from Pakistan². Pure-tone audiometric tests, between 125 and 8,000 Hz up to 120 dB, revealed a maximum audio threshold in both ears of affected individuals of 105 dB at 1,000 Hz. Onset of hearing-related problems was between 10 and 12 y of age

with profound hearing loss evident at 14 to 16 y. We obtained blood samples from 16 hearing and 8 deaf individuals after obtaining informed consent and in accordance with institutional guidelines for human subjects.

Bacterial clone contigs, genomic sequencing and isolation of *TMPRSS3* cDNAs. A BAC/cosmid contig spanning MX1-D21S171 was constructed (see <http://www.dmb.med.keio.ac.jp/seqpub/map/APECED.html>). Genomic sequencing and analysis was as described⁹, and primers were designed to predicted exons (Table B, see http://genetics.nature.com/supplementary_info/). We amplified *TMPRSS3* cDNA fragments with primer pairs TM1-F/TM1-R and TM2-F/TM2-R. We carried out 5'- and 3'-RACE with primer pairs TMRA-R1/AP1, TMRA-R2/AP2, TMRA-F1/AP1 and TMRA-F2/AP2. RT-PCR was carried out on thymus, spleen, lymph node, liver and placenta cDNA pools (MTC cDNA and Marathon-Ready cDNA, Clontech) using 35 cycles of 94 °C for 30 s, 65 °C for 1 min, and 72 °C for 2 min. PCR products were cloned into pBluescript II(SK+) (Stratagene) and sequenced. The sequence of predicted proteins of *TMPRSS3* were analyzed using web-based tools including TMPRED (transmembrane prediction; http://ulrec3.unil.ch/software/TMPRED_form.html), and domain searches using the Pfam databases (<http://www.sanger.ac.uk/Software/Pfam/search.shtml>).

Multiple-tissue cDNA panel and northern-blot analysis. Expression analysis of *TMPRSS3* was performed using the Human Multiple Tissue cDNA (MTC) panels (Clontech; I, II, fetal, and immune system panels) containing cDNAs from 27 human tissues and a cDNA prepared from fetal cochlea (18 weeks) mRNA. *TMPRSS3* cDNAs were amplified with primer pairs TMa-F/TMabc-R (*TMPRSS3a*), TMb-F/TMabc-R (*TMPRSS3b*), TMc-F/TMabc-R (*TMPRSS3c*) and TM1-F/TMd-R (*TMPRSS3d*) using 20 pg of each cDNA as template, whereas the control PCR (a G3PDH amplimer) was amplified from 2 pg of cDNA. Northern blots containing 2 µg poly(A)⁺ RNA from 23 different adult human tissues (Clontech Human MTN Blots 1-3) were hybridized with a ³²P-labeled probe (nt 1–517 of *TMPRSS3a*).

Mutation analyses. We determined the genomic structure of *TMPRSS3* by comparing the genomic sequence with the cDNA sequences. Each *TMPRSS3* exon and each flanking regions were amplified from patient and unrelated control DNAs (for primers see Table C, http://genetics.nature.com/supplementary_info/) and analysed as described⁶. PCR products

containing exons 4 and 5 were amplified from a DFN8 and a normal sample and the fragments were cloned into the exon-trapping vector pSPL3 (Gibco-BRL). Transfections, RNA isolation and RT-PCR were performed as described²⁷ between oligonucleotides 7614-TMP4eF (5'-GCTCATCTTTAAGTGTATCG-3') and 7615-TMP5eR (5'-ATGGTCTTCCACGAAGCAGCTG-3'). Products were cloned into pTOPO (Invitrogen) and the 4-nt insertion was assayed by colony hybridization with oligonucleotides 7737-E4E5con (5'-AGTACCGCTGTGTCGGGT-3', normal) and 7738-E4E5deaf (5'-CCGCTGTGCCAGTCCGGGT-3', mutant 4-nt underlined).

FISH analyses. BAC KB169B4 (containing *TMPRSS3*) and the mutant exon 11 PCR product containing 18 β -satellite repeats were labeled using the Biotin and DIG-nick translation mixes (Roche), respectively, and detected with Alexa Fluor 568 conjugate of Streptavidin (Molecular Probes) and sheep-anti-digoxigenin-fluorescein antibodies (Roche), respectively. Metaphase spreads were prepared using cultured lymphoblastic cells (from normal, heterozygote and affected individuals) and hybridized with a mixture of both probes using ChromaHyb 600 hybridization buffer (Quantum Biotechnologies).

GenBank accession numbers. Genomic sequence of BAC KB169B4, AP001623; human *TMPRSS3a*, *TMPRSS3b*, *TMPRSS3c* and *TMPRSS3d* cDNAs, AB038157, AB038158, AB038159 and AB038160, respectively.

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