Mutations in the human α-tectorin gene cause autosomal dominant non-syndromic hearing impairment

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The tectorial membrane is an extracellular matrix of the inner ear that contacts the stereocilia bundles of specialized sensory hair cells. Sound induces movement of these hair cells relative to the tectorial membrane, deflects the stereocilia, and leads to fluctuations in hair-cell membrane potential, transducing sound into electrical signals. α-tectorin is one of the major non-collagenous components of the tectorial membrane[1,2]. Recently, the gene encoding mouse α-tectorin (*Tecta*) was mapped to a region of mouse chromosome 9, which shows evolutionary conservation with human chromosome 11q (ref. 3), where linkage was found in two families, one Belgian (*DFNA12*; ref. 4) and the other, Austrian (*DFNA8;* unpublished data), with autosomal dominant non-syndromic hearing impairment. We determined the complete sequence and the intron-exon structure of the human *TECTA* gene. In both families, mutation analysis revealed missense mutations which replace conserved amino-acid residues within the zona pellucida domain of *TECTA*. These findings indicate that mutations in *TECTA* are responsible for hearing impairment in these families, and implicate a new type of protein in the pathogenesis of hearing impairment.

As mouse *Tecta* is only expressed in the inner ear[2], and human *TECTA* maps to the genetic linkage interval for both *DFNA8* and *DFNA12* (ref. 3), we considered *TECTA* a candidate gene for the non-syndromic hearing impairment in both families[3]. We therefore determined the sequence and the intron-exon structure of *TECTA* by genomic sequencing. Human genomic *TECTA* sequence was aligned with the mouse *Tecta* cDNA sequence. In regions where the homology between the mouse cDNA and the human genomic sequence diverged, the presence of splice-site consensus sequences were evaluated, and consensus values (CV) were calculated[5]. We found a total of 23 exons, ranging in size from 65 to 602 bp.

The composite DNA sequence, comprising all exons of *TECTA*, defines a single open reading frame of 6465 bp, displaying 88% identity to mouse *Tecta*. *TECTA* encodes a protein of 2155 amino acids, with 95% identity to mouse α-tectorin (Fig. 1). Nearly all structural features of TECTA are conserved between man and mouse (Fig. 1). Alpha-tectorin has an amino-terminal hydrophobic signal sequence for translocation across

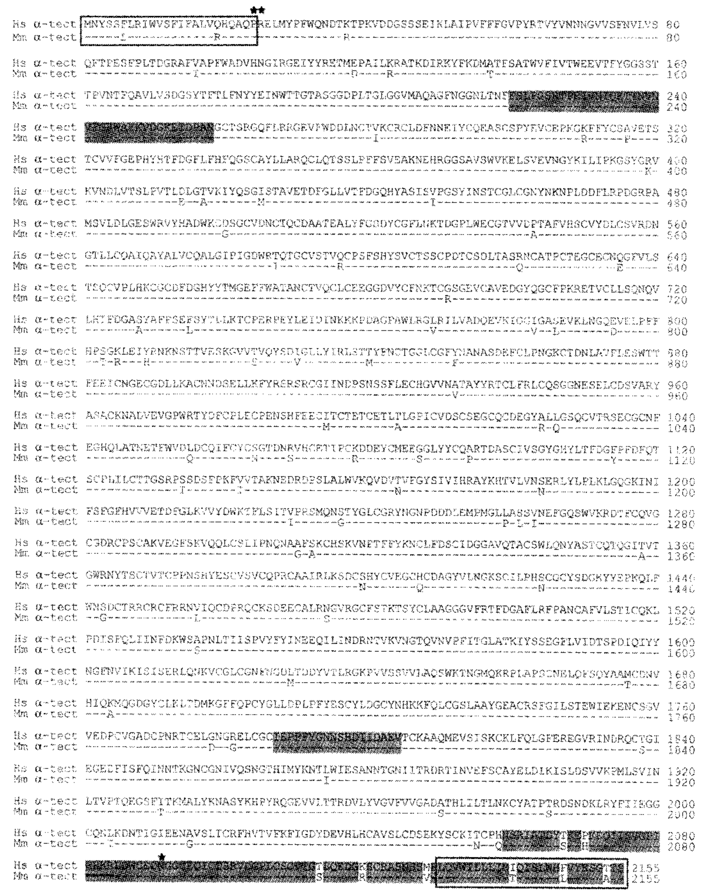

Fig. 1 Amino-acid sequence alignment of human (Hs) and mouse (Mm) α-tectorin. Identical amino-acid sequences are indicated with (-). Positions showing no homology to any known protein sequence are darkly shaded. The first 219 amino acids show homology with the G1 domain of entactin. Thirty-nine amino acids separate this domain from a 1528–amino-acid domain (amino acids 259–1786) which is homologous with zonadhesin. The third conserved region (amino acids 1805 to 2057) is the zona pellucida domain. The NH2- and COOH-terminal hydrophobic sequences are boxed. Two stars (★★) indicate the cleavage site of the signal peptide. One star (★) indicates the most likely acceptor of the glycosylphosphatidylinositol anchor. The tetrabasic putative endoproteinase cleavage site is underlined.

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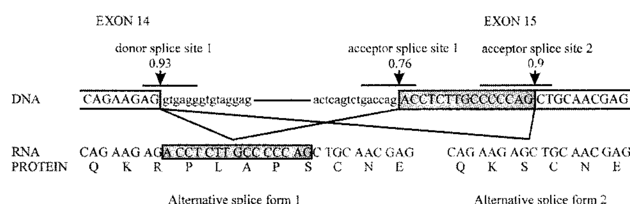

Fig. 2 Alternative splicing of *TECTA*. Intron sequences are given in lower-case letters; exon sequences are given in upper-case letters and are boxed. Splice-site consensus sequences with their CV value are indicated above the DNA sequence. Use of the first acceptor splice-site gives rise to 15 extra nucleotides in the mRNA, leading to 5 extra amino acids (RPLAP). The protein lacks the RPLAP amino acids when the second acceptor splice site is used.

the membrane and a carboxy-terminal hydrophobic region characteristic of precursors for glycosylphosphatidylinositol-linked membrane-bound proteins. The protein is probably released from the membrane by proteinase cleavage at a conserved tetrabasic sequence upstream of the predicted acceptor for the glycosylphosphatidylinositol anchor (residue 2091; Fig. 1; ref. 2). α-tectorin is further processed into three polypeptides: a module containing a region homologous to the G1 domain of entactin[6], a module similar to zonadhesin[7], and a module consisting of a zona pellucida domain[8]. These three polypeptides are crosslinked to each other by disulfide bridges and interact with β-tectorin to form the non-collagenous matrix of the tectorial membrane.

In the mouse, RT-PCR of *Tecta* cDNA amplified two splice variants, the larger variant encoding a protein containing five amino acids (RPLAP; ref. 2) not found in the smaller variant. The exact nature of the alternative splicing, however, could not be determined. In the human *TECTA* genomic sequence, exon 15 was found to contain two possible 5′ splice sites 15 bp apart, with CV values of 0.76 and 0.90, respectively (Fig. 2). Use of the first splice site would lead to the insertion of an RPLAP peptide in the same position as in the mouse. These data suggest that in man as well as in mouse, both splice sites are used, giving rise to two isoforms with and without the RPLAP peptide (Fig. 2).

We found no gross rearrangements in *TECTA* by Southern blot of DNA from either family studied. We then conducted exon-by-exon scanning using Single Stranded Conformation Polymorphism (SSCP) analysis. Mutation analysis of *TECTA* in the Austrian family revealed an A→G missense mutation at nt 5876 (Table 1, Fig. 3). This mutation replaces the tyrosine at residue 1870 with a cysteine (Y1870C). The Y1870C mutation segregated in 8 affected family members and was not found in any of the 6 unaffected family members, 50 Belgian controls or 50 Austrian controls living in the same region as the *DFNA8* family.

Mutation analysis of all 23 exons of *TECTA* in the Belgian *DFNA12* family revealed two mutations in exon 17. The first mutation, C→T at nucleotide position 5725, results in phenylalanine replacing leucine residue 1820 (L1820F; Table 1, Fig. 3). The second mutation, G→A at nt 5738, leads to a substitution of aspartic acid for glycine at residue 1824 (G1824D; Table 1, Fig. 3). These two mutations are only 12 base pairs apart (Fig. 3). Eighteen affected members of the *DFNA12* family had both mutations, but neither was present in any of the 40 unaffected family members or 100 controls. It is possible that one mutation is a rare polymorphism, while the other one is the disease-causing mutation. Alternatively, they might have a synergistic effect, neither being capable of producing disease by itself.

To investigate the evolutionary conservation of the amino acids changed by the missense mutations found in the *DFNA8/DFNA12* families, we searched the GenBank database for protein sequences homologous to the zona pellucida domain of TECTA (residues 1805–2057) using BLASTX. Alignment of the 10 genes with the highest homology in the BLAST results showed that all three amino acids changed by the missense mutations were evolutionarily conserved (Fig. 4; refs 2,9–16).

The mutations in the zona pellucida domain of α-tectorin may have dominant-negative phenotypes that disrupt the interactions between the different tectorin polypeptides, and as a consequence, disrupt the structure of the tectorial membrane. A deficient tectorial membrane is expected to lead to inefficient transmission of sound to the mechanosensitive stereociliary bundles of the hair cells, resulting in hearing impairment. It is also possible that a mutation causes mRNA instability or the degradation of α-tectorin, reducing the amount of this protein in the tectorial membrane.

The hearing impairment in both families is prelingual in onset. The fact that the human tectorial membrane is formed between the twelfth and twentieth week of embryonic development is consistent with a defect of the tectorial membrane in these families[17]. Furthermore, α- and β-tectorin are only expressed transiently during cochlear development in the mouse. CT scans of the temporal bones of affected members of the *DFNA8* family and magnetic resonance imaging of the inner ear of *DFNA12* patients (unpublished results) did not reveal any gross structural abnormalities. These *in vivo* imaging methods are inadequate to visualize the structure of the tectorial membrane. Definitive proof of the disease-causing nature of the α-tectorin mutations must, therefore come from further experiments investigating the effects of the missense mutations in α-tectorin on the structure and the function of the tectorial membrane.

Table 1 • *TECTA* mutations in *DFNA12/DFNA8* families

Exon	Domain	DNA	Protein	Family
17	ZP	5725C→T	L1820F	Belgian (*DFNA12*)
17	ZP	5738G→A	G1824D	Belgian (*DFNA12*)
18	ZP	5876A→G	Y1870C	Austrian (*DFNA8*)

ZP, zona pellucida domain

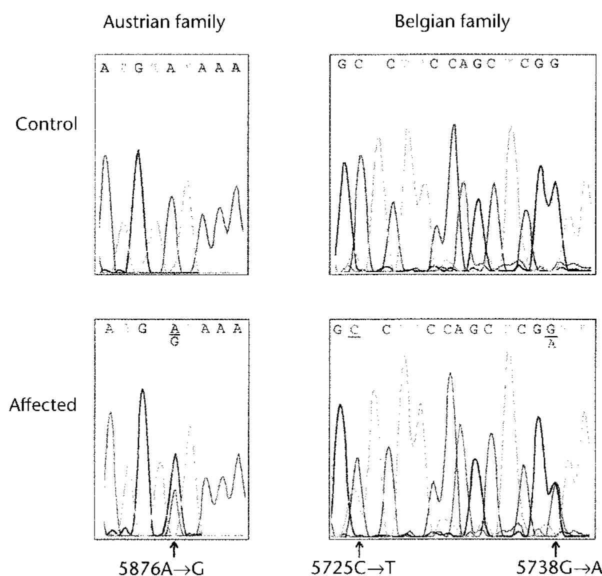

Fig. 3 DNA sequences with *TECTA* mutations in *DFNA8/DFNA12* families. Electropherograms for the regions immediately surrounding the *DFNA8/DFNA12* mutations are shown. For each family, an affected patient and a control person is depicted. Arrows indicate the positions of the mutations.

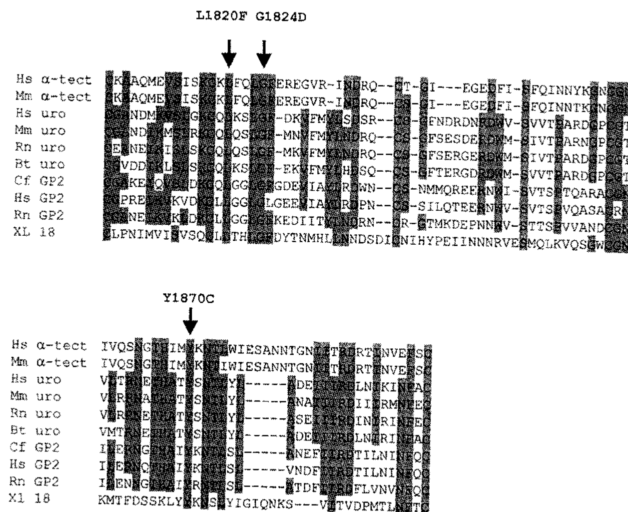

Fig. 4 Multiple amino-acid alignment of proteins homologous to the α-tectorin zona pellucida domain. The alignment includes α-tectorin from human (Hs α-tect) and mouse (Mm α-tect; ref. 3), human uromodulin (Hs uro; ref. 5) mouse uromodulin (Mm uro; ref. 6), rat uromodulin (Rn uro; ref. 7), bovine uromodulin (Bt uro; ref.8), dog glycoprotein 2 (Cf GP2; ref. 9), human glycoprotein 2 (Hs GP2; ref. 10), rat glycoprotein 2 (Rn GP2; ref. 11) and frog thyroid-regulated glycoprotein 18 (Xl18; ref. 12). Only a short region surrounding the three missense mutations in the zona pellucida domain is shown. Positions with conservation in at least 6 of 12 sequences are shaded. Arrows indicate the 3 mutations in *DFNA8*/*DFNA12*.

Methods

DFNA12 and _DFNA8_ families. The Belgian *DFNA12* family has already been described clinically[18] and genetically[4]. All 8 affected members of the Austrian *DFNA8* family showed a moderate-to-severe hearing deficit (60–80 dB) involving all frequencies. The hearing impairment was prelingual and the patients reported no change in hearing over time. Linkage results have localized the gene responsible for *DFNA8* on the long arm of chromosome 11, in the same region as that for *DFNA12*. A detailed description of the *DFNA8* family will be published elsewhere.

Identification of cosmid clones. Cosmids containing *TECTA* DNA sequences were identified by screening an arrayed chromosome-11-specific cosmid library[19] with the mouse *Tecta* cDNA sequence as a probe. Five positive clones were obtained from the Resource Centre of the German Human Genome Project, and grown overnight in LB medium containing 25 μg/ml kanamycin. Cosmid DNA samples were digested with restriction enzymes, electrophoresed through a 0.8% agarose gel and transferred to a Hybond N$^+$ membrane (Amersham) using standard procedures. The membranes were consecutively hybridized with four overlapping *Tecta* cDNA fragments.

Shotgun cloning of cosmid clones. DNA from four selected chromosome-11-specific cosmid clones (ICRFc107F1132D1, ICRFc107c05177D1, ICRFc107A0652D1, ICRFc107F06171D1) was sonicated into fragments of 400 bp to 1.5 kb, blunt-ended and shotgun-cloned into a plasmid. From each cosmid, 768 plasmid subclones were picked in two 384-well microtiter plates. Replicas were made on Hybond N+ membranes (Amersham) using a 384-pin replicator (Genetix). The membranes were hybridized with four overlapping fragments of the mouse *Tecta* cDNA as probes, and positive plasmid clones were sequenced.

Mutation analysis. All 23 exons were amplified by PCR using primers flanking the different exons (primers sequence available from author on request) if the corresponding PCR products were smaller than 200 bp. Otherwise, additional primers were designed to generate several overlapping fragments comprising the whole exon. SSCP analysis was carried out using 0.5×MDE gels (FMC) as described[20]. DNA was sequenced on an ABI 377 automated DNA sequencer (Perkin Elmer), using Thermo Sequenase (Amersham) and Big Dye (Perkin Elmer) dye terminator cycle sequencing kits. Rapid mutation screening was carried out by restriction enzyme digestion of PCR-amplified exons followed by polyacrylamide gel electrophoresis. To analyse theY1870C mutation in exon 18, a modified forward primer was designed (5′–CCAATGGCACGCATATCATGT–3′) to create an *Nde*I site (Boehringer). To analyse the L1820F substitution in exon 17, a second modified forward primer was designed (5′–GTGTCCATATCTAAGTGCGAG–3′) to create an artificial restriction site for *Sac*I (Boehringer) in the wild-type allele. The G1824D mutation in exon 17 creates a new restriction site for *Taq*I (Life Technologies).

GenBank accession numbers. *TECTA* genomic sequences, AF055114 to AF055136.

Acknowledgements

This study was supported by a grant from the University of Antwerp and a grant from the Flemish 'Fonds voor Wetenschappelijk Onderzoek' (F.W.O.). K.V. holds a pre-doctoral research position with the 'Vlaams instituut voor de bevordering van het wetenschappelijk-technologisch onderzoek in de industrie' (I.W.T.), and G.V.C. holds a research position with the F.W.O.. P.K.L., G.P.R. and D.C.H. are supported by the U.K. Medical Research Council. R.J.H.S. is supported by an NIDCD grant. K.K. and W.K. are supported in part by N.I.H. grants.

Received 11 February; accepted 31 March, 1998.

1. Richardson, G.P., Russell, I.J., Duance, V.C. & Bailey, A.J. Polypeptide composition of the mammalian tectorial membrane. *Hear. Res.* **25**, 45–60 (1987).
2. Legan, P.K., Rau, A., Keen, J.N. & Richardson, G.P. The mouse tectorins. Modular matrix proteins of the inner ear homologous to components of the sperm-egg adhesion system. *J. Biol. Chem.* **272**, 8791–8801 (1997).
3. Hughes, D.C., Legan, P.K., Steel, K.P. & Richardson, G.P. Mapping of the alpha tectorin gene to mouse chromosome 9 and human chromosome 11 : A candidate for human autosomal dominant non-syndromic deafness. *Genomics* **48**, 46–51 (1998).
4. Verhoeven, K. *et al.* A gene for autosomal dominant non-syndromic hearing loss (DFNA12) maps to chromosome 11q22-24. *Am. J. Hum. Genet.* **60**, 1168–1174 (1997).
5. Krawczak, M., Reiss, J. & Cooper, D.N. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum. Genet.* **90**, 41–54 (1992).
6. Durkin, M.E. *et al.* Amino-acid sequence and domain structure of entactin. Homology with epidermal growth factor precursor and low density lipoprotein receptor. *J. Cell Biol.* **107**, 2749–2756 (1988).
7. Hardy, D.M. & Garbers, D.L. A sperm membrane protein that binds in a species-specific manner to the egg extracellular matrix is homologous to von Willebrand factor. *J. Biol. Chem.* **270**, 26025–26028 (1995).
8. Bork, P. & Sander, C. A large domain common to sperm receptors (Zp2 and Zp3) and TGF-beta type III receptor. *FEBS Lett.* **300**, 237–240 (1992).
9. Hession, C. *et al.* Uromodulin (Tamm-Horsfall glycoprotein) : a renal ligand for lymphokines. *Science* **237**, 1479–1484 (1987).
10. Prasasan, K. *et al.* Nucleotide sequence and peptide motifs of mouse uromodulin (Tamm-Horsfall protein)-the most abundant protein in mammalian urine. *Biochim. Biophys. Acta* **1260**, 328–332 (1995).
11. Fukuoka, S., Freedman, S.D. & Scheele, G.A. A single gene encodes membrane-bound and free forms of GP-2, the major glycoprotein in pancreatic secretory (zymogen) granule membranes. *Proc. Natl. Acad. Sci. USA* **88**, 2898–2902 (1991).
12. Yu, H., Papa, F. & Sukhatme, V.P. Bovine and rodent Tamm-Horsfall protein (THP) genes: cloning, structural analysis, and promotor identification. *Gene Expr.* **4**, 63–75 (1994).
13. Fukuoka, S., Freedman, S.D., Heron, Y., Sukhatme, V.P. & Scheele G.A. Gp-2/THP gene family encodes self-binding glycosylphosphatidylinositol-anchored proteins in apical secretory compartments of pancreas and kidney. *Proc. Natl. Acad. Sci. USA* **89**, 1189–1193 (1992).
14. Wong, S.M. & Lowe A.W. Sequence of the cDNA encoding human GP-2, the major membrane protein in the secretory granule of the exocrine pancreas. *Gene* **171**, 311–312 (1996).
15. Hoops, T.C. & Rindler, M.J. Isolation of the cDNA encoding glycoprotein-2 (GP-2), the major zymogen granule membrane protein, homology to uromodulin/ Tamm-Horsfall protein. *J. Biol. Chem.* **266**, 4257–4263 (1991).
16. Brown, D.D. *et al.* The thyroid hormone-induced tail resorption program during *Xenopus laevis* metamorphosis. *Proc. Natl. Acad. Sci. USA* **93**, 1924–1929 (1996).
17. Sulik, K.K. in *Hereditary Hearing Loss and its Syndromes* Ch. 4, (eds Gorlin R.J., Toriello, H.V. & Cohen, M.M.) 22–23 (Oxford Univ. Press, Oxford, 1995).
18. Govaerts, P.J. *et al.* A new autosomal dominant locus (DFNA12) is responsible for a non-syndromic, mid-frequency, prelingual and non-progressive sensorineural hearing loss. *Am. J. Otol.* (In press).
19. Lerach, H. *et al.* in *Genome Analysis* 1, (eds Davies, K.E. & Tilghman, S.M.) 39-81 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1990).
20. Orita, Y., Sekiya, T. & Hayashi, K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* **5**, 874–879 (1989).