

ARTICLE

Mid-frequency DFNA8/12 hearing loss caused by a synonymous *TECTA* mutation that affects an exonic splice enhancer

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Autosomal dominant hearing loss is highly heterogeneous. Hearing impairment mainly involves the mid-frequencies (500–2000 Hz) in only a low percentage of the cases. In a Dutch family with autosomal dominant mid-frequency/flat hearing loss, genome-wide SNP analysis combined with fine mapping using microsatellite markers mapped the defect to the DFNA8/12 locus, with a maximum two-point LOD score of 3.52. All exons and intron–exon boundaries of the *TECTA* gene, of which mutations are causative for DFNA8/12, were sequenced. Only one heterozygous synonymous change in exon 16 (c.5331G>A; p.L1777L) was found to segregate with the hearing loss. This change was predicted to cause the loss of an exonic splice enhancer (ESE). RT-PCR using primers flanking exon 16 revealed, besides the expected PCR product from the wild-type allele, a smaller fragment only in the affected individual, representing part of an aberrant *TECTA* transcript lacking exon 16. The aberrant splicing is predicted to result in a deletion of 37 amino acids (p.S1758Y/G1759_N1795del) in α -tectorin. Subsequently, the same mutation was detected in two out of 36 individuals with a comparable phenotype. Owing to the position of the protein deletion just N-terminal of the zona pellucida (ZP) domain of α -tectorin, it is likely that the deletion of 37 amino acids may affect the proteolytic processing, structure and/or function of this domain, which results in a clinical phenotype comparable to that of missense mutations in the ZP domain. In addition, this is the first report of a synonymous mutation that affects an ESE and causes hereditary hearing loss.

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Introduction

Autosomal, dominant, nonsyndromic hearing loss (ADNSHL) is highly heterogeneous. To date, 54 loci for ADNSHL (DFNA) have been mapped, for 22 of which the causative gene has been identified.¹ Postlingual hearing loss affecting the high frequencies is the clinical manifestation in the majority of cases.² Occasionally however, hereditary hearing loss involves mainly low- or

mid-frequencies, the latter for instance in the case of DFNA8/12, DFNA13, DFNA21, DFNA31, DFNA44 and DFNA49. In the majority of these traits, a certain level of progression is observed.² Only for DFNA8/12 and DFNA44, the causative genes have been identified. DFNA44 hearing loss is caused by mutations in the *CCDC50* gene, encoding an effector of EGF-mediated cell signaling.³ DFNA8/12 hearing loss is caused by missense mutations in *TECTA* (DFNA8/12) encoding the tectorial membrane protein α -tectorin. Depending on the exons carrying the mutation and thus the affected protein domains, *TECTA* mutations cause either mid-frequency or high-frequency hearing loss.⁴ Here, we report on a Dutch family in which individuals suffer from autosomal dominant hearing loss with either a U-shaped or a flat audiogram. Whole-genome SNP genotyping mapped the genetic defect underlying the hearing loss in this family to the DFNA8/12 locus. Sequence analysis of the corresponding *TECTA* gene and further studies revealed a heterozygous synonymous mutation that results in aberrant splicing as the cause of the hearing loss.

Materials and methods

Subjects

A Dutch family (W04-096) suffering from prelingual mid-frequency/flat, nonprogressive hearing loss was ascertained. Seventeen individuals participated in this study, of which eleven showed clinical symptoms of hearing impairment. The first symptoms of hearing loss were reported in the range of <1–30 years. Medical history was taken from all participants, paying attention to hearing impairment and vestibular symptoms. Furthermore, concomitant disease, use of medication and any other possible cause of acquired deafness were ruled out. After micro-otoscopic examination, pure tone audiometry was performed. Clinically affected individuals also underwent speech audiometry. In addition, 36 individuals affected with flat/mid-frequency hearing loss participated in this study as well as 150 ethnically matched controls. Written informed consent was obtained from all individuals, and this study was approved by the local ethics committee.

Linkage analysis

Genomic DNA of all the participating individuals was extracted from peripheral blood lymphocytes according to standard protocols.⁵ All individuals of family W04-096 were genotyped using Affymetrix 10 K SNP arrays (Affymetrix GeneChip Human Mapping 10K Array Xba142 2.0; Affymetrix, Santa Clara, CA, USA) containing 10,204 highly informative SNP markers. Multipoint linkage analysis was performed with Allegro version 1.2c in the EasyLinkage software package^{6,7} using the deCODE SNP map and Caucasian allele frequencies. An autosomal

dominant mode of inheritance with 95% penetrance was assumed, and the disease allele frequency was estimated at 0.001. For fine mapping, microsatellite markers (D11S925; D11S4089; D11S4107; D11S4167; D11S4094 and D11S4144) were used in all available family members and analyzed using the GeneMapper program (Applied Biosystems, Foster City, CA, USA). Two-point LOD scores for these markers were calculated with the SuperLINK program in the EasyLinkage software package. Allele frequencies for each of the markers were considered to be equal. The genomic localization of the markers was derived from the Marshfield map and the UCSC human genome database (build hg18, March 2006; <http://www.genome.ucsc.edu>).

Mutation analysis

All 23 exons and intron–exon boundaries of the *TECTA* gene (NM_005422) were amplified under standard PCR conditions. Primer sequences are available on request. Sequence analysis was performed with the ABI PRISM Big Dye Terminator Cycle Sequencing V2.0 Ready Reaction kit and the ABI PRISM 3730 DNA analyzer (Applied Biosystems). The occurrence of the observed mutation (*TECTA* exon 16: c.5331G>A) in family W04-096, a selection of patients suffering from mid-frequency/flat hearing loss, and in the Dutch, normal hearing population was analyzed with *Bse*YI restriction analysis.

RNA analysis

Total RNA of affected individuals and ethnically matched control individuals was isolated from EBV-transformed lymphoblasts using the Rneasy Midi Kit (Qiagen, Hilden, Germany). Subsequently, cDNA synthesis was performed according to standard procedures using random hexamers. The part of *TECTA* cDNA carrying the mutation was amplified using forward primer 5'-CAACGAGCTGCAGTTC TCAC-3' (located in exon 15) and reverse primer 5'-CACAATCCCTTTGGTGTG-3' (located in exon 17), or using forward primer 5'-AACTTCAACGGGGACCTAAC-3' (located in exon 14) and reverse primer 5'-GATGTTGCCA GTGTTGTTGG-3' (located in exon 18) with a total number of 35 PCR cycles. PCR products were analyzed using agarose gel electrophoresis, excised from gel, purified and sequenced as described above.

Bioinformatic analyses

To address the predicted effect of the synonymous change detected in this study on splice efficiency, software tools at the following URLs were used. For general splice site prediction: http://www.fruitfly.org/seq_tools/splice.html, <http://www.cbs.dtu.dk/services/NetGene2> and http://www.tigr.org/tdb/GeneSplicer/gene_spl.html. For prediction of exonic splice enhancers:

exonic splice enhancers (ESE) finder (<http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home>).

Results and discussion

Mid-frequency hearing loss in family W04-096 linked to the DFNA8/12 locus

Members of family W04-96 suffered from early onset autosomal dominant hearing loss. Clinical assessment of all participating family members revealed 11 affected individuals. Audiometric profiling in this family showed in some patients a typical U-shaped configuration of the audiogram, characteristic of mid-frequency hearing loss, whereas in other patients, a more flat audiometric profile was observed. Figure 1a shows two representative examples

of hearing loss in the family, a U-shaped audiogram in individual II:2 and a flat audiogram in individual III:1. The shape of the audiogram observed in the other affected individuals is shown in Figure 1c. A more detailed description of the clinical characteristics of hearing loss in this family will be presented in a separate article (de Heer *et al*,⁸ submitted).

All individuals in the pedigree were genotyped using an Affymetrix 10 k SNP array. Linkage analysis resulted in only one region with a multipoint LOD score >2 , on chromosome 11 flanked by SNPs rs951657 and rs728178

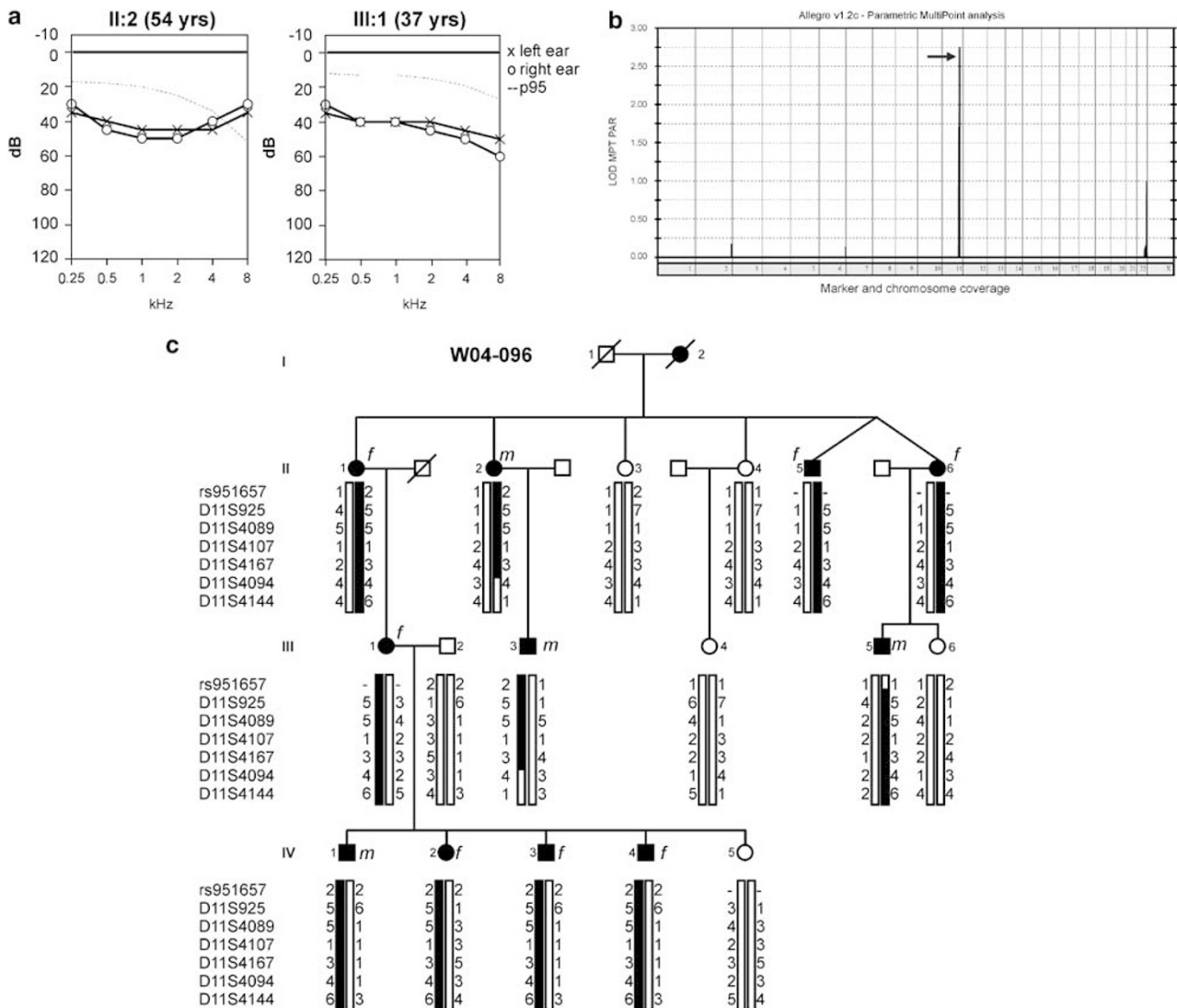


Figure 1 (a) Pure-tone air-conduction hearing thresholds for two affected individuals of family W04-096, for both the left and the right ears. Individual II:2 shows a typical U-shaped audiogram, whereas individual III:1 shows a more flat audiogram. The type of hearing loss observed in the remainder of the family is indicated in Figure 1c. The p95-line indicates that 95% of the population has thresholds lower than these values. (b) LOD score calculations using the 10 k SNP array genotyping data. The only region with a nearly significant LOD score on chromosome 11 is indicated by an arrow. (c) Pedigree and haplotype analysis of family W04-096 for microsatellite markers on chromosome 11q24.1. Both SNPs that determined the linkage interval (rs951657 in III:5 and rs728178 in II:2 and III:3) are also shown. For each individual, the type of hearing loss is indicated by an m (for mid-frequency hearing loss) or an f (for a flat audiogram).

Table 1 Two point LOD scores at DFNA8/12 locus

Marker	LOD score at $\theta =$				
	0.00	0.10	0.20	0.30	0.40
D11S925	3.52	2.89	2.19	1.40	0.59
D11S4089	2.94	2.36	1.73	1.08	0.48
D11S4107	3.40	2.78	2.10	1.34	0.56
D11S4167	0.33	0.25	0.16	0.07	0.01
D11S4094	1.29	0.95	0.58	0.19	-0.09
D11S4144	-4.08	2.19	1.79	1.20	0.53

Two-point LOD scores calculated for microsatellite markers surrounding the DFNA8/12 locus in family W04-096. Significant LOD scores were reached for markers D11S925 and D11S4107 at $\theta = 0$.

(Figure 1b). Fine mapping with microsatellite markers on chromosome 11 redefined the critical region to an ~9.9 cM-region between SNP rs951657 and marker D11S4144 (Figure 1c) with a maximum two-point LOD score of 3.52 for marker D11S925 (Table 1). The linkage interval encompasses the *TECTA* gene in which mutations cause autosomal dominant hearing impairment DFNA8/12.

Affected individuals of family W04-096 carry a mutation in *TECTA*

Next, one affected individual (II:6) of family W04-096 was analyzed for mutations in the *TECTA* gene. Sequence analysis revealed only one heterozygous nucleotide change that had not been previously described, in exon 16 of the *TECTA* gene (c.5331G>A). At the protein level, this change, however, does not result in the substitution of an amino acid (p.L1777L; Figure 2a), indicating that the α -tectorin protein encoded by the mutant allele would not differ from the wild-type protein. Although thus far, only missense mutations within the zona pellucida (ZP) domain of α -tectorin have been shown to cause a mid-frequency type of hearing impairment,⁴ the synonymous change found in exon 16 was still considered to be a candidate causative mutation for a number of reasons. First, the c.5331G>A change segregated with the hearing loss and was not detected in over 250 ethnically matched control alleles (data not shown). Second, the linkage analysis clearly showed only one genomic region, in which the *TECTA* gene resides, with a significant LOD score (Figure 1b). *TECTA* is one of the few genes known to be involved in the rather rare form of mid-frequency hearing loss, implicating a high probability of this gene to harbor the genetic defect in family W04-096. Finally, also changes that do not directly alter the amino-acid sequence of a certain protein may be involved in human disease, for instance, by influencing splicing events.⁹⁻¹¹ Exonic and intronic splice enhancers or silencers are splice factors that bind to a specific consensus sequence within a pre-mRNA molecule, and in this way they regulate the splicing process.¹² Using several bioinformatic tools, the effect of the synonymous change on the splice sites was analyzed.

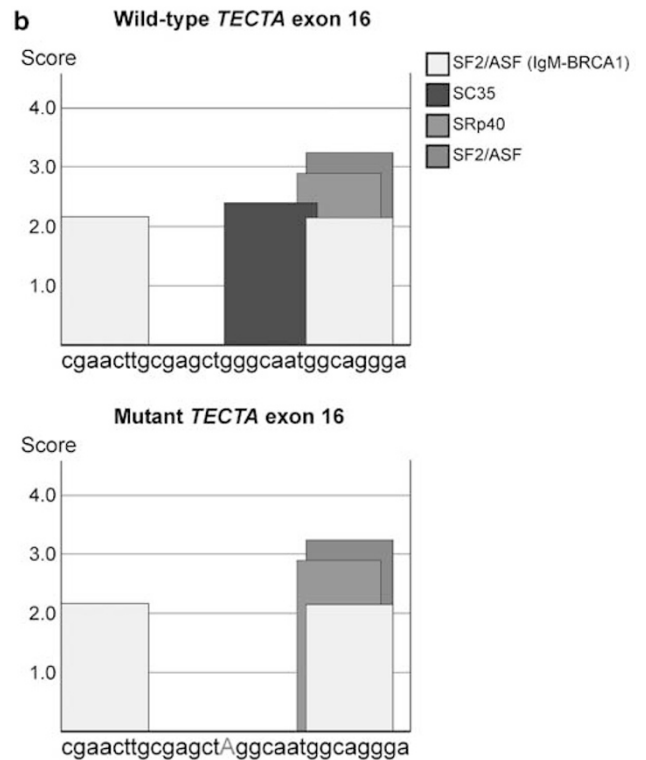
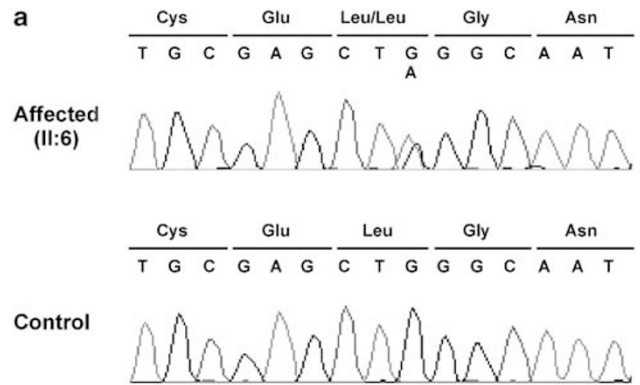


Figure 2 (a) Partial sequence of the *TECTA* gene showing the synonymous c.5331G>A change in an affected family member (II:6) and in a control individual. The mutation does not directly alter the amino-acid composition of the corresponding protein, as depicted above the sequence trace. (b) Graphical representation of *in silico* ESE finder analysis. Using the wild-type *TECTA* sequence surrounding the synonymous change, a number of different splice enhancers are predicted to bind (upper panel). If the synonymous change is introduced (changed adenine depicted in capital and red), the splice enhancer SC35 is predicted to lose its ability to bind (lower panel). The nucleotides that were analyzed with ESE finder correspond to nucleotides 5317–5345 of *TECTA* cDNA.

No major changes were detected in the strength of both the acceptor and donor splice sites. Subsequently, as the sequence variant in exon 16 of *TECTA* is located >50 bp away from both splice sites, the presence of consensus

sequences within the exon that may represent ESE was studied. With the ESE finder software, several splicing elements were predicted to recognize their consensus sequence within the wild-type *TECTA* exon 16 sequence (Figure 2b, upper panel). When the nucleotide change detected in family W04-096 was introduced, one of these ESE-binding sites was lost (SC35; Figure 2b, lower panel), indicating that the c.5331G>A change may influence the binding of elements regulating splicing of *TECTA* pre-mRNA. To determine whether this change occurs more frequently in hearing-impaired individuals with a comparable audiometric profile, 36 individuals were screened for the presence of this mutation. In two patients, the c.5331G>A change was indeed found to be present heterozygously, supporting the causative nature of this mutation. The mutation was also segregating with the hearing loss in the families of these two patients, an affected father of the proband in family W02-032 and an affected mother and brother of the proband in family W06-249. Haplotype analysis revealed the change to be present on the same allele as compared to family W04-096, indicating a founder effect.

To determine whether the synonymous change affects splicing of *TECTA* mRNA *in vivo*, cDNA was synthesized from total RNA derived from lymphoblasts of all patients for whom a cell line was available. In total, RT-PCR analysis

was performed for six affected individuals (W04-096 (II:5), the proband and his mother from W02-032, the proband and her two children from W06-249 and three unrelated control individuals). Using a forward primer located in exon 15 and a reverse primer located in exon 17 of the *TECTA* gene, part of the cDNA molecule including exon 16 carrying the silent mutation was amplified. Agarose gel electrophoresis showed that in all affected individuals and in the three control individuals, a PCR product of 577 bp was present (Figure 3a), which was expected based on the wild-type sequence. However, an additional smaller PCR product of 466 bp was detected only in the affected individuals (Figure 3a), which suggests that a differential splicing event had occurred. The sequence analysis of this smaller product revealed that exon 16 is absent in the *TECTA* transcript (Figure 3b). In addition, sequencing of the 577-bp PCR product derived from individual II:5 showed only a guanosine and no adenosine at nucleotide position 5331 (data not shown). This suggests that the large majority, if not all transcripts, of the mutant allele lack exon 16. To exclude the possibility that the synonymous change alters the splicing efficiency of the neighboring exons 15 and 17, RT-PCR analysis was performed with primers located in exon 14 (fw) and exon 18 (rev). Besides the two expected products (the one containing and the one lacking exon 16), no additional products of aberrant

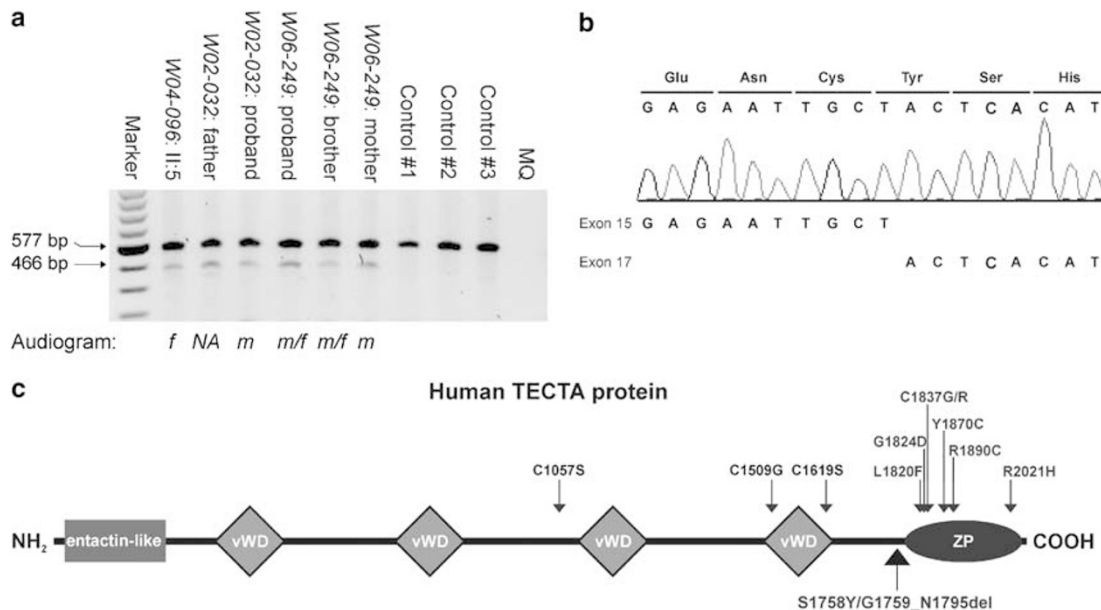


Figure 3 (a) RT-PCR on lymphocyte RNA of six affected individual and three unrelated control individuals, using primers located in exons 15 and 17 of *TECTA*. In all individuals, a PCR product of 577 bp is present, whereas only in the affected individuals, an additional product of 466 bp is detected. The identity of the affected individuals and their type of hearing loss (m for mid-frequency, f for flat; NA, not available) are indicated above and below the graph, respectively. M, 100 bp marker; MQ, negative control for PCR. (b) The sequence analysis of the 466-bp PCR product presented in panel a. As indicated by the genomic sequences of exons 15 and 17 of *TECTA* below the sequence trace, exon 16 is clearly absent from this transcript. (c) Graphical representation of the α -tectorin protein. Important functional domains, such as the entactin-like domain at the N-terminal part of the protein, four von Willebrand factor type D domains (vWD) in the central zona adhesin domain and the zona pellucida (ZP) domain at the C-terminus are indicated. Missense mutations causing DFNA8/12 are indicated by arrows. The ones that cause typical mid-frequency hearing loss are all located in the ZP domain and indicated in blue. The mutation described in this study is depicted below the protein.

splicing were observed (data not shown). Together, these results show that the synonymous change indeed affects the splicing of the *TECTA* transcript *in vivo*.

As a consequence of the skipping of exon 16, 111 nucleotides (based on the RNA sequence) are absent in the *TECTA* transcript derived from the mutant allele. Together, these 111 nucleotides encode 37 amino acids, indicating that no frameshift would occur by the loss of exon 16. At the protein level, the first amino acid to be affected is a serine residue at position 1758 that now becomes a tyrosine by the joining of exons 15 and 17 (Figure 3b). In addition, amino acids 1759–1795 are deleted from the mutant α -tectorin protein, after which the normal reading frame continues from residue serine 1796 onward (p.S1758Y/G1759_N1795del).

Pathogenesis of the c.5331G>A. mutation in *TECTA*

The wild-type α -tectorin protein consists of 2155 amino acids, and is one of the three major noncollagenous components of the tectorial membrane in the inner ear.^{13,14} Several studies have revealed that the tectorial membrane is thought to play an important role in ensuring an optimal cochlear feedback as well as providing the principal drive to the inner hair cells at their optimal frequency.^{15,16} Targeted deletion of the *Tecta* gene in mice results in a complete detachment of the tectorial membrane from the organ of corti,¹⁶ implying an essential role for α -tectorin in tectorial membrane structure and functioning.

In humans, mutations in *TECTA* cause both autosomal recessive and dominant hearing loss, DFNB21 and DFNA8/12, respectively. All recessive DFNB21 mutations in *TECTA* result in premature stop codons that are predicted to cause either truncated α -tectorin protein products or nonsense-mediated degradation of the *TECTA* mRNA, and are thus considered to be loss-of-function mutations.^{17–19} In contrast, the *TECTA* mutations thus far reported to cause dominant hearing loss all substitute highly conserved amino acids. The α -tectorin protein contains three important functional domains.¹³ At the N-terminus, a module resembling the G1 domain of entactin is present. The large central part of α -tectorin resembles the sperm protein zonadhesin and contains four von Willebrand type D (vWD) domains. At the C-terminus, a domain exhibiting similarity to the ZP domains of uromodulin and GP2 is present (Figure 3c). Following a proteolytic event in α -tectorin, three polypeptides are formed each containing one of these functional domains that subsequently interact with each other and with other proteins (for instance β -tectorin) to form the extracellular matrix of the tectorial membrane.²⁰ The various missense mutations in *TECTA* that cause DFNA8/12 can be subdivided into classes with a clear genotype–phenotype correlation. Mutations affecting residues in the central part of α -tectorin cause high-frequency hearing loss, whereas mutations affecting

residues in the ZP domain mainly affect mid-frequencies.^{4,20–23} In addition, if mutations substitute cysteine residues that are important in the formation of disulfide bridges, the hearing loss has a progressive nature regardless of the protein domain affected.^{24–26} An overview of the various *TECTA* mutations that cause DFNA8/12 is presented in Figure 3c. The synonymous change described in this study, similar to missense mutations in the ZP domain, causes mid-frequency or flat threshold hearing impairment (de Heer *et al*,⁸ submitted). The 37 amino acids that are absent in the mutant protein owing to the aberrant splicing event are located just N-terminally of the ZP domain. Although the precise positions of the proteolytic cleavage events resulting in the three polypeptides are not exactly known, the 37 deleted residues may harbor the cleavage site necessary for separation of the zonadhesin and the ZP domain or may affect its cleavage indirectly.^{13,27} Alternatively, the folding and thus the functional properties of the ZP domain may be altered.

Conclusion

In conclusion, this study describes the identification of a synonymous change in *TECTA* as the cause of nonsyndromic mid-frequency/flat hearing impairment in three Dutch families. Owing to this mutation, a predicted ESE-binding site is lost, resulting in an aberrant splicing event in the affected individuals and a deletion of 37 amino acids of the α -tectorin protein. This is the first report of such a synonymous change being the cause of hereditary hearing loss. It is to be expected that synonymous changes affecting splicing will be identified more frequently. Therefore, characterization of such mutations will result in a better prediction of how nucleotide changes may affect splicing, and it will, in general, lead to a better understanding of how various mutations cause disease and of genotype–phenotype correlations.

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