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DFNA8/12 Caused by *TECTA* Mutations is the Most Identified Subtype of Non-syndromic Autosomal Dominant Hearing Loss

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Abstract

The prevalence of DFNA8/DFNA12 (DFNA8/12), a type of autosomal dominant non-syndromic hearing loss (ADNSHL), is unknown as comprehensive population-based genetic screening has not been conducted. We therefore completed unbiased screening for *TECTA* mutations in a Spanish cohort of 372 probands from ADNSHL families. Three additional families (Spanish, Belgian and English) known to be linked to DFNA8/12 were also included in the screening. In an additional cohort of 835 American ADNSHL families, we preselected 73 probands for *TECTA* screening based on audiometric data. In aggregate, we identified 23 *TECTA* mutations in this process. Remarkably 20 of these mutations are novel, more than doubling the number of reported *TECTA* ADNSHL mutations from 13 to 33. Mutations lie in all domains of the α-tectorin protein,

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including those for the first time identified in the entactin domain, the vWFD1, vWFD2 and vWFD3 repeats, and the D1-D2 and TIL2 connectors. While the majority are private mutations, four of them – p.Cys1036Tyr, p.Cys1837Gly, p.Thr1866Met and p.Arg1890Cys – were observed in more than one unrelated family. For two of these mutations founder effects were also confirmed. Our data validate previously observed genotype-phenotype correlations in DFNA8/12 and introduce new correlations. Specifically, mutations in the N-terminal region of α-tectorin (entactin domain, vWFD1 and vWFD2) lead to mid frequency NSHL, a phenotype previously associated only with mutations in the ZP domain. Collectively, our results indicate that DFNA8/12 hearing loss is a frequent type of ADNSHL.

Keywords

DFNA8; DFNA12; *TECTA*; mid-frequency hearing loss; high-frequency hearing loss

INTRODUCTION

Sensorineural hearing loss (SNHL) is the most common sensory defect. In developed countries, it is diagnosed in 1 of every 500 newborns (Marazita, et al., 1993; Morton, 1991; Smith, et al., 2005) and increases in prevalence with age, affecting 10% of 60 year olds and 50% of octogenarians (Petit, 1996). Although the proportion of late-onset SNHL that is genetic has not been determined, monogenic post-lingually acquired SNHL is most commonly inherited in an autosomal dominant manner (autosomal dominant non-syndromic hearing loss, ADNSHL). ADNSHL is genetically and clinically heterogeneous – 60 loci have been mapped, 24 genes have been cloned and a variety of audioprofiles have been reported (Van Camp and Smith, 2011). With few exceptions these genes have been associated with hearing loss in a small number of families by linkage analysis and comprehensive mutational searching in populations has not been conducted; hence the contribution of the majority of genes to the whole of ADNSHL is still unknown.

One of these ADNSHL genes is the α-tectorin gene [*TECTA*; MIM# 602574]. The encoded protein, α-tectorin, is one of the main non-collagenous proteins of the tectorial membrane (TM), a ribbon-like strip of extracellular matrix that lies over the stereocilia of the hair cells and is critical for the mechanical transmission and amplification of sound (Verhoeven, et al., 1998). α-tectorin is a large modular glycoprotein that is posttranslationally cleaved to produce three covalently associated fragments linked by disulfide bridges: the entactin domain (ENT); the large zonadhesin region (ZA) containing two partial and three full von Willebrand factor type C (vWFC V0) or D (vWFD V1, V2, V3, V4) domains; the C-terminal zona pellucida (ZP) domain; and three trypsin inhibitor like cysteine rich domains (TIL1,2,3) (Fig. 1) (Legan, et al., 1997; Maeda, et al., 2001; Rau, et al., 1999) .

Missense mutations of *TECTA* cause ADNSHL (DFNA8/12) while nonsense mutations cause autosomal recessive NSHL (DFNB21) (Table 1). The audioprofile associated with the former depends on the domain and residue affected (Alloisio, et al., 1999; Balciuniene, et al., 1999; Collin, et al., 2008; Iwasaki, et al., 2002; Meyer, et al., 2007b; Moreno-Pelayo, et al., 2001; Pfister, et al., 2004; Plantinga, et al., 2006; Sagong, et al., 2010; Verhoeven, et al., 1998). The established genotype-phenotype correlations indicate that missense mutations in the ZP domain lead to mid-frequency sensorineural hearing loss (MFSNHL) while missense mutations in the ZA region cause high-frequency sensorineural hearing loss (HFSNHL). If cysteine residues are affected the loss is progressive; if other residues are affected the loss is stable. No mutations have been reported in the entactin domain.

In this study we have used unbiased and biased (phenotype-driven audioprofile analysis by AudioGene) genetic screening to investigate the prevalence of DFNA8/12 in two large cohorts of ADNSHL patients, one from Spain and the other from America. Our results confirm previous genotype-phenotype correlations for *TECTA* and establish novel ones. We more than double the number of deafness-causing *TECTA* mutations making SNHL at the DFNA8/12 locus a prevalent subtype of ADNSHL.

MATERIALS AND METHODS

Sample Collection and Clinical Evaluation

An initial cohort of 835 probands of American ADNSHL families were subjected to biased preselection by visual examination of audiometric data to select 73 families for further study based on the presence of MFSNHL or HFSNHL. Information on family history of ADNSHL was not available for seven of these families. This cohort was then screened using AudioGene (http://audiogene.eng.uiowa.edu/) (Hildebrand, et al., 2009a; Hildebrand, et al., 2009b; Hildebrand, et al., 2008b). AudioGene, a support vector machine trained on a dataset of 1,929 audiograms from 17 different known ADNSHL loci, exploits specific characteristics of an auditory phenotype to make phenotypically driven predictions of the causative genotype, a process we term ‘audioprofiling’. We have previously reported its successful implementation to identify new families with high-frequency SNHL at the DFNA2 and DFNA9 loci (Hildebrand, et al., 2009b; Hildebrand, et al., 2008b).

A second cohort of 372 ADNSHL Spanish families was included in the study without preselection. Three additional families (1 Spanish, 1 Belgian, 1 English) suitable for linkage analysis were also included in the study. All family members involved in the study gave written informed consent.

Ten milliliters of whole blood was obtained from all patients by venipuncture and genomic DNA was extracted using routine methods (Grimberg, et al., 1989) Clinical history ruled out environmental factors as the cause of the hearing loss in the probands and physical examination excluded syndromic features. No other clinically significant features, including balance or visual problems, were reported by affected persons. Tympanometry excluded middle ear pathology and pure tone audiometry with air and bone conduction at frequencies ranging 250-8000 Hz was completed according to standard protocols. Transient-evoked otoacoustic emissions (TEOAEs) in the frequency bands of 0.5–1, 1–2, 2–3, 3–4 and 4–5 kHz, and auditory brainstem responses (ABRs) were measured in three persons from the last generation of family S726 (III:2 and III:4, at the age of 15 and 4 years, respectively; and III: 5 at the age 3 years and 7 months) (Table 1; Fig. 2). All procedures were approved by human research institutional review boards at the University of Iowa, Iowa City, Iowa, USA and the Hospital Ramón y Cajal, Madrid, Spain.

PCR, Sequencing and DHPLC Analysis

The human *TECTA* [RefSeq: NM_005422.2; MIM# 602574] gene was amplified using previously reported gene-specific primers (Meyer, et al., 2007a; Meyer, et al., 2007b). Amplification reactions were cycled using a standard protocol on a GeneMate Genius thermocycler (ISC BioExpress, Kaysville, UT) or a GeneAmp PCR® System 9700 (Applied Biosystems, Foster City, CA). Sequencing was completed with a BigDye™ v3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Sequencing products were read using an ABI 3730s sequencer (Perkin Elmer, Waltham, MA).

For probands of the Spanish cohort PCR amplimers of each exon of the *TECTA* gene were screened for mutations by Denaturing High Performance Liquid Chromatography (DHPLC)

on a Wave™ DNA fragment analysis system (Transgenomic™, Omaha, NE) according to the manufacturer's protocol. The different DHPLC heteroduplex profiles were characterized by sequencing a product of a second PCR amplification round.

For mutations, nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. All *TECTA* mutations identified in this study will be incorporated into a database of all reported hearing loss mutations from all known NSHL genes that will soon be made publicly available on the hereditary hearing loss homepage (http://hereditaryhearingloss.org).

Genotyping and Linkage Analysis

Genome-wide screening on the S1091 family (Spain) was performed with 394 microsatellite markers distributed across the genome (ABI Prism Linkage Mapping Set 2, Applied Biosystems, Foster City, CA). The order and genetic distances of additional markers used for fine mapping of the critical interval were taken from the Genethon human linkage map and from the Marshfield chromosome 11 map (http://research.marshfieldclinic.org/genetics). Linkage analysis was performed using the LINKAGE 5.1 software package setting the frequency of the deafness gene to 0.0001 and considering marker allele frequencies to be equal to each other. For the Belgian-K family (Belgium) a genome-wide scan with a set of 6×10^3 SNPs (HumanLinkage-12 array, Illumina, Essex, UK) was performed. In the case of the UK-E family (UK) linkage analysis was carried out using microsatellite markers spanning the critical regions of selected candidate loci (DFNA6, DFNA8/12, DFNA9, DFNA20 and DFNA48) and statistically significant linkage was only obtained for DFNA8/12.

To explore the evolutionary origin of three *TECTA* mutations – R1890C, C1837G and T1866M – probands from the relevant families (S488, S1080, 10050, W04-077, S063, S324, S726, S1385 and 504260) were genotyped for seven polymorphic microsatellite markers that scan the DFNA8/12 region (D11S4111, D11S925, D11S4089, D11S4107, D11S4167, D11S1336 and D11S934). Fluorescently labelled alleles were analyzed in an ABI PRISM 3100 automated DNA genetic analyzer (Applied Biosystems, Foster City, CA).

ConSeq and ARTA Analyses

Conservation scores for substituted amino acids in α-tectorin when compared to 50 similar protein sequences was determined using the ConSeq program (http://conseq.tau.ac.il/) (Berezin, et al., 2004). Any conservation score greater than 1 standard deviation above the mean for the α-tectorin amino acid sequence was regarded as highly conserved.

For the construction of age-related typical audiograms (ARTA) in family Belgian-K, air conduction threshold values were plotted versus age for each frequency separately and visually inspected. Progression of the hearing impairment was analyzed at each frequency by quadratic regression analysis. The regression equations were used to construct ARTAs per decade between 20 and 70 years of age as described (Huygen, et al., 2002).

RESULTS

Mutation Screening of the *TECTA* Gene

We used two approaches to study the prevalence of DFNA8/12 hearing loss in two large groups of ADNSHL families. For one approach, based on reported genotype-phenotype correlations for DFNA8/12 hearing loss (Alloisio, et al., 1999; Balciuniene, et al., 1999; Collin, et al., 2008; Iwasaki, et al., 2002; Meyer, et al., 2007b; Moreno-Pelayo, et al., 2001;

Pfister, et al., 2004; Plantinga, et al., 2006; Sagong, et al., 2010; Verhoeven, et al., 1998), we identified 73 probands with either 'cookie-bite' MFSNHL audioprofiles or congenital-early childhood HFSNHL profiles and filtered this group using AudioGene, which predicted that 64 of these families (87.6%) had DFNA8/12 hearing loss. *TECTA* mutations were identified in nine for a positive predictive value of 14.1% (9/64) and a negative predictive value of 100% (55/55; Table 1, Fig. 1a).

For the second approach, we enrolled without phenotypic preselection a cohort of 372 unrelated ADNSHL Spanish probands and *TECTA* mutations were identified in 15 families (Table 1, Fig. 1a). *TECTA* mutations were also found in the three additional families (Spanish, Belgium and English) included in the study that we mapped to DFNA8/12. As several *TECTA* mutations appeared repeatedly, in total we found 23 different pathogenic mutations, 20 of which are novel. According to Conseq scores the mutations affect highly conserved amino acids of the α-tectorin protein (Fig 1b). Segregation with hearing loss was confirmed in all cases for which DNA samples were available and none of the mutations were detected in control populations (>100 Spanish controls or 104 ethnically matched CEPH controls (Centre d'Etude du Polymorphisme Humain; (Cox, et al., 1996)).

Among the novel missense mutations, two were found in the entactin domain, eleven were found in the ZA region and four were detected in the ZP domain. We also found two splice site mutations which were predicted to abrogate the 5′splice site of intron 16 by the ESEfinder and MaxEntscan software (Cartegni, et al., 2003; Smith, et al., 2006), likely leading to the in-frame skipping of exon 16 and resulting in a shorter isoform (Collin, et al., 2008). One of these latter mutations, c.5383+5delGTGA, was detected in *cis* with the p.Asn886Ser in all patients of the UK-E family (Table 1). In addition, the deletion of the nucleotide T at position 1124 (c.1124delT) was found in the heterozygous state in persons II:1 and II:3 of family S1203 showing MFSNHL. This mutation generates a frameshift after codon 375, which results in the addition of four amino acids and a premature termination of the polypeptide chain (p.Val375Alafs*4), and was not found in person III:1 from the same family who has a different audioprofile (Table 1, Supp. Fig. S1). It is well known that inactivating mutations in the *TECTA* gene are the cause of DFNB21, a recessive form of hereditary hearing loss. Direct sequencing of all exons of *TECTA* and flanking intronic regions in subjects II:1 and II:3 revealed no other accompanying mutations. Based on the available data we cannot unambiguously rule out dominant inheritance of the c.1124delT allele in affected subjects II:1 and II:3. Furthermore, they may still be considered coincidental carriers for that mutation if the presence of an accompanying mutation is not detectable by the methods used in this study.

The list of mutations we identified also includes three previously described variants – p.Cys1837Gly, p.Thr1866Met and p.Arg1890Cys (Moreno-Pelayo, et al., 2001; Plantinga, et al., 2006; Sagong, et al., 2010) (Supp. Fig. S2, Table 1).

Linkage Mapping and Haplotype Analysis of DFNA8/12 Families

Most families enrolled in this study were screened for *TECTA* mutations by direct sequencing (American cohort) or by DHPLC (Spanish cohort); only three were suitable for linkage analysis. By using this approach we mapped the deafness gene segregating in these families to the DFNA8/12 locus with mutation screening of *TECTA* identifying mutations p.Cys1517Arg (Spanish family S1091), p.Asn465Lys (Belgian-K family), and p.Asn886Ser and c.5383+5delGTGA in *cis* (UK-E family).

Four of 23 mutations were detected in multiple families. The p.Cys1036Tyr and p.Cys1837Gly were found in two (S236, S694) and two (S324, S726) Spanish families, respectively; p.Thr1866Met in one American simplex case (504260) and one Spanish family

(S1385); and p.Arg1890Cys was detected in two Spanish (S488, S1080) and one American (10050) pedigree. The origin could be investigated for three of these mutations. Haplotype analysis showed that the S488, S1080 and 10050 families share a core haplotype (203-204, in base pairs) for *TECTA* intragenic markers D11S4089 and D11S4107 consistent with an ancestral founder for the p.Arg1890Cys mutation; however this core haplotype was not present in the Dutch family (W04-077) in which this mutation was first reported (Plantinga, et al., 2006) (Table 2). This suggests a common founder for this mutation in the Spanish and American populations but not the Dutch population. p.Cys1837Gly segregates on a common haplotype (208-173-203-200-102-246, in base pairs) shared by all three families that carry this mutation, including family S063 in which this mutation was originally identified. A single core haplotype (204-192-199-204-101-248-180, in base pairs) was also associated with the p.Thr1866Met mutation in the S1385 family, which is derived from a mating between genetically unrelated hearing impaired subjects (I:1 and I.2), although the disease haplotype associated with the American simplex case (504260) carrying this variant could not be unambiguously defined (Table 2, Fig. 3). Based on this data we confirmed one unique and two different founder effects for p.Cys1837Gly and p.Arg1890Cys, respectively.

Genotype-Phenotype Correlations - Mutations in the Entactin Domain and Zonadhesin Region

The p.Asp197Asn and p.Phe211Ser mutations in families 11420 and S206 are the first reported variants in the entactin domain. Disruption of this domain in both instances appears to be associated with stable MFSNHL (Table 1; Supp. Fig. S3A and B). The three previously reported ZA region mutations are located in the vWFD2-D3 inter-repeat connector (p.Cys1057Ser), and the vWFD4 repeat (p.Cys1509Gly, p.Cys1619Ser), and are all associated with HFSNHL (Table 1; Fig. 1) (Alloisio, et al., 1999; Balciuniene, et al., 1999; Collin, et al., 2008; Pfister, et al., 2004). This relationship is consistent with the novel p.Ala1098Val and p.Cys1517Arg mutations we identified, which are also located in the vWFD2-D3 connector and in the vWFD4 repeat, respectively. The remaining DFNA8/12 mutations we identified are the first to be reported in the vWFD1 repeat (p.Ser362Cys, p.Asn465Lys); in the vWFD1-D2 interdomain (p.Thr562Met); in the vWFD2 repeat (p.Thr815Met, p.Asn886Ser); in the trypsin inhibitor like cysteine rich domain 2 (TIL2), which is located in the vWFD2-D3 connector (p.Cys1036Tyr); and in the vWFD3 repeat (p.Asp1136His, p.Pro1248Leu) (Table 1, Fig 1). The majority of these mutations, with the exception of p.Asn886Ser, p.Asn1136His and p.Pro1248Leu, are associated with MFSNHL.

Genotype-Phenotype Correlations – Mutations in the Zona Pellucida Domain

The p.Pro1791Arg and the splice site mutations c.5383+2T>G and c.5383+5delGTGA affect the ZA-ZP interdomain; their impact on the structure and function of the α-tectorin protein is unknown. The P1791 residue is highly conserved, and substitution of an alkaline arginine sidechain for a bulky proline sidechain is likely to be deleterious. Interestingly, two of these mutations (p.Pro1791Arg and c.5383+2T>G) are associated with MFSNHL, a phenotype similar to the one resulting from the synonymous change Leu1777Leu which leads to exon 16 skipping and is also located in the ZA-ZP interdomain (Collin, et al., 2008).

The novel mutations identified in the ZP domain, p.His1867Arg, p.Cys1898Arg, p.Arg1947Cys and p.Ile2009Thr, also lead to MFSNHL, although p.Ile2009Thr affects the high-frequencies as well (Iwasaki, et al., 2002; Meyer, et al., 2007b; Moreno-Pelayo, et al., 2001; Plantinga, et al., 2006; Verhoeven, et al., 1998). Patients II:2, II:4 and II:5 of the S1385 family (Fig. 3) are homozygous for the p.Thr1866Met mutation and represent the first cases of homozygosity for a dominant *TECTA* mutation. Not surprisingly their hearing loss is more severe than that of older affected relatives who carry only a single mutant allele.

Another interesting observation is the progressive deterioration of TEOAEs in heterozygous carriers of the p.Cys1837Gly mutation in the S726 family (Fig. 2). The youngest mutation carrier, III:5, who was asymptomatic at the time of this study has normal TEOAEs in both ears. However, III:4 and III:2 have abnormal amplitude measurements, indicating that outer hair cell function is affected. In patient III:4, with mild hearing loss at age 4 years, TEOAEs were present for only two frequencies (2 and 4 kHz) in the right ear and for any frequency in the left ear. Patient III:2 had moderate-to-severe hearing loss at age 15 years and no TEOAEs. ABRs were present bilaterally in these three patients for stimulation intensities higher than hearing thresholds without distortion or alteration of the wave latency and interlatency measurements.

DISCUSSION

Efforts to determine the prevalence of the different types of ADNSHL have been hampered by the extreme clinical and genetic heterogeneity of NSHL, which mandates a serial gene-by-gene sequencing approach unless comprehensive genetic testing platforms are used. It is important to note that the OtoChip™ genetic testing platform that contains 19 deafness genes does not include the *TECTA* gene (Kothiyal, et al., 2010). However, the recently developed OtoSCOPE™ platform that screens all NSHL genes does include the *TECTA* gene and will soon be offered for clinical testing (Shearer, et al., 2010). In this study, we have conducted the first comprehensive genetic screen of *TECTA* in two large cohorts of patients to establish the mutation spectrum and genotype-phenotype correlations associated with this type of ADNSHL.

Prior to this study, only one Spanish and one American DFNA8/12 family had been reported (Table 1) (Meyer, et al., 2007b; Moreno-Pelayo, et al., 2001). Our identification of two additional American DFNA8/12 families and seven additional American patients for which family history data are not available suggests that hearing loss at the DFNA8/12 locus rivals in absolute numbers the prevalence of hearing loss at the DFNA6/14/38 (seven American cases) (Bespalova, et al., 2001; Cryns, et al., 2002; Gurtler, et al., 2005; Hildebrand, et al., 2008a) and DFNA2 (six American cases) loci (Coucke, et al., 1999; Hildebrand, et al., 2008b; Talebizadeh, et al., 1999, Arnett et al. 2011). In previous studies only two cases of DFNA20/26 and two of DFNA50 hearing loss were identified in the Spanish population by comprehensive genetic screenings in *ACTG1* [MIM# 102560] and *miR-96* [MIM# 611606] genes, respectively (Mencia, et al., 2009; Morin, et al., 2009). The identification of 16 novel Spanish familial cases with *TECTA* mutations in addition to the initial reported case makes DFNA8/12 hearing loss one of the most common forms of ADNSHL in the Spanish population and suggests that its global prevalence is about 4% of all ADNSHL (4.5%, 17/374 familial cases). The high number of novel mutations identified (20/23, representing 86.9% of the diagnosed cases) suggests that a significant proportion of DFNA8/12 cases in our cohorts is due to private mutations. This may also be true worldwide as of the 33 DFNA8/12 mutations now reported only four (p.Cys1036Tyr, p.Cys1837Gly, p.Thr1866Met and p.Arg1890Cys) have been observed in multiple unrelated families and in only one (p. Cys1837Gly) was a unique founder effect confirmed (Table 1 and 2).

Our data reveal that mutations are not mainly concentrated in the ZP domain, but widely distributed throughout the α-tectorin protein structure, allowing us to establish novel genotype-phenotype correlations. Mutations for the first time reported in TIL2, vWFD2, vWFD1-D2 connector, and the vWFD1 repeat are associated with MFSNHL, conversely to all previous ZA mutations that lead to HFSNHL (Table 1). These distinct genotype-phenotype correlations (Balciuniene, et al., 1999; Plantinga, et al., 2006) are not unprecedented as mutations in *COL11A2* [MIM# 120290] at the DFNA13 locus also result in MFSNHL or HFSNHL (Kunst, et al., 2000; McGuirt, et al., 1999) and induce collagenous

TM abnormalities (McGuirt, et al., 1999). It is unclear why disruption of different repeats in the ZA region leads to distinct audioprofiles, although it may indicate that the role of vWFD repeats in α-tectorin assembly and maturation differ. It is possible that ZA region mutations disrupt processing of the three polypeptides of α-tectorin since the ZA region is homologous to the sperm membrane protein zonadhesin, which is known to interact with the zona pellucida by cleavage of the protein into two disulphide-linked polypeptides (Killick, et al., 1995). If processing of α-tectorin is similar, ZA region mutations might interfere with polypeptide assembly thus disrupting the extracellular matrix in the TM and impacting distinctly different sound frequency ranges.

Mutations of the entactin domain also cause MFSNHL. Together with type IV collagen, laminin and heparan sulfate proteoglycans, entactin is a major component of basement membranes where it facilitates cell adhesion by binding to calcium ions (Chung, et al., 1993; Durkin, et al., 1988). It has been predicted that entactin interacts with laminin and type IV collagen by acting as a bridge and inducing their deposition in the extracellular matrix (Dziadek, et al., 1985). Thus it is possible that the entactin domain of α-tectorin facilitates assembly and modeling of the extracellular matrix of the TM and the mutations identified could impact more drastically its role during mechanotransduction of mid-frequency sound. Consistent with this observation is the severe elevation of hearing threshold in the mid-(5-20 kHz) frequency region in transgenic mice homozygous for a recessive deletion in the entactin domain (Legan, et al., 2000).

Collectively, our results suggest that mutations in the Nt-region (N-terminal-region) of α-tectorin (entactin, vWFD1 and vWFD2) are associated with MFSNHL, a phenotype previously linked to mutations in the ZP domain. However, two exceptions to the above genotype-phenotype correlations are the p.Val317Glu mutation in the vWFC-D1 connector (Sagong, et al., 2010), and the p.Asn886Ser mutation in the vWFD2 repeat, both of which induce HFSNHL. Patients with the p.Asn886Ser mutation also carry in cis the c. 5383+5delGTGA mutation in the ZA-ZP inter-domain, which could be contributing to the HFSNHL phenotype. The p.Val317Glu mutation lies very close to the vWFC domain and as no mutations have been identified in this domain, it is unclear how its disruption may impact processing or maturation of the ZA region. Whether this mutation could initially result in pure mid-frequency hearing loss is unknown as carriers of this mutation were clinically examined for the first time at the sixth decade of age. Therefore, the progressive deterioration in high frequency hearing observed in normal age-matched controls likely contributes to the observed downsloping audiometric profile.

Previous studies have shown that the progression of DFNA8/12 hearing loss appears to be related to the residue mutated since in all cases a cysteine is altered. We provided TEOAE data from family S726 carrying the p.Cys1837Gly mutation suggesting that the progressive hearing loss due to this *TECTA* mutation parallels a progressive loss of outer hair cell function. Consistent with this observation is temporal bone histopathology from two unrelated family members with mid-frequency hearing loss that showed loss of inner and outer hair cells and atrophy of the stria vascularis at the time of death (77 and 82 years of age) (Bahmad, et al., 2008). It is likely that hair cell loss and strial damage arises secondary to TM dysfunction. Transgenic mice for the p.Cys1509Gly mutation, which causes progressive HFSNHL in humans, also show altered otoacoustic emissions secondary to the anatomic malformation of the TM (Xia, et al., 2010). This pathophysiological aspect has not been observed in previously published mice with *Tecta* mutations, including the p.Tyr1870Cys mutation responsible for stable hearing loss (Legan, et al., 2000; Legan, et al., 2005). Whether the progression is exclusive to cysteine mutations remains a possibility as clinical data for many patients in this study were limited to a single timepoint making it impossible to confirm or refute progression of the hearing loss. However, two notable

exceptions were noticed. Serial audiograms from patient IV:1 in family S694 who carries the ZA region p.Cys1036Tyr mutation indicate a stable hearing loss (Supp. Fig. S3A). In contrast, the age-related typical audiograms (ARTA) analyses performed on the Belgian-K family, in which a cysteine is not mutated (p.Asn465Lys), indicate the hearing loss is progressive (Supp. Fig. S4).

All ZP mutations with the exception of p.Ile2009Thr lead to MFSNHL. The common p.Arg1890Cys mutation located in the ZP domain identified in both cohorts in this study was originally reported in cis with the p.Thr83Met substitution, which is not located in a known domain and is not predicted to affect splicing (Plantinga, et al., 2006). However, as p.Thr83Met was not present in controls, an effect on phenotype cannot be ruled out. It is therefore important to note that the hearing thresholds of families 10050, S488 and S1080 (Supp. Fig. S3B), all segregating the p.Arg1890Cys mutation (Table 2), are very similar to those reported in the original Dutch family, suggesting that the impact of the p.Thr83Met mutation is negligible.

We also found that members of family S1385 were homozygous for the p.Thr1866Met mutation, the first example of homozygosity for a dominant *TECTA* mutation. Their hearing loss suggests a dosage effect on TM function – family members homozygous for the mutant allele have more severe hearing loss than that of older affected relatives who are heterozygous for the mutant allele. Consistent with this observation are morphological and phenotypic data from transgenic mice carrying the p.Tyr1870Cys ZP domain mutation (Legan, et al., 2005). In heterozygous mice, the TM is inadequately attached to the limbal zone and has a distinctive ‘hump back’ morphology; in comparison, in homozygous mice the phenotype is more drastic – the TM is completely detached, similar to transgenic mice homozygous for a deletion in the entactin domain, the p.Cys1509Gly ZA missense mutation or a spontaneous, recessive, missense mutation (p.Ala349Asp) (Legan, et al., 2000; Moreno-Pelayo, et al., 2008).

The substantial increase in genotypic and phenotypic data yielded by this study will lead to better diagnostic and prognostic information for DFNA8/12 patients, who apparently represent a substantial proportion of ADNSHL cases. The number of dominant *TECTA* mutations has increased over two-fold worldwide (from 13 to 33) based on this report and collectively these data suggest that DFNA8/12 is one of the most frequent subtypes of autosomal dominant hearing loss.

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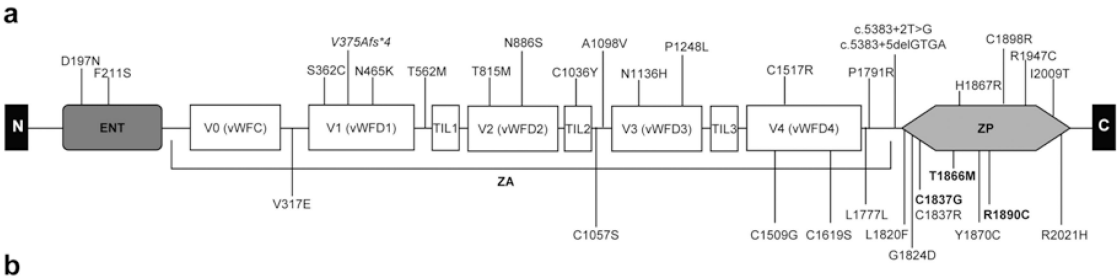

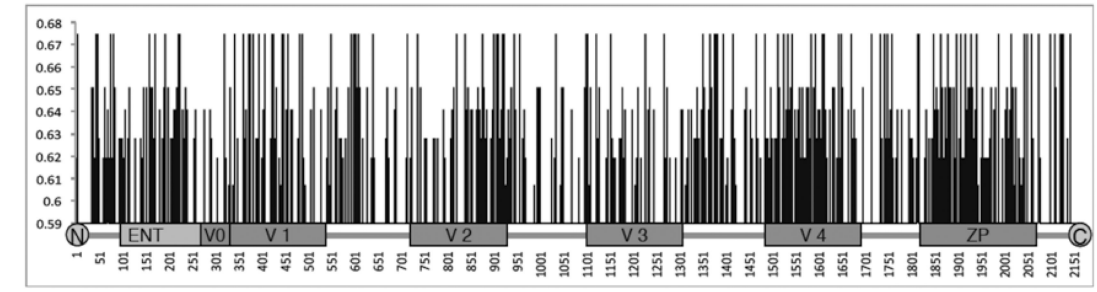

c

Domain	Amino Acids	Exons
ENT-Like	98-252	3-5
V0 (VWFC)	260-314	5-6
V1 (VWFD 1)	321-540	6-7
TIL1	597-650	8
V2 (VWFD 2)	712-929	8-9
TIL2	984-1036	10
V3 (VWFD 3)	1099-1317	10-11
TIL3	1372-1425	12
V4 (VWFD 4)	1486-1694	13-15
ZP	1805-2059	17-21

Figure 1.

a Scheme depicting the different domains of the α-tectorin protein. The novel pathogenic mutations and those previously identified are shown at the top and at the bottom, respectively. The mutations described in this study among the previously identified are highlighted in bold. **b** Highly conserved amino acids of the α-tectorin protein based on Conseq scores, showing conserved protein domains (to scale). **c** Domains and regions of the α-tectorin protein. The amino acids and exons that comprise each domain are indicated. ENT, entactin domain; V0 (vWFC), von Willebrand factor C domain; V1 (vWFD1), von Willebrand factor D domain 1; V2 (vWFD2), von Willebrand factor D domain 2; V3 (vWFD3), von Willebrand factor D domain 3; V4 (vWFD4), von Willebrand factor D domain 4; ZP, zonapellucia domain; ZA, zonadhesin region; TIL1, trypsin inhibitor like cysteine rich domain 1; TIL2, trypsin inhibitor like cysteine rich domain 2; TIL3, trypsin inhibitor like cysteine rich domain 3.

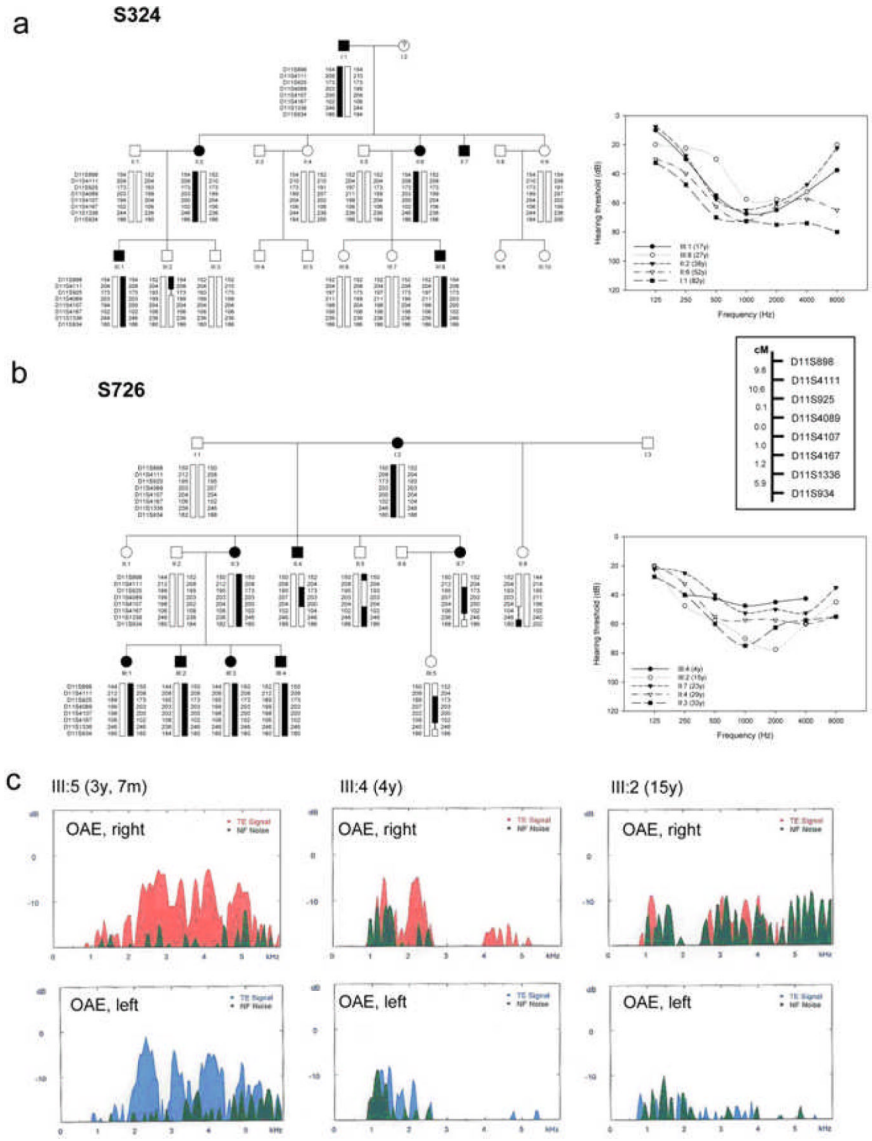

Figure 2.

a - b Pedigrees of the Spanish families S324 and S726. Black and white symbols indicate the affected and the unaffected subjects, respectively. Haplotypes are represented by bars, with haplotype associated with hearing loss in black. The numbers placed beside chromosomes are the allelic sizes for each microsatellite marker. The box shows the relative order and genetic distances of the microsatellite markers spanning the DFNA8/12 region. On the right are audiograms showing the hearing threshold values obtained from five different patients of each family - III:1, III:8, II:2, II:6 and I:1 of S324 and III:4, III:2, II:7, II:4 and II: 3 of S726. Each graph point represents the average hearing loss for the right and left ears. **c** Transient evoked otoacoustic emissions (TEOAEs) of patients III:5, III:4 and III:2 of family S726.

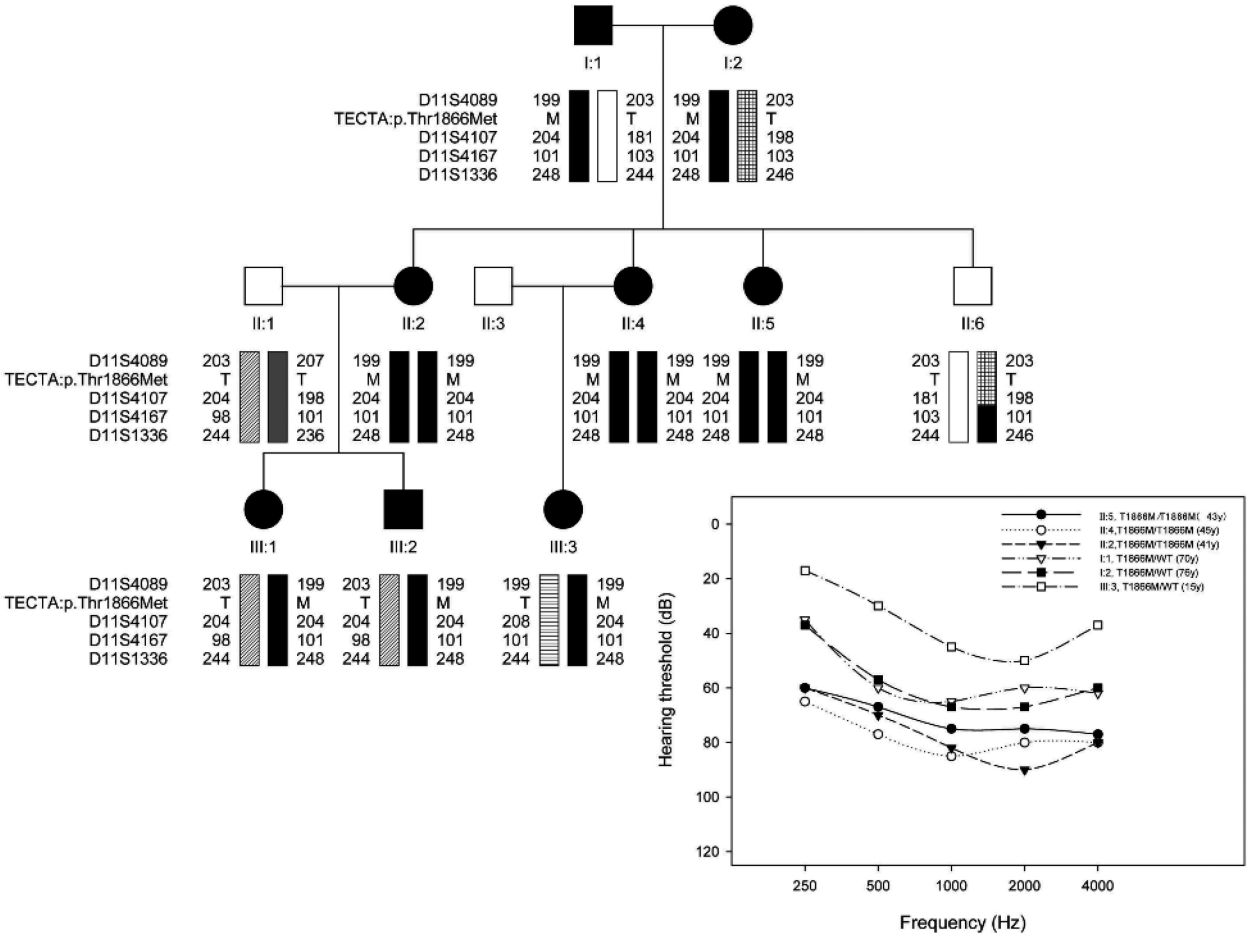

Figure 3.

Pedigree of the Spanish family S1385. The marks and symbols are as described in Fig. 2. The relative order of the *TECTA* gene and the microsatellite markers within the DFNA8/12 genetic interval is indicated. The mutation found in the family is notated as TECTA:p.Thr1866Met. Audiograms show the mean of the hearing threshold values obtained from the left and right ears in the patients II:5, II,4, II:2, I:1, I:2 and III:3 at the time of recording.

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Table 1

All known *TECTA* (DFNA8/12 or DFNB21) mutations including those identified in this study.

Inheritance pattern	Exon	Mutation[#]	Protein change	Domain[a]	Time of onset	Progression	Frequencies	Family	Family origin	Reference
AD	**4**	**c.589G>A**	**p.Asp197Asn**	**ENT**	**Postlingual**	**Stable**	**Mid**	**11420**	**American**	**This work**
AD	**5**	**c.632T>C**	**p.Phe211Ser**	**ENT**	**Postlingual**	**Stable**	**Mid**	**S206**	**Spanish**	**This work**
AD	6	c.950T>A	p.Val317Glu	ZA (none)	Postlingual	Unknown	High		Korean	Sagong et al. 2010
AD	**6**	**c.1084A>T**	**p.Ser362Cys**	**ZA (VWFD1)**	**Postlingual**	**Unknown**	**Mid**	**10180**	**American**	**This work**
AD	**6**	**c.1124delT**	**p.Val375Alafs*4**	**ZA (VWFD1)**	**Postlingual**	**Unknown**	**Mid**	**S1203**	**Spanish**	**This work**
AD	**7**	**c.1395T>G**	**p.Asn465Lys**	**ZA (VWFD1)**	**Postlingual**	**Progressive**	**Mid**	**Belgian-K**	**Belgian**	**This work**
AD	**7**	**c.1685C>T**	**p.Thr562Met**	**ZA (none)**	**Postlingual**	**Unknown**	**Mid**	**10680**	**American**	**This work**
AD	**9**	**c.2444C>T**	**p.Thr815Met**	**ZA (VWFD2)**	**Prelingual**	**Unknown**	**Mid**	**515250**	**American**	**This work**
AD	**9**	**c.2657A>G**	**p.Asn886Ser**	**ZA (VWFD2)**	**Prelingual**	**Progressive**	**High**	**UK-E**	**UK**	**This work***
AD	**10**	**c.3107G>A**	**p.Cys1036Tyr**	**ZA (TIL2)**	**Postlingual**	**Stable**	**Mid Mid**	**S236 S694**	**Spanish Spanish**	**This work**
AD	10	c.3169T>A	p.Cys1057Ser	ZA (none)	Postlingual	Progressive	High		Swedish	Balciuniene et al 1999
AD	**10**	**c.3293C>T**	**p.Ala1098Val**	**ZA (none)**	**Postlingual**	**Unknown**	**High**	**S052**	**Spanish**	**This work**
AD	**10**	**c.3406G>C**	**p.Asp1136His**	**ZA (VWFD3)**	**Postlingual**	**Unknown**	**High**	**S984**	**Spanish**	**This work**
AD	**11**	**c.3743C>T**	**p.Pro1248Leu**	**ZA (VWFD3)**	**Prelingual**	**Unknown**	**High**	**S1360**	**Spanish**	**This work**
AD	13	c.4525T>G	p.Cys1509Gly	ZA (VWFD4)	Unknown	Progressive	High		Turkish	Pfister et al 2004
AD	**13**	**c.4549T>C**	**p.Cys1517Arg**	**ZA (VWFD4)**	**Postlingual**	**Progressive**	**High**	**S1091**	**Spanish**	**This work**
AD	14	c.4856G>C	p.Cys1619Ser	ZA (VWFD4)	Postlingual	Progressive	High		French	Alloisio et al 1999
AD	16	c.5331G>A	p.Leu1777Leu	ZA (none)	Prelingual	Stable	Mid		Dutch	Collin et al 2008
AD	**16**	**c.5372C>G**	**p.Pro1791Arg**	**ZA (none)**	**Prelingual**	**Unknown**	**Mid**	**505510**	**American**	**This work**
AD		**c.5383+2T>G**		**ZA (none)**	**Prelingual**	**Stable**	**Mid**	**S1615**	**Spanish**	**This work**
AD		**c.5383+5del GTGA**		**ZA (none)**	**Prelingual**	**Progressive**	**High**	**UK-E**	**UK**	**This work***
AD	17	c.5458C>T	p.Leu1820Phe	ZP	Postlingual	Stable	Mid		Belgian	Verhoeven et al 1998 *

Inheritance pattern	Exon	Mutation[#]	Protein change	Domain[a]	Time of onset	Progression	Frequencies	Family	Family origin	Reference
AD	17	c.5471G>A	p.Gly1824Asp	ZP	Postlingual	Stable	Mid		Belgian	Verhoeven et al. 1998 *
AD	17	c.5509T>G	p.Cys1837Gly	ZP	Postlingual	Progressive	Mid		Spanish	Moreno-Pelayo et al. 2001
AD	**17**	**c.5509T>G**	**p.Cys1837Gly**	**ZP**	**Postlingual**	**Progressive**	**Mid**	**S324 S726**	**Spanish Spanish**	**This work**
AD	17	c.5509T>C	p.Cys1837Arg	ZP	Postlingual	Progressive	Mid		American	Meyer et al. 2007
AD	18	c.5597C>T	p.Thr1866Met	ZP	Postlingual	Stable	Mid		Korean	Sagong et al. 2010
AD	**18**	**c.5597C>T**	**p.Thr1866Met**	**ZP**	**Postlingual Postlingual**	**Progressive Unknown**	**Mid Mid**	**S1385 504260**	**Spanish American**	**This work**
AD	**18**	**c.5600A>G**	**p.His1867Arg**	**ZP**	**Postlingual**	**Progressive**	**Mid**	**S798**	**Spanish**	**This work**
AD	18	c.5609A>G	p.Tyrl870Cys	ZP	Prelingual	Stable	Mid		Austrian	Verhoeven et al. 1998
AD	18	c.5668C>T	p.Arg1890Cys	ZP	Prelingual	Stable	Mid		Dutch	Plantinga et al. 2006
AD	**18**	**c.5668C>T**	**p.Arg1890Cys**	**ZP**	**Prelingual**	**Stable**	**Mid**	**S488 S1080 10050**	**Spanish Spanish American**	**This work**
AD	**18**	**c.5692T>C**	**p.Cys1898Arg**	**ZP**	**Postlingual**	**Unknown**	**Mid**	**520510**	**American**	**This work**
AD	**19**	**c.5839C>T**	**p.Arg1947Cys**	**ZP**	**Postlingual**	**Unknown**	**Mid**	**CDS-3151**	**American**	**This work**
AD	**20**	**c.6026T>C**	**p.Ile2009Thr**	**ZP**	**Postlingual**	**Stable**	**High**	**S193**	**Spanish**	**This work**
AD	20	c.6062G>A	p.Arg2021His	ZP	Prelingual	Stable	Mid		Japanese	Iwasaki et al. 2002
AR	3	c.266delT	p.Leu89Argfs*34	ENT	Prelingual	Stable	Mid		Iranian	Meyer et al. 2007
AR	5	c.651dupC	p.Asn218Glnfs*31	ENT	Prelingual	Stable	All freq.		Iranian	Naz et al. 2003
AR		c.2941+1G>A		ZA	Prelingual	ND	All freq.			Mustapha et al. 1999
AR		9.6 Kb del		ZA	Prelingual	Stable	Mid		Iranian	Meyer et al. 2007
AR	15	c.5211C>A	p.Tyr1737*	ZA	Prelingual	Stable	Mid		Iranian	Meyer et al 2007
AR	20	c.6037delG	p.Glu2013Argfs*6	ZP	Prelingual	Stable	Mid		Pakistani	Naz et al. 2003
AR	21	c.6203-6218del	p.Lys2068Argfs*38	ZP	Prelingual	Stable	All freq.		Iranian	Alasti et al. 2008

In bold mutations identified in this work

[a] ENT: Entactin domain, ZA: Zonadhesin region, ZP: zona pellucida domain.

[*] This mutation was present in the same family

[#] TECTA gene sequence [RefSeq: NM_005422.2]. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Table 2

Haplotypes associated with the *TECTA* mutations R1890C, C1837G and T1866M

Marker	S488	S1080	10050	W04-077[2]	S063	S324	S726	S1385	504260	Genotype for CEPH individual 134702	Heterozygosity[1]
D11S4111	210	212	214	210	**208**	**208**	**208**	**204**	206/206	208/210	84%
D11S925	**175**	**175**	190	196	**173**	**173**	**173**	**192**	175/198	173/173	84%
D11S4089	**203**	**203**	**203**	211	**203**	**203**	**203**	**199**	**199**/207	199/207	74%
Mutation	**R1890C**	**R1890C**	**R1890C**	**R1890C**	**C1837G**	**C1837G**	**C1837G**	**T1866M**	**T1866M**		
D11S4107	**204**	**204**	**204**	198	**200**	**200**	**200**	**204**	198/202	198/202	78%
D11S4167	**106**	**106**	101	100	**102**	**102**	**102**	**101**	108/110	98/108	78%
D11S1336	**244**	**244**	236	236	**246**	**246**	**246**	**248**	236/242	236/244	67%
D11S934	180	186	184	180	**186**	**186**	180	**180**	180/180	180/186	84%

Relative order and physical distances are as follows: D11S4111- 5 Mb -D11S925- 161 Kb -D11S4089- 59.4 Kb -D11S4107- 1.1 Mb -D11S4167- 488 kb-D11S1336- 3.4 Mb- D11S934. Markers comprising the shared core haplotype are depicted in bold face and those depicted in italics are intragenic to *TECTA* gene [RefSeq: NM_005422.2]. The marker heterozygosity and allele frequencies were calculated from a sample group of 50 Spanish normal-hearing subjects. To allow other laboratories to compare their data with those reported in this work, we provide allele sizes for individual 134702, available from CEPH (Dib et al. 1996). We have included for this analysis the family W04-077 in which the mutation R1890C was originally identified (Platinga et al 2006).