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Progressive Sensorineural Hearing Loss and Normal Vestibular Function in a Dutch DFNB7/11 Family with a Novel Mutation in *TMC1*

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Key Words
Cochlear implant · Sensorineural hearing loss, progressive · Audioprofile · DFNB7/11 · *TMC1*

Abstract
In a Dutch family with autosomal recessive hearing loss, genome-wide single-nucleotide polymorphism analysis mapped the genetic defect to the DFNB7/11 locus. A novel homozygous A-to-G change in the TMC1 gene was detected near the splice donor site of intron 19 (c.1763+3A→G) segregating with the hearing loss in this family. One of the 6 transmembrane domains and the actual TMC channel domain are predicted to be absent in the mutant protein. The sensorineural hearing impairment in this DFNB7/11 family has a postlingual onset. Audiometric analysis initially showed a steeply downward-sloping threshold configuration. The progressive phenotype in this family resembles the phenotype previously described for families with dominant TMC1 mutations (DFNA36) rather than that of families with recessive TMC1 mutations (DFNB7/11) which invariably cause severe-to-profound prelingual hearing impairment.

Introduction

Approximately 1 in 1000 children are affected by severe-to-profound hearing loss at birth or will develop this during childhood [Morton, 1991]. At least 50% of childhood hearing impairment in developed countries is attributed to genetic defects [Toriello et al., 2004]. Approximately 70% of these cases are nonsyndromic, i.e. not associated with other distinctive clinical features. Autosomal recessive inheritance is the most prevalent pattern of inheritance (70–80%) in cases with nonsyndromic hearing loss [Morton, 1991].

The autosomal recessive nonsyndromic forms of hearing impairment (DFNB) are generally severe with a congenital or prelingual onset. Many of the autosomal dominant nonsyndromic forms (DFNA) are postlingual, exhibit a variable age of onset, variable audiometric presentations, severity, and rate of progression.

Nonsyndromic hearing impairment is genetically extremely heterogeneous; already more than 60 DFNB loci

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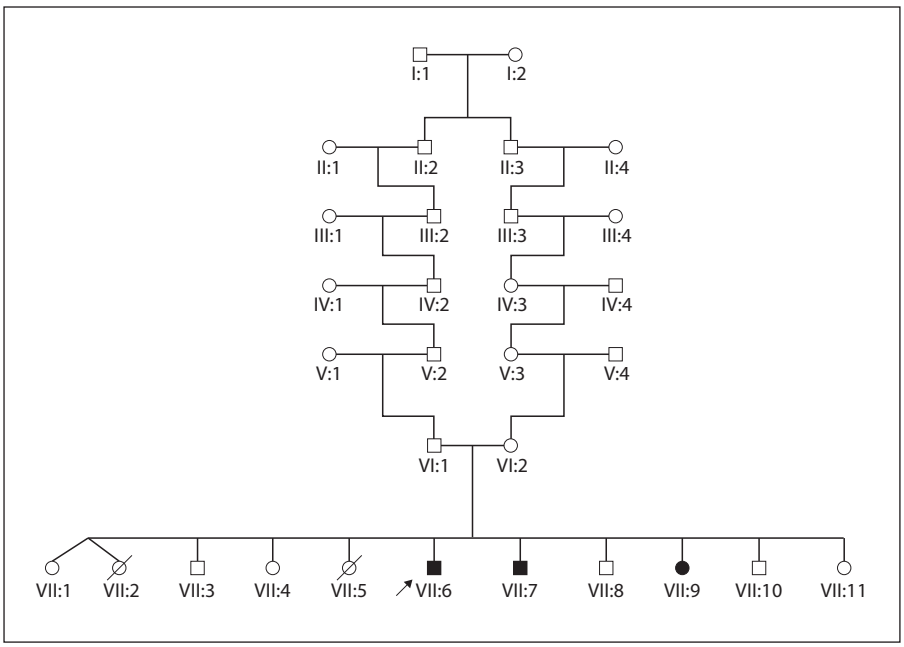

Fig. 1. Pedigree of Dutch family W06-792 with the novel *TMC1* mutation (c.1763+3A→G). The pedigree has been modified for privacy reasons. Squares = males; circles = females; black symbol = clinically affected; empty symbol = clinically unaffected; slash = deceased individual; arrow = proband. The generation is indicated in the symbol legend.

and 30 of the causative genes have been identified [Ahmed et al., 2008; Collin et al., 2008b; van Camp and Smith, 2008].

Different mutations in the transmembrane channel-like 1 gene *(TMC1)* can cause prelingual autosomal recessive profound deafness (DFNB7/11) as well as postlingual progressive autosomal dominant (DFNA36) sensorineural hearing loss [Kurima et al., 2002]. *Tmc1* is expressed in the inner and outer hair cells of the murine cochlea and in the hair cells in the vestibular labyrinth. The precise function of TMC1 remains to be determined, but the transmembrane proteins encoded by the *TMC* gene family are thought to act as ion channels, ion pumps or transporters [Keresztes et al., 2003; Kurima et al., 2003].

This report provides a detailed audiometric and vestibular phenotype description of affected members of a Dutch family with autosomal recessive hearing loss (DFNB7/11). Remarkably, the nonsyndromic sensorineural hearing impairment in this family starts as a moderate impairment and shows progression. The affected persons have been ascertained during childhood and their audiometric characteristics until young adulthood have been previously described [Cremers, 1979; McKusick, 1992]. Genome-wide homozygosity mapping, as part of a project to trace genes involved in autosomal recessive sensorineural hearing loss in the Dutch population, followed by sequence analysis of *TMC1,* revealed a novel mutation (c.1763+3A→G) to be causative of the hearing loss in this family. After identification of the underlying genetic defect, we performed a detailed clinical follow-up study to improve genotype-phenotype correlations.

Patients and Methods

Patients

In 1979, we reported on a family with 11 siblings including 3 individuals who suffered from a remarkable type of nonsyndromic, progressive sensorineural hearing impairment. An autosomal recessive pattern of inheritance was shown [Cremers, 1979]. We updated the clinical characteristics and follow-up of these 3 affected family members as part of a project in which we unravel the causes of autosomal recessive nonsyndromic hearing loss in the Dutch population. The pedigree of this family (W06-792) [Cremers, 1979] was extended (fig. 1). Informed consent was obtained from all participating individuals. The study was approved by the local medical ethics committee (Commissie Wetenschappelijk Onderzoek bij Mensen; project No. 9504-0521).

Most of the participating family members underwent puretone audiometry. In the affected individuals, speech audiometry and vestibular testing was carried out as well. Also, microotoscopy was performed again. Peripheral blood samples were obtained for DNA and RNA isolation. To allow individual longitudinal analysis, previous medical records and audiograms were retrieved. Computerized tomography of the temporal bone was performed as part of the cochlear implant procedures.

Genotyping

Genomic DNA from peripheral blood lymphocytes of all participating individuals was extracted as described by Miller et al.

[1988]. After exclusion of the involvement of *GJB2* via linkage analysis with variable number tandem repeat (VNTR) markers, genotyping was performed for the 3 affected individuals by using Affymetrix 250K single-nucleotide polymorphism (SNP) arrays (*NspI* array; Affymetrix, Santa Clara, Calif., USA) and homozygous regions determined by the CNAG software program [Nannya et al., 2005]. Chromosomal segments were accepted to be homozygous if the loss-of-heterozygosity (LOH) score was ≥8. The LOH score is a measure of the likelihood of a stretch of SNPs to be homozygous based on the population SNP allele frequencies. Confirmation of homozygosity of the candidate region was achieved by VNTR markers (D9S1822, D9S1837 and D9S1876). The genomic localization of the markers was derived from the UCSC (University of California, Santa Cruz) human genome database (http://genome.ucsc.edu/).

Mutation Analysis

Primers for all coding exons and the intron-exon boundaries of *TMC1* were designed and the exons of this gene amplified under standard PCR conditions. Primer sequences are listed in online supplement table 1 (www.karger.com/doi/10.1159/000313282). Sequence analysis was performed as described before [Collin et al., 2008a] using the same primers that were used for PCR. Primer sequences are listed in online supplement table 1. The occurrence of the observed mutation (c.1763+3A→G) in family W06-792, as well as in a panel of 80 Dutch probands with (presumably) recessive hearing loss and in 177 Dutch normally hearing individuals, was tested with *Sau*96I restriction digestion.

RNA Analysis

RNA of 1 of the affected individuals of family W06-792 was isolated from peripheral blood collected and isolated using the Paxgene Blood RNA Kit (Qiagen Benelux BV, Venlo, The Netherlands). Subsequently, RNA was reverse transcribed into cDNA according to standard procedures using random hexamer primers. A nested PCR approach to cDNA was used to amplify part of the *TMC1* transcript containing exons 18–20, by using forward primer 5′-TGACCACCTACGTCACAATCC-3′ and reverse primer 5′-AAAGATGGTGGGAGGGACAC-3′. For the nested PCR, forward primer 5′-CAATCCTCATTGGGGACTTTC-3′ and reverse primer 5′-GCATTGTGGACAGGAAGAGG-3′ were employed. PCR products were purified from agarose gels and subsequently sequenced with the forward and reverse primers used in the nested PCR as described above.

Minigene Construction and Analysis

A plasmid containing the genomic region encompassing exons 3–5 of *RHO* inserted at the EcoRI/SalI sites in the pCI-NEO vector was used for in vivo splicing assays [Gamundi et al., 2008]. The plasmid was digested with EcoNI and PflMI (New England Biolabs, Beverly, Mass., USA) according to the manufacturer's protocol, resulting in the removal of exon 4 and part of the flanking intronic sequences. Subsequently, a fragment containing *TMC1* exon 19 and approximately 600 bp of flanking intronic sequences on each side were amplified from genomic DNA of a patient carrying the c.1763+3A→G mutation and of a wild-type control, and also digested with EcoNI and PflMI. The primer sequences are listed in online supplement table 1. Purification of the digested PCR products and plasmids was performed after size separation of the digests on agarose gels with NucleoSpin Extract II columns (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Subsequently, the fragments and plasmids were ligated by using the Rapid DNA Ligation Kit (Roche, Mannheim, Germany).

HEK293T cells (80% confluency) in a 6-well plate were transfected with 1 μg of plasmid DNA in duplicate, using Effectene Transfection Reagent (Qiagen Benelux BV) according to the manufacturer's protocol. After transfection, the cells were incubated for 24 h at 37°C and RNA isolation was performed using the NucleoSpin RNA II Kit (Macherey-Nagel) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA according to standard procedures using random hexamer primers. The *RHO* and *TMC1* exons were amplified from the cDNA with forward primer 5′-cggaggtcaacaacgagtct-3′ and reverse primer 5′-aggtgtaggggatgggagac-3′, which are located in *RHO* exon 3 and exon 5, respectively. PCR fragments were sequenced as described [Collin et al., 2008a].

Audiometry and Data Analysis

Pure-tone and speech audiometry were performed in a sound-shielded room, according to the International Organization for Standardization (ISO) norms [International Organization for Standardization, 1985, 1989]. The threshold was fixed at an arbitrary value of 130 dB hearing level (HL) when measurements were out of scale. The individual 95th percentile threshold values of presbyacusis (P95) were derived in relation to the participant's gender and age at each frequency, using the ISO 7029 method [International Organization for Standardization, 1984]. Individuals were considered to be clinically affected when the best ear showed air conduction thresholds beyond the P95 for at least 2 frequencies. Bone conduction thresholds were also measured to exclude conductive or mixed hearing impairment.

To describe the level of hearing impairment, the GENDEAF (European Thematic Network on Genetic Deafness) criteria were used: mild = 20–40 dB; moderate = 41–70 dB; severe = 71–95 dB, and profound = >95 dB [Mazzoli et al., 2003].

Binaural mean pure-tone thresholds (air conduction level in dBHL) were calculated and plotted for each frequency for each individual.

Speech audiometry was performed in a quiet environment using standard monosyllabic Dutch word lists. Maximum monaural phoneme recognition scores were derived from individual performance versus intensity plots. Phoneme recognition scores (averaged over both ears) were plotted against age and the binaural mean pure-tone average at the frequencies of 1, 2 and 4 kHz ($PTA_{1,2,4\,kHz}$) [Bom et al., 2001].

Vestibular Examination and Data Analysis

All 3 mutation carriers underwent vestibular examination at ages of 46–55 years. The horizontal vestibuloocular reflex was evaluated in the dark with the eyes open, using electronystagmography and computer analysis. Saccades, smooth-pursuit eye movements and optokinetic nystagmus responses were evaluated, as well as gaze-evoked and spontaneous nystagmus. Vestibular stimulation included velocity steps of 90°/s and caloric tests, as described previously [Marres et al., 1997; Verhagen et al., 1992]. One of the affected individuals (VII:9) also underwent vestibular examination elsewhere, which included caloric testing and sinusoidal rotation at 0.1 Hz and 50°/s maximum velocity.

Evaluation of the Effect of Cochlear Implantation on Phoneme Recognition

Pre- and postimplant performance was tested by open-set word testing, using a meaningful monosyllabic word list. The words were presented through a loudspeaker at a fixed distance of 1 m from the patient at a normal conversational level of 70 dB sound pressure level (SPL). Subject responses were scored as percentage of correct phonemes. The reference groups were composed of postlingually implanted adults, and their test results, after 1 year of use, were analyzed. The first group (n = 70), implanted with a CII/HR90K device by Advanced Bionics, showed a mean phoneme score of 64% (SD = 23%). The second group (n = 65), implanted with a Nucleus 24RCA by Cochlear, showed a mean phoneme score of 72% (SD = 18%) [Verhaegen et al., 2008].

Results

The pedigree of family W06-792 (fig. 1) shows 3 clinically affected members in 1 sibship; a distant consanguinity had previously been traced by a genealogical search [Cremers, 1979]. All 3 affected individuals were diagnosed with progressive sensorineural hearing loss. The parents and the other siblings had normal hearing.

There was no evidence of any nongenetic cause of the hearing impairment, and a syndromic type of hearing impairment was excluded because no other physical (urine analysis, neurological, ophthalmological and hematological examination) abnormalities were found [Cremers, 1979]. This was confirmed by a recent update of the medical history. No substantial noise exposure was reported. The 3 affected individuals had noticed first symptoms of hearing impairment at ages of 4, 7 and 11 years (VII:9, VII:6 and VII:7, respectively), while the first audiometric tests were performed 2–5 years later. Otoscopy did not reveal any anomalies. All 3 persons affected made use of a conventional hearing aid before the age of 20 years. Tinnitus was not reported by any of them.

Mapping of the Genetic Defect to the DFNB7/11 Locus

To find the locus carrying the genetic defect that causes the hearing loss in family W06-792, a 250K SNP array was used to genotype the 3 affected individuals. The genotypes were analyzed for homozygous regions shared by all affected individuals since the pedigree suggests recessive inheritance and the parents are distantly related. The 3 affected individuals shared only 1 homozygous region on chromosome 9 (71.4–72.7 Mb) flanked by SNP_A-1861230 and SNP_A-4216934. VNTR marker analysis confirmed a segregation of this region with the disease: only the 3 affected individuals were homozygous for this region, whereas both parents and all the nonaffected siblings showed different haplotypes (data not shown), with a maximum 2-point LOH score of 2.14 for marker D9S1876. Nine known genes resided within the homozygous region, among which was *TMC1,* which is associated with autosomal recessive (DFNB7/11) and dominant (DFNA36) nonsyndromic hearing impairment [Kurima et al., 2002].

Mutation Analysis of TMC1

For 1 affected family member (VII:6), the coding region and the intron-exon boundaries of the *TMC1* gene were analyzed for the presence of a mutation by DNA sequencing. A homozygous A-to-G transition was detected in intron 19 close to the boundary of exon 19 (c.1763+3A→G), as shown in figure 2a.

A unique *Sau*96I restriction site was created by the mutation. To verify segregation of the mutation in the family, a restriction digestion of the PCR fragment containing exon 19 and flanking DNA was performed with *Sau*96I. In the 3 affected individuals, both mutant alleles were digested, in the parents 1 of their alleles. The unaffected siblings were either heterozygous carriers or had 2 wild-type alleles (data not shown for privacy reasons).

In the 177 Dutch control individuals, 1 heterozygous carrier of the c.1763+3A→G change was identified, but the change was not found in a homozygous state in any of the control individuals. Furthermore, this mutation was not present in any of the 80 Dutch probands with (presumably) recessive hearing loss, indicating that it is not a frequent cause of hearing impairment in the Dutch population. The mutation detected in family W06-792 has not previously been reported (table 1; fig. 2d).

Effect of Splice Site Mutation on Splicing

Several splice site prediction programs (http://www.fruitfly.org/seq_tools/splice.html and http://www.cbs.dtu.dk/services/NetGene2/) predicted the actual splice donor site to be lost by the mutation. In addition, a weak alternative splice donor site was predicted to be present 47 bp downstream in intron 19. To determine *TMC1* splicing in vivo, RNA was isolated from blood of 1 of the affected individuals followed by RT-PCR analysis of *TMC1* RNA. The nested PCR on the cDNA for exons 18–20 of *TMC1* resulted in a PCR product with an increased size in 1 affected individual as compared to that in a control sample, implying the use of a different splice site in these individuals (fig. 2b). Sequence analysis of this PCR fragment showed that exon 19 was extended with 47 bp of intron 19 (fig. 2c). By additional experiments with all 3 affected individuals, we found both the

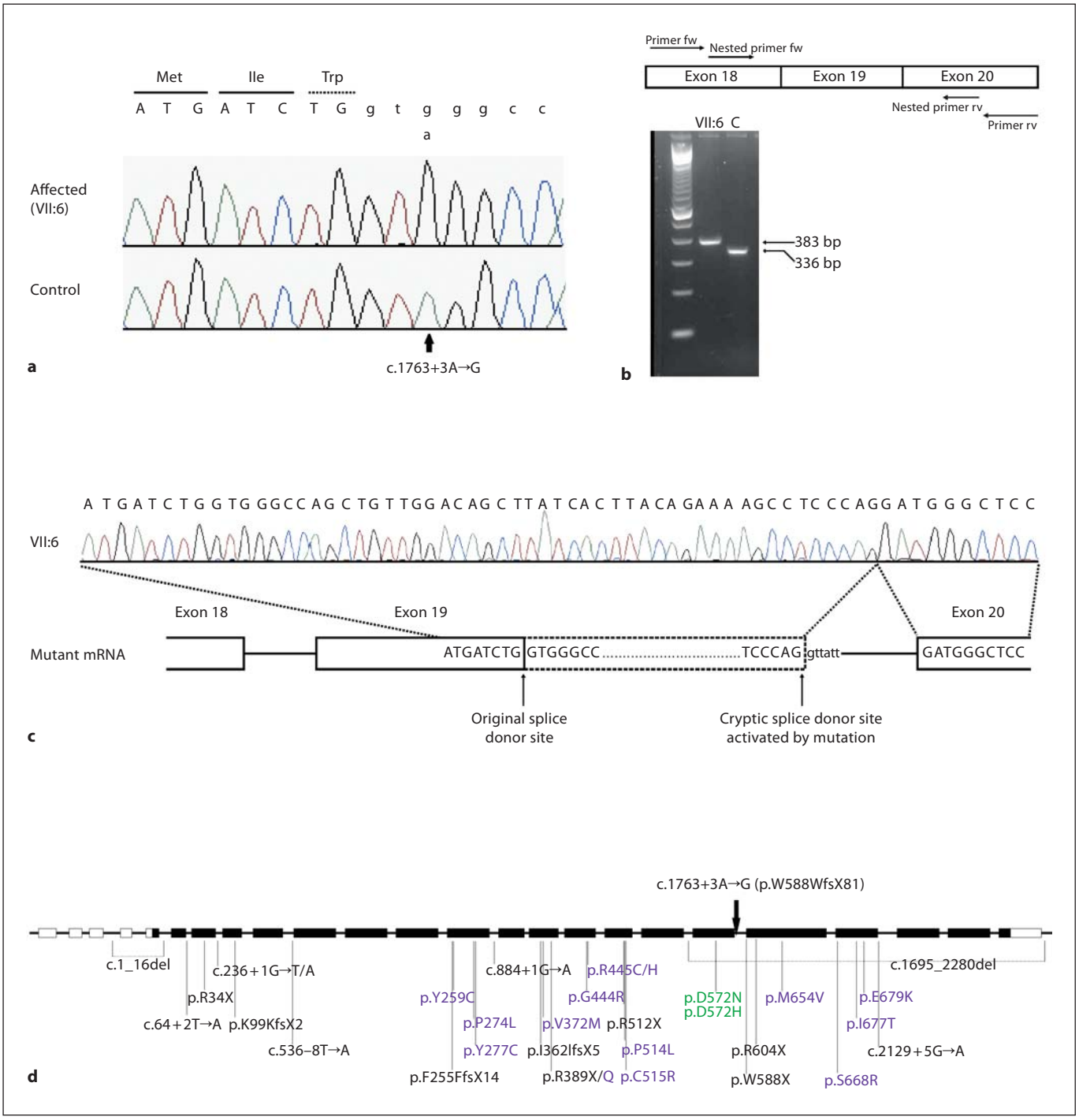

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Fig. 2. a Partial sequence of the *TMC1* gene showing the splice site mutation c.1763+3A→G in an affected family member (VI:6) and in a control individual. **b** Nested RT-PCR on lymphocyte RNA of an affected individual (VII:6) and an unrelated control individual, using primers located in exons 18 and 20 of *TMC1*. In the affected individual, a PCR product of 383 bp was present, while in the control sample, a smaller band of 336 bp was detected. **c** Sequence analysis of the 383-bp PCR product presented in **b**. Due to the mutation, a cryptic splice site was used, resulting in the addition of 47 nucleotides to exon 19 of the mutant *TMC1* transcript. **d** Overview of previously described *TMC1* mutations causing DFNA36 or DFNB7/11. Rectangles: exons of the *TMC1* gene, with the protein-coding part indicated in black. The various mutations are presented below the gene. Protein-truncating and splice site mutations are depicted in black, while missense changes are presented in purple (online version only). The 2 missense changes causing DFNA36 (D572N and D572H) are depicted in green (online version only).

Table 1. *TMC1* mutations described in DFNA36 and DFNB7/11 families

Ethnic origin of family	Sequence variant		Onset	Phenotype	Reference
	cDNA (NM_138691)	protein			
DFNA36					
North America	c.1714G→C	p.D572H	postlingual	profound 2nd decade: high frequencies, 4th–5th decade: all frequencies	Kitajiri et al. [2007a]
North America	c.1714G→A	p.D572N	postlingual	severe-profound 1st decade: mid and high frequencies 2nd decade: all frequencies	Hilgert et al. [2009]; Kurima et al. [2002]; Makishima et al. [2004]
DFNB7/11					
The Netherlands	c.1763+3A→G	p.W588WfsX81	postlingual	profound 1st decade: high frequencies 2nd–3rd decade: all frequencies	present study
Pakistan	c.1_16del	n.d.	prelingual	severe-profound	Kurima et al. [2002]
Turkey	c.64+2T→A	n.d.	congenital/ prelingual	severe-profound	Sirmaci et al. [2009]
Pakistan, Tunisia, Lebanon, Jordan, Iran, Turkey	c.100C→T	p.R34X	prelingual	severe-profound	Kitajiri et al. [2007b]; Kurima et al. [2002]; Tlili et al. [2008]; Sirmaci et al. [2009]; Yang et al. [2002]
Pakistan, Iran	c.236+1G→T/A	n.d.	prelingual	severe-profound	Kitajiri et al. [2007b]; Hilgert et al. [2008]
India	c.295del	p.K99KfsX2	prelingual	severe-profound	Kurima et al. [2002]
Pakistan	c.536–8T→A	n.d.	prelingual	profound	Kurima et al. [2002]; Santos et al. [2005b]
Turkey	c.767delT	p.F255FfsX14	prelingual	severe-profound	Hilgert et al. [2008]
Turkey	c.776A→G	p.Y259C	prelingual	profound	Kalay et al. [2005]
Turkey	c.821C→T	p.P274L	prelingual	profound	Kalay et al. [2005]
Pakistan	c.830A→G	p.Y277C	prelingual	severe-profound	Santos et al. [2005b]
Pakistan	c.884+1G→A	n.d.	prelingual	severe-profound	Kurima et al. [2002]
Turkey	c.1083_1087del	p.I362IfsX5	prelingual	profound	Kalay et al. [2005]
Pakistan	c.1114G→A	p.V372M	prelingual	severe-profound	Santos et al. [2005b]
Sudan, Tunisia, Lebanon, Jordan	c.1165C→T	p.R389X	prelingual	profound	Hilgert et al. [2008]; Meyer et al. [2005]; Tlili et al. [2008]
Turkey	c.1166G→A	p.R389Q	prelingual	severe-profound	Hilgert et al. [2008]
Turkey	c.1330G→A	p.G444R	congenital/ prelingual	severe-profound	Sirmaci et al. [2009]
Turkey	c.1333C→T	p.R445C	congenital/ prelingual	severe-profound	Sirmaci et al. [2009]
Turkey, Pakistan	c.1334G→A	p.R445H	prelingual	profound	Kalay et al. [2005]; Santos et al. [2005b]
Pakistan	c.1534C→T	p.R512X	prelingual	severe-profound	Kurima et al. [2002]
Pakistan	c.1541C→T	p.P514L	prelingual	severe-profound	Kitajiri et al. [2007b]
Pakistan	c.1543T→C	p.C515R	prelingual	severe-profound	Kitajiri et al. [2007b]
Tunisia	c.1764G→A	p.W588X	prelingual	profound	Tlili et al. [2008]
Greece	c.1810C→T	p.R604X	prelingual	profound	Hilgert et al. [2008]
India	c.1960A→G	p.M654V	prelingual	severe-profound	Kurima et al. [2002]
Pakistan	c.2004T→G	p.S668R	prelingual	severe-profound	Kitajiri et al. [2007b]; Santos et al. [2005b]
Turkey	c.2030T→C	p.I677T	prelingual	severe-profound	Sirmaci et al. [2009]
Pakistan	c.2035G→A	p.E679K	prelingual	severe-profound	Santos et al. [2005b]
Sudan	c.2129+5G→A	n.d.	prelingual	not reported	Hilgert et al. [2008]; Meyer et al. [2005]; Tlili et al. [2008]
Turkey	c.1695_2280del	n.d.	congenital/ prelingual	severe-profound	Sirmaci et al. [2009]

n.d. = Not determined.

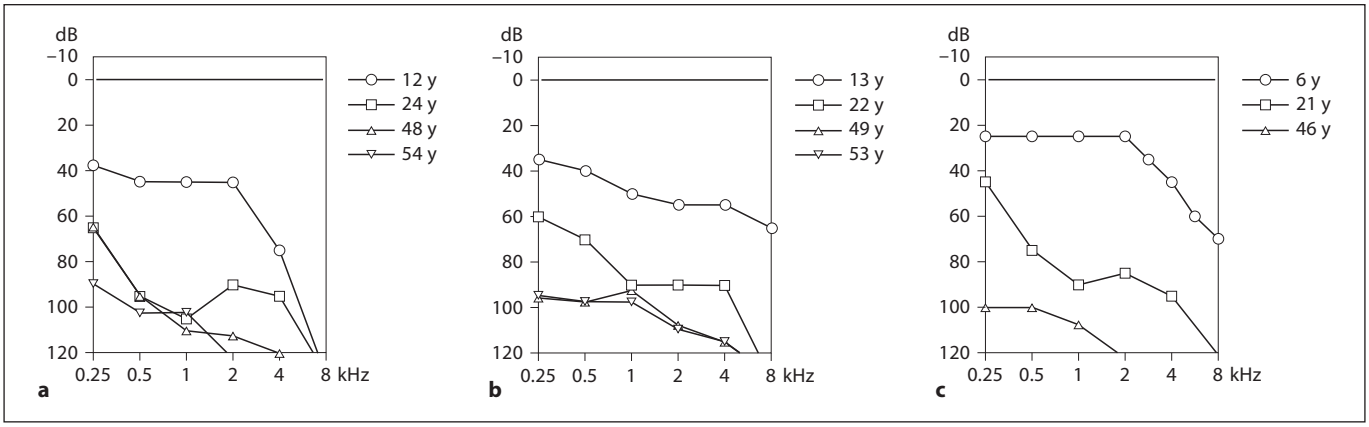

Fig. 3. Longitudinal binaural mean air conduction threshold data of the 3 affected individuals (W06-792). Age (y) is shown by symbol. Some measurements have been omitted for clarity. **a** VII:6 male. **b** VII:7 male. **c** VII:9 female.

correctly spliced and aberrantly spliced *TMC1* mRNA in the blood of these patients, while in the controls, only the correctly spliced mRNA was detected. To further determine the effect of the c.1763+3A→G mutation on splicing, we employed a minigene approach, which showed correct splicing of the wild-type *TMC1* exon 19, while the c.1763+3A→G mutation completely abolished the normal splicing signal.

The 47 bp extension of exon 19 causes a frameshift, a premature stop codon in exon 20 and the incorporation of 81 aberrant amino acids (p.W588WfsX81) in the protein. Protein domain prediction by SMART (http://smart.embl-heidelberg.de) revealed that the TMC domain and most of the C-terminal transmembrane region are absent from the predicted mutant protein. This may cause either nonsense-mediated degradation of *TMC1* mRNA or reduced protein stability. Alternatively, the 81 aberrant amino acids may cause the mutant TMC1 protein to acquire different functional properties.

Audiometric Analysis

Pure-Tone Thresholds versus Age

Figure 3 shows the audiograms of the 3 affected individuals. The hearing losses were symmetrical (data not shown). Hearing impairment started in the high frequencies. The thresholds deteriorated rapidly with increasing age, and hearing impairment progressed to (overall) severe-profound, and ultimately to residual hearing (fig. 3). The audiograms of the heterozygous mutation carriers showed normal hearing.

In figure 4, the binaural mean threshold data from the patients are separately plotted against age for each frequency. The initial slope was estimated by visually fitting a slope tangent into each frequency panel of the data panel up to the age of 30 years.

Readings of the thresholds from the graphs at the ages of 10, 20, 30, 40 and 50 years were separately derived for each frequency from the plots shown in figure 4. These were used to construct age-related typical audiograms (ARTA) [Huygen et al., 2003]. The ARTA for the present DFNB7/11 family are shown together with the ARTA derived for the DFNA36 traits reported by Makishima et al. [2004] and Kitajiri et al. [2007a] in figure 5. Similar readings of the graphs have been applied by us to their published threshold data.

The present DFNB7/11 trait showed considerable progression, especially during the first 3 decades of life, as reflected by estimated annual threshold deterioration values in the range of 2.5–5 dB/year (fig. 4). Although this progression was generally higher than the progression shown by the p.D572H DFNA36 trait described by Kitajiri et al. [2007a] (data not shown), it was generally lower than the progression reported by Makishima et al. [2004] for the p.D572N DFNA36 trait, as is reflected by the ARTA shown in figure 5.

Speech Recognition Scores

The onset age of the deterioration in speech recognition (90% score) may have occurred in the first decade, and the score deteriorated by about 2–3% per year up to the age of about 30 years (fig. 6a). It may be questioned whether person VII:6 ever attained a score of 90% or higher.

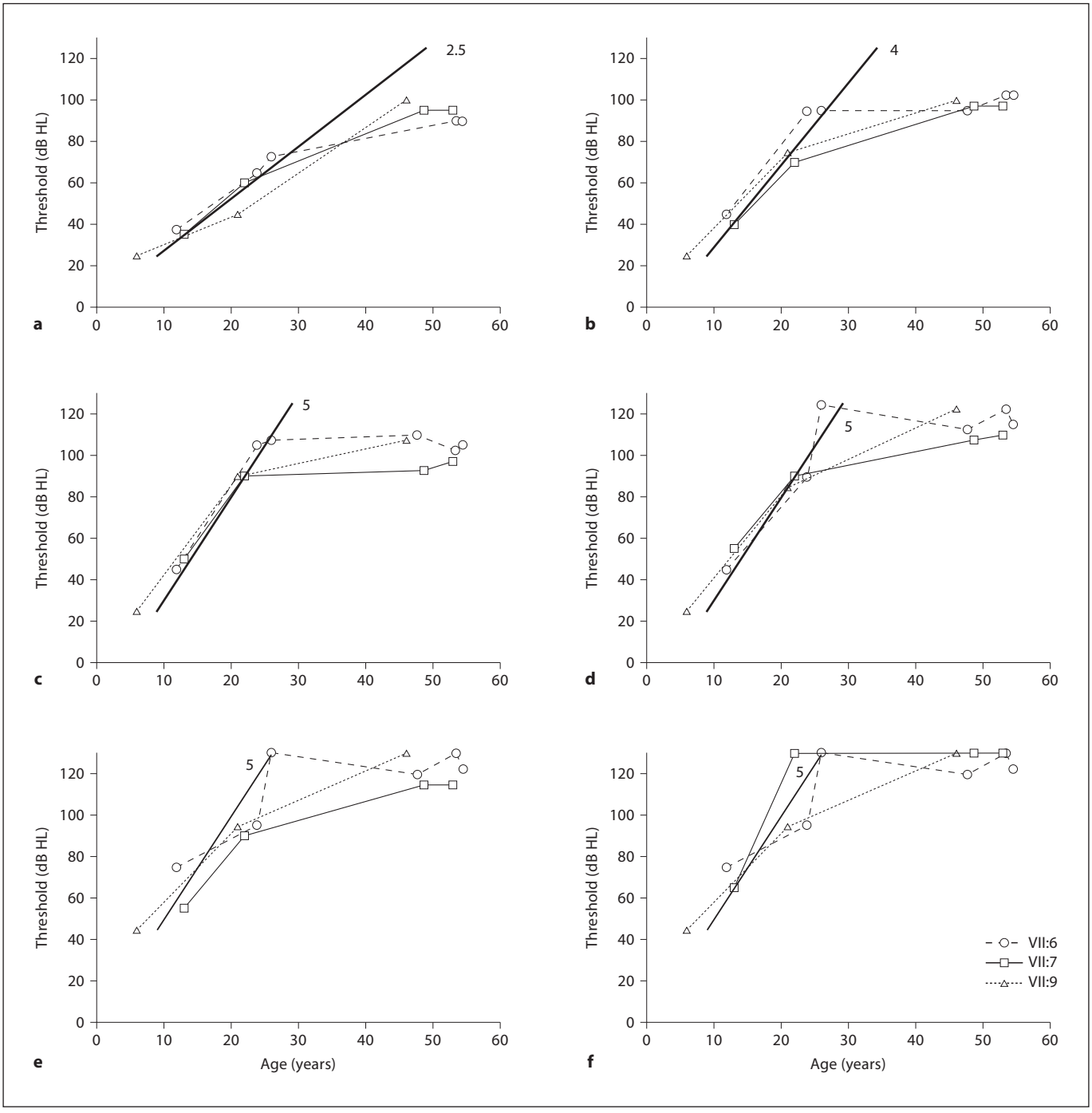

Fig. 4. Longitudinal analysis of binaural mean air conduction thresholds of affected individuals (dB HL) versus age. Individual longitudinal data (circles, triangles and squares) with connecting hairlines are plotted for the frequencies 0.25 (**a**), 0.5 (**b**), 1 (**c**), 2 (**d**), 4 (**e**) and 8 kHz (**f**). The trend lines are added in bold to calculate the approximate annual threshold deterioration (in decibel per year) per frequency. The annual threshold deterioration value up to the age of 30 years is shown next to this trend line.

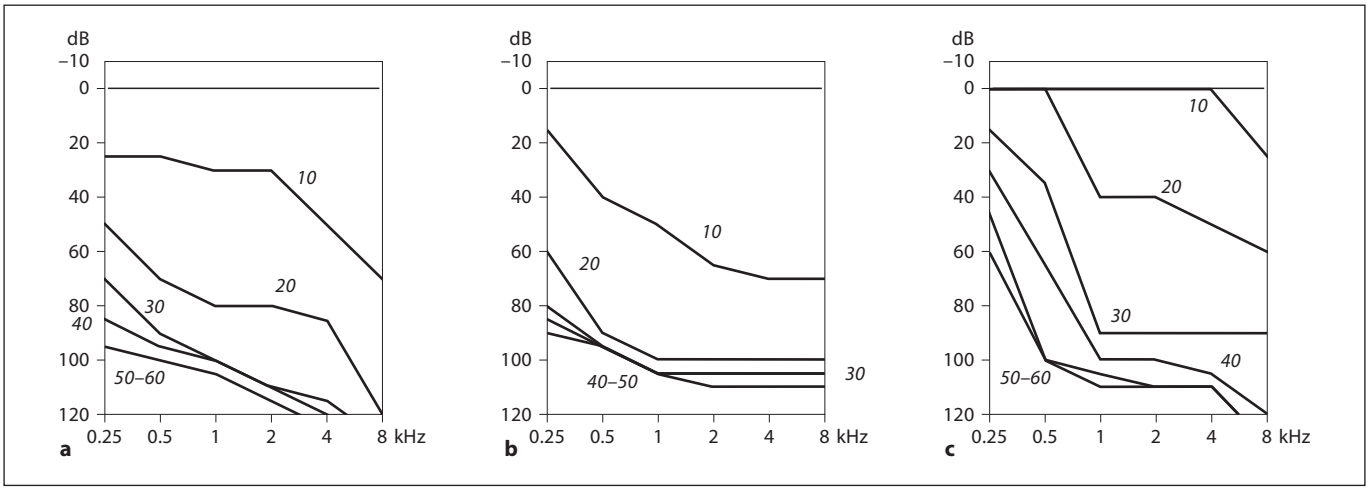

Fig. 5. Numbers in italics: age (in years) in decade steps. **a** ARTA derived for the present DFNB7/11 family with a c.1763+3A→G mutation in *TMC1*. **b**, **c** For comparison, the ARTA pertaining to the DFNA36 families are shown [Kitajiri et al., 2007a; Kurima et al., 2002; Makishima et al., 2004]. **b** DFNA36 D572N. **c** DFNA36 D572H.

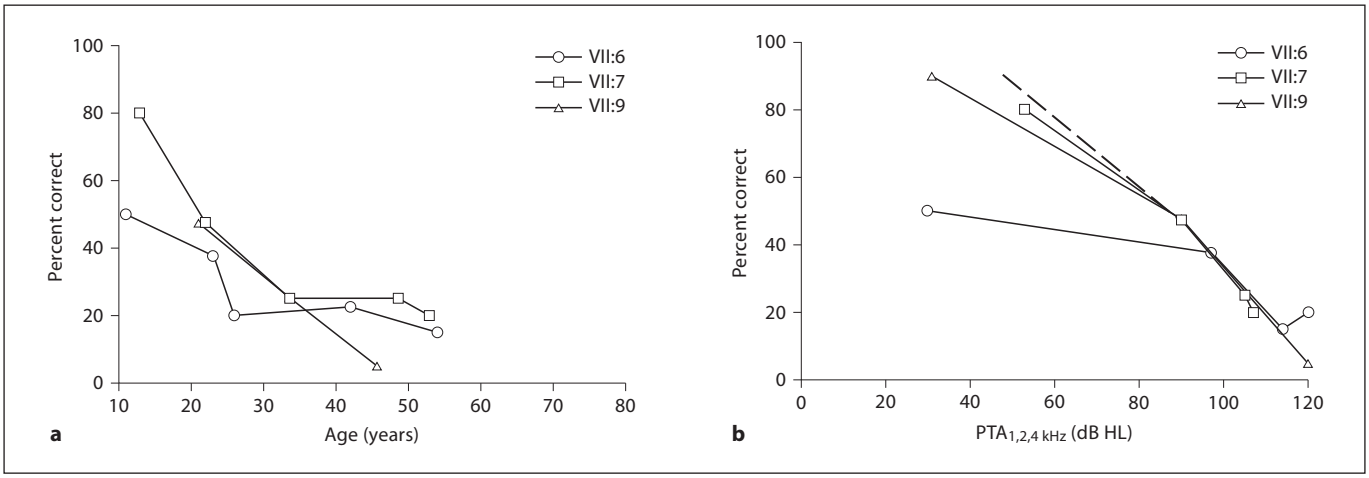

Fig. 6. Binaural mean phoneme recognition scores (percent correct) plotted against age (**a**) and binaural mean $PTA_{1,2,4\ kHz}$ (**b**). **b** Dashed line: binaural mean P50 phoneme score plotted against the binaural mean $PTA_{1,2,4\ kHz}$ for the 45 patients with only presbyacusis that were covered in the report by de Leenheer et al. [2002].

The onset level (90% score) was at best in the range of a $PTA_{1,2,4\ kHz}$ of 20–30 dB HL in individuals VII:7 and VII:9. The score deteriorated by about 0.8–1% per dB HL in these persons. In patient VII:6, the score may have deteriorated at an age of <11 years by about 2–3% per dB HL (fig. 6a). For the sake of comparison, this figure includes an indication of the P50 for presbyacusis which was obtained from the data pertaining to 45 patients with only presbyacusis that were covered in the report by de Leenheer et al. [2002]. The data for persons VII:7 and VII:9 were very close to the P50 for the presbyacusis patients. This is similar for person VII:6, except for the outlying value of this person's first measurement.

Vestibular Examination

No vestibular symptoms appeared to be present in the patients (data not shown). Vestibular function was tested in all 3 affected individuals and was found to be normal at ages of 46–55 years.

Evaluation of Effect of Cochlear Implantation on Phoneme Recognition

The affected individuals have recently been selected for cochlear implantation. Computerized tomography of the temporal bone was performed as part of the preoperative cochlear implantation selection procedure. It showed a normal configuration of the temporal bone, and normal middle and inner ear structures in all 3 individuals.

Two patients underwent cochlear implantation in our hospital, and the cochlear implant was uneventfully and completely inserted into the right side in both cases. Individual VII:6 was implanted with a Nucleus Freedom by Cochlear and person VII:7 with a CII/HR90K device by Advanced Bionics. Individual VII:9 was implanted in another university medical center with a Nucleus 24RCA by Cochlear, and complete insertion was reported.

Person VII:6 was tested 3 months after the activation of the speech processor and, at 70 dB SPL, showed a 65% phoneme score with the cochlear implant. This is equivalent to a word score of about 37%. Before implantation, aided speech reception thresholds with a conventional aid on the right had been tested and no speech reception had been found at 70 dB SPL, and the maximum speech reception (phonemes) of about 30% had been found at 90 dB SPL. Individual VII:7 was tested after 6 months of use and, at 70 dB SPL, showed 88% speech reception with the cochlear implant, which is equivalent to a word score of about 74%. Before implantation, aided thresholds with a conventional aid on the right had been tested and a speech reception of 10% had been found at 70 dB SPL; the maximum speech reception (phonemes) of about 25% had been found at 90 dB SPL. Person VII:9 was tested after 12 months of use and showed a speech reception of 50% (phonemes) at 70 dB SPL, and a word score of about 27% was found. Before implantation, no speech reception (phonemes) had been found at 120 dB SPL.

The results in all 3 patients are within the normal range of those of our 2 reference groups (12 months of use) [Verhaegen et al., 2008]; moreover, final results are expected to be even better since persons VII:6 and VII:7 were evaluated after 3 months and 6 months of use, respectively.

Discussion

This report describes a Dutch family whose hearing impairment is caused by a novel mutation in the known deafness gene *TMC1*. Mutations in this gene have previously been described to cause autosomal dominant progressive hearing loss (DFNA36) as well as severe-to-profound prelingual hearing loss inherited in an autosomal recessive manner (DFNB7/11). Interestingly, the sensorineural hearing impairment in this family was progressive but inherited in an autosomal recessive pattern.

TMC1 is predicted to encode a multiple-membrane-spanning protein whose topologic organization resembles that of ion channels, pumps or transporters [Keresztes et al., 2003; Kurima et al., 2002, 2003], but its precise function remains unknown. In the murine cochlea, *Tmc1* is strongly expressed in both outer and inner hair cells in early stages of development [Kurima et al., 2002; Vreugde et al., 2002]. This expression pattern suggests that *Tmc1* plays a role in the normal function of the cochlear hair cells rather than in the early embryonic development of these cells [Kurima et al., 2002]. Besides the cochlear hair cells, *Tmc1* is also expressed in the vestibular neurosensory epithelium [Noguchi et al., 2006].

To unravel the physiological role of Tmc1 in more detail, others have extensively studied the characteristics of two mouse models for hearing impairment caused by a defect in *Tmc1* [Kurima et al., 2002; Vreugde et al., 2002]. The *Beethoven (Bth)* mouse mutant carries a heterozygous mutation in *Tmc1,* resulting in the substitution of a lysine for a methionine residue (p.M412K). In $Tmc1^{Bth/+}$ mice, progressive hair cell degeneration, mainly affecting the inner hair cells, started from postnatal day 20 onwards, and these mice are therefore considered to be a model for progressive hearing loss resembling DFNA36. Before degeneration initiated, the hair cells seemed to function normally [Vreugde et al., 2002]. The *deafness (dn)* mice carry a deletion of almost 1.7 kb of genomic DNA, encompassing exon 14 of the *Tmc1* gene which is predicted to result in a frameshift and premature termination of the Tmc1 protein. The *dn* mouse is considered to be a model for recessive hearing impairment DFNB7/11 [Kurima et al., 2002]. In homozygous $Tmc1^{dn/dn}$ mice, cochlear hair cells develop normally, but cochlear responses are completely lacking [Bock and Steel, 1983; Deol and Kocher, 1958; Steel and Bock, 1980]. Besides early hair cell degeneration of the organ of Corti, occasionally a degeneration of the vestibular saccular macule is observed in the $Tmc1^{dn/dn}$ mice 10 days after birth [Bock and Steel, 1983; Deol and Kocher, 1958]. Despite this observation, the phenotype of both mutant mice *($Tmc1^{dn/dn}$* and *$Tmc1^{Bth/+}$)* does not seem to be associated with vestibular deficits [Deol and Kocher, 1958; Kurima et al., 2002; Vreugde et al., 2002]. Taken together, the studies performed on the *Beethoven* and *deafness* mice support a role

for *Tmc1* in the inner and outer hair cells, either in proper trafficking of other membrane proteins in these cells or in regulating the differentiation of immature hair cells into fully functional auditory receptors [Marcotti et al., 2006].

Whereas a clear auditory phenotype was present in heterozygous $Tmc1^{Bth/+}$ mice, heterozygous $Tmc1^{dn/+}$ mice did not show any obvious defects [Marcotti et al., 2006]. These data indicate that the p.M412K mutation in the *Beethoven* mice has a dominant-negative or a gain-of-function effect rather than causing haploinsufficiency. This is further supported by the fact that Tmc1 is thought to act as a multimeric entity whose function may be disturbed by the incorporation of mutant subunits [Marcotti et al., 2006]. In addition, genetic modifiers may influence the phenotype associated with the $Tmc1^{Bth}$ allele since at least one quantitative trait locus has been mapped that influences the phenotype associated with the $Tmc1^{Bth}$ allele in a series of experiments crossing *Beethoven* mice with several other mouse strains [Noguchi et al., 2006]. In contrast to the $Tmc1^{Bth}$ allele, the $Tmc1^{dn}$ allele seems to be a true functional null allele.

In humans, only 3 DFNA36 families have been reported so far. The hearing loss in these families is postlingual with an onset in the first or second decade of life and progression to profound deafness [Hilgert et al., 2009; Kitajiri et al., 2007a; Kurima et al., 2002; Makishima et al., 2004]. Intriguingly, the affected individuals of these families all carry a missense mutation in *TMC1* with the same amino acid substituted (aspartic acid at position 572), indicating a critical and specific role for this amino acid in the normal function and pathology of TMC1.

More than 50 DFNB7/11 families have been reported that originated from Pakistan, Sudan, Turkey, Iran, Lebanon/Jordan, Tunisia, India and Greece (table 1) with more than 30 different mutations in the *TMC1* gene [Hilgert et al., 2008; Kalay et al., 2005; Kitajiri et al., 2007b; Kurima et al., 2002; Meyer et al., 2005; Santos et al., 2005b; Sirmaci et al., 2009; Tlili et al., 2008; Yang et al., 2002]. Some of the mutations recurred in different families, suggesting a founder effect [Kitajiri et al., 2007b; Kurima et al., 2002]. All reported DFNB7/11 families thus far presented with a congenital or prelingual severe-to-profound hearing loss [Hilgert et al., 2008; Kalay et al., 2005; Kitajiri et al., 2007b; Kurima et al., 2002; Meyer et al., 2005; Santos et al., 2005b; Sirmaci et al., 2009; Tlili et al., 2008; Yang et al., 2002], affecting all frequencies [Tlili et al., 2008]. In the present family, hearing loss was noticed in the 1st to 2nd decade of life [Cremers, 1979], started at the high frequencies and rapidly progressed to residual HLs ('corner audiogram') already in the 2nd decade. From the 2nd decade onwards, hearing aids were necessary for the patients for proper communication. The phoneme recognition scores were very similar to those in presbyacusis patients. This seems to favor a primary involvement of outer hair cells [Felder and Schrott-Fischer, 1995], but does not exclude an involvement of inner hair cells [Nelson and Hinojosa, 2006]. Overall, the type of hearing impairment shown by the present DFNB7/11 family resembled the progressive type of hearing loss described in the 3 DFNA36 traits rather than the profound-to-severe type of hearing impairment that has been reported for the previously outlined DFNB7/11 traits. Generally, recessively inherited forms of hearing impairment are not progressive although, in addition to the DFNB7/11 family described here, also DFNB8, DFNB13, DFNB30, DFNB59, DFNB77 and several mutations in DFNB1 are reported to cause progressive hearing loss [Grillet et al., 2009; Janecke et al., 2002; Mustapha et al., 1998; Santos et al., 2005a; Schwander et al., 2007; Veske et al., 1996; Walsh et al., 2002]. Beside *TMC1,* also *MYO3A* (DFNB30), *PJVK* (DFNB59) and *LOXHD1* (DFNB77) are expressed in hair cells, suggesting that intrinsic defects in hair cell function might be responsible for progressive recessive hearing loss [Grillet et al., 2009; Schwander et al., 2007; Walsh et al., 2002].

The mutation c.1763+3A→G in the present DFNB7/11 family was shown to alter *TMC1* splicing in at least part of the transcripts, and thereby to lead to the addition of 47 bp in the RNA after exon 19, resulting in a frameshift and a premature stop codon after the addition of 81 codons. This mutation would regularly be considered a *TMC1* null allele, like many other mutations previously described to cause DFNB7/11. Why would yet another null allele cause a different type of hearing impairment compared to other protein-truncating *TMC1* mutations? A likely explanation is that the mutation does not completely abolish normal splicing of *TMC1* mRNA in the inner ear, which is supported by the finding of both the normally and aberrantly spliced *TMC1* mRNA in lymphocytes of the patients. As such, a certain amount of normal TMC1 protein might still be present in the cochlear hair cells, sufficient for hearing at young age, and thus the mutation would be hypomorphic, as has been described for a synonymous change in exon 7 of the *Cdh23* gene in mice [Noben-Trauth et al., 2003]. This variant results in the skipping of exon 7 and leads to susceptibility to age-related hearing loss rather than causing the severe-to-profound hearing loss observed in both mice and humans carrying other recessive mutations in

Cdh23/CDH23. An alternative explanation for the progressive hearing loss as a result of the c.1763+3A→G mutation in the present DFNB7/11 family could be that the mutant *TMC1* transcript is not completely degraded via nonsense-mediated decay and that the remaining truncated protein, including the 81 aberrant amino acids, still has some residual function sufficient for hearing at young age.

In summary, in this report we describe a DFNB7/11 family with a novel mutation in *TMC1*. Hearing impairment developed postlingually in the high frequencies and progressed towards severe to profound in the 2nd decade of life, affecting all frequencies. Remarkably, this type of hearing loss has not previously been described for recessive hearing loss caused by many different mutations in *TMC1*. Notably, the progressive phenotype in this family was more similar to the phenotypes described for dominant *TMC1* mutations (DFNA36). As such, this report contributes to genotype-phenotype correlations that may be essential for understanding the pathology of hearing impairment caused by mutations in *TMC1*.

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