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Molecular genetics of *MARVELD2* and clinical phenotype in Pakistani and Slovak families segregating DFNB49 hearing loss

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Abstract

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Conflict of interest

The authors declare no conflict of interest.

Pathogenic mutations of *MARVELD2*, encoding tricellulin, a tricellular tight junction protein, cause autosomal recessive non-syndromic hearing loss (DFNB49) in families of Pakistan and Czech Roma origin. In fact, they are a significant cause of prelingual hearing loss in the Czech Roma, second only to *GJB2* variants. Previously, we reported that mice homozygous for p.Arg497* variant of *Marveld2* had a broad phenotypic spectrum, where defects were observed in the inner ear, heart, mandibular salivary gland, thyroid gland and olfactory epithelium. The current study describes the types and frequencies of *MARVELD2* alleles and clinically reexamines members of DFNB49 families. We found that *MARVELD2* variants are responsible for about 1.5% (95% CI: 0.8 – 2.6) of non-syndromic hearing loss in our cohort of 800 Pakistani families. The c.1331+2T>C allele is recurrent. In addition, we identified a novel large deletion in a single family, which appears to have resulted from non-allelic homologous recombination between two similar Alu short interspersed elements. Finally, we observed no other clinical manifestations co-segregating with hearing loss in DFNB49 human families, and hypothesize that the additional abnormalities in the *Marveld2* mutant mouse indicates a critical non-redundant function for tricellulin in other organ systems.

Keywords

Deafness; genetic etiology; inner ear; locus heterogeneity; moderate hearing loss; tight junction; *MARVELD2*; tricellulin

Introduction

Mutations in *MARVELD2* cause autosomal recessive nonsyndromic deafness at the *DFNB49* locus (Riazuddin et al. 2006). Seven different pathogenic variants of human *MARVELD2* have been identified in families segregating moderate to profound hearing loss, with no obvious vestibular phenotype (Chishti et al. 2008; Riazuddin et al. 2006; Safka Brozkova et al. 2012; Yang et al. 2013). *MARVELD2* encodes tricellulin, a tricellular tight junction protein (Ikenouchi et al. 2005; Riazuddin et al. 2006). Tight junctions are a type of apical junctional complex that occur between epithelial cells. Bicellular tight junctions are formed when two cells meet and tricellular tight junctions are formed where three cells converge (Tsukita et al. 2008; Yap et al. 1998). Tight junctions regulate ionic and other molecular traffic via the paracellular route or through the intercellular spaces between epithelial cells (Ikenouchi et al. 2005; Schneeberger and Lynch 2004; Steed et al. 2010; Tsukita et al. 2001). In the inner ear, epithelial cells delimit the fluid-filled compartments and the tight junctions between adjacent cells prevent intermixing of the extracellular fluids (Gulley and Reese 1976; Jahnke 1975). Several tight junction proteins are found in the inner ear epithelia and variants in many of these have been associated with hearing loss in humans and mouse mutants (Ben-Yosef et al. 2003; Borck et al. 2011; Citi et al. 1988; Gow et al. 2004; Higashi et al. 2013; Kitajiri et al. 2004a; Kitajiri et al. 2004b; Lee et al. 2012; Morozko et al. 2014; Nakano et al. 2009; Nayak et al. 2013; Raphael and Altschuler 1991; Riazuddin et al. 2006; Wilcox et al. 2001).

Mice with a *Marveld2* p.Arg497* knock-in variant display hearing loss by postnatal day 16 (P16) and are profoundly deaf by P30 (Nayak et al. 2013). These mice lose their cochlear

hair cells in a temporal pattern that coincides with the observed hearing loss. Similar to the affected human subjects, these mice have no vestibular phenotype and have an apparently normal stria vascularis. Intriguingly, the tricellulin mutant mice also showed phenotypic changes in the heart, thyroid, mandibular salivary glands and olfactory epithelium (Nayak et al. 2013). Besides hearing loss, the overall body and several other organs weights were higher in the knock-in mice, along with altered histology of the granular ducts of the mandibular salivary glands, rostral olfactory epithelium and cardiac myocytes (Nayak et al. 2013).

Given the wide distribution of tricellulin in human epithelial cells, one would have predicted that the hearing loss would have been accompanied by multiple other disorders and deafness would be part of a syndrome rather than a nonsyndromic hearing loss (Chishti et al. 2008; Riazuddin et al. 2006; Safka Brozkova et al. 2012) as we initially reported. The goal of this study was to determine the spectrum of mutant alleles, and revisit families and obtain a more comprehensive clinical evaluation of DFNB49 families from Pakistan and Slovakia to determine if individuals homozygous for *MARVELD2* mutation also have other clinical symptoms in addition to the hearing loss, similar to that observed in the tricellulin knock-in mice.

Materials and methods

Family participation

This study was approved by IRBs at the National Centre of Excellence in Molecular Biology (NCEMB), Lahore, Pakistan, at the National Institutes of Health, USA (Combined Neuroscience IRB; OH-93-N-016), at the University of Maryland, USA and at the University Hospital in Bratislava, Slovakia. Written informed consent was obtained from adult subjects and parents of minor subjects. The study participants comprised of twelve DFNB49 families, which were identified through linkage studies of 800 families segregating moderate to profound, prelingual- or congenital-onset, recessive deafness ascertained through the NCEMB including the original 550 families screened by Riazuddin and co-authors (Riazuddin et al. 2006).

Clinical evaluation

We obtained a detailed clinical history for each affected individual of the DFNB49 families to determine the neonatal course, any developmental delays, and signs of polyuria, polydipsia, goiter and hypotonia. For all affected individuals, we also performed a physical examination to evaluate motor skills, body weight, stature, and morphology. Hearing was evaluated on few participating individuals in audiology clinics by pure tone audiometry at octave frequencies with intensities up to 110 dBHL. Vestibular function was evaluated by tandem gait and Romberg testing (Khasnis and Gokula 2003). In seven subjects from family PKDF1069 and three from the Slovak family, we determined serum and urinary chemistry values to define aspects of metabolic and renal function. We also performed complete blood count to determine the hematopoietic abnormalities. Electrocardiogram and echocardiograms were obtained to determine the structure and function of the heart. Plain and contrast Computed Tomography (CT) scans of the brain were obtained for assessment

of brain lesion and bony abnormalities including temporal bone, pituitary gland and peripheral nervous system.

Genotype and mutational analysis

Genomic DNA was extracted from 10 ml of peripheral venous blood as described (Grimberg et al. 1989). Three fluorescently labeled microsatellite markers (*D5S2036*, *D5S637* and *D5S1982*) linked to chromosome 5q13.2 were PCR-genotyped as described (Ahmed et al. 2001). Primers for polymerase chain reaction (PCR) amplification and *MARVELD2* sequencing were described previously (Riazuddin et al. 2006). For sequencing, 15 µl of PCR product was treated with 0.3U of shrimp alkaline phosphatase and 3 U of exonuclease I at 37°C for 1 hr, followed by incubation at 80°C for 15 minutes. DNA sequencing reactions contained 3.2 pmol of primer, 0.6 ml of Big Dye Terminator Ready Reaction Mix (Applied Biosystems, CA, USA), and 2 µl of 5 x dilution buffer (400 mM Tris-HCl pH 9 and 10 mM MgCl₂) in a 10 µl reaction. Cycling conditions were 96°C for 2 minutes and 45 cycles of 96°C for 10 s, 55°C for 10 s, and 60°C for 4 minutes. Sequencing reaction products were ethanol precipitated and the pellets were resuspended in 10 µl of deionized formamide and sequence obtained using an ABI 3730 XL (Applied Biosystems).

To map the deletion breakpoints encompassing *MARVELD2* exons 4 and 5 in family PKDF1069, we performed long-range PCR analysis using the DNA samples from the participating individuals of PKDF1069 family. For long-range PCR, the analysis reaction contained the kit recommended concentrations of primers, dNTPs, PCR buffer and LA-Taq polymerase (Clontech Laboratories Inc., CA, USA). For genotyping of deletion segregating in family PKDF1069 we used the forward primer (5'-CTGGCCTCAACCTCACTTCTT-3') and reverse primer (5'-ACTATAGCAATGCCCTCGTTCA-3'). Cycling conditions were 96°C for 2 minutes and 30 cycles of 96°C for 30 s, 60°C for 30 s, and 68°C for 9 minutes. For sequencing of the long-range PCR products we used the same parameters as described above.

Co-segregation of the mutations with hearing loss in each family was demonstrated for all subjects participating in this study. Control DNA samples from ethnically matched Pakistanis were sequenced for novel variant of *MARVELD2*. We also estimated the contribution of *MARVELD2* mutations to recessive, severe to profound, congenital or prelingual-onset deafness using a sample size of 800 Pakistani families and 95% confidence interval (CI).

Fluorescently tagged tricellulin expression constructs

A wild type tricellulin construct (TRIC^{WT}) in pEGFP C2 vectors (Invitrogen) was a gift from Alan Fanning, PhD, Department of Cell Biology and Physiology, University of North Carolina, Chapel Hill, North Carolina, USA. The TRIC^{C395-Q501del} construct was made using the In-Fusion® HD cloning kit and the TRIC^{WT} cDNA as template. Primer pairs were designed to amplify the N-terminal region encoding the transmembrane domain [Primer pair Inf-exon2F (5'-GGACTCAGATCTCGAGGATGTCAAATGATGGAAGATCCAG-3') and Exon3R (5'-AATTCTCTCATGTTCCATTTTCCTTTTCGATGACAAT-3')] fused to that encoding the C-terminal end [(Primer pair Exon6F (5'-

TCGAAAAGGAAAATGGAACATGAGAGAATTTTC AAGAA-3') and Inf-exon7R (5'-GAAGCTTGAGCTCGAGTCAAGAATAACCTTGTACA TCCCAAT-3')], such that the resulting construct lacked the region encoded by exons 4 and 5. The sequences of primers Exon3R and Exon6F are partially complimentary and are a fusion of exon3 and exon6 which results in the deletion of exons 4 and 5.

For the PCR reaction, the annealing temperature was 60°C, with 90 seconds extension at 72°C for primers Inf-exon2F and Exon3R and 30 seconds extension for primers Exon6F and Inf-exon7R. Meanwhile the pEGFP C2 vector was linearized by restriction enzyme XhoI (Fermentas) and dephosphorylated. Both PCR products and the linearized vector were gel-purified and ligated together in the recommended molar ratio, using the In-Fusion® HD enzyme as per the manufacturers protocol. The ligation reaction was used to transform Stellar competent cells (Clontech Laboratories) and resulting clones were sequence verified before using for transfection of cells.

Cell culture conditions and transfection

Madin-Darby Canine Kidney Epithelial (MDCK) cells were transiently transfected using Eugene-6 (Roche Applied Science, Indianapolis, IN) with 1.5 µg of the desired construct per well in a 6-well dish. After transfection, cells were incubated for 48 hours at 37°C followed by fixation with 4% paraformaldehyde. For visualization of the bicellular tight junctions anti-ZO1 rabbit polyclonal (Life Technologies) antibody was used at 1:100 dilution. A Zeiss LSM700 confocal microscope was used for imaging and the digital images were adjusted in Photoshop 6.0 (Adobe) for optimal contrast and brightness. For quantification, the GFP fluorescence intensities at three tricellular and bicellular tight junctions per image were measured using ImageJ. The anti-ZO-1 signal intensity peak was used as the reference point to locate the tight junctions. The ratio of the average GFP fluorescence intensity at tricellular tight junctions to bicellular tight junctions was calculated for each image to determine if the protein was enriched specifically at either type of tight junctions. Statistical analyses were performed using the Student's *t*-test. All average data on the graph are shown as mean ± standard error of mean (S.E.M.). *P* values less than 0.05 were considered significant.

Results

Mutation analysis of *MARVELD2*

We identified five families co-segregating deafness (Figs. 1 and 2) with microsatellite markers linked to the *DFNB49* locus (Riazuddin et al. 2006). The Slovak Roma family has two affected members who are homozygous for a pathogenic allele of *MARVELD2* (Fig. 1). Sequence analysis of *MARVELD2* revealed three probable homozygous pathogenic variants co-segregating with prelingual, severe to profound, sensorineural hearing loss in all five of these families (Figs. 1 and 2). The previously reported splice junction variants (c.1331+2T>C and c.1183-1G>A) were found in four families (Fig. 1). Both of these alleles are known to cause aberrant splicing of the *MARVELD2* mRNA, resulting in premature stop codons (Riazuddin et al. 2006). In the affected individuals of another *DFNB49* family (PKDF1069), several attempts at PCR amplification of exons 4 and 5 of *MARVELD2* did not produce an amplicon, while we were able to amplify the neighboring exons 3 and 6. These

observations prompted us to search for an intragenic deletion of *MARVELD2* exons segregating in family PKDF1069. We performed long-range PCR to amplify 5 to 15 kb products using several combinations of primers from upstream of *MARVELD2* exon 4 and downstream of exon 5 using DNA samples from the affected individuals and their normal hearing parents. Eventually, we identified an 8,095 bp deletion encompassing both exons 4 and 5 of *MARVELD2* segregating with hearing loss in family PKDF1069 (Fig. 2). The proximal breakpoint of this deletion is in intron 4 at chr5:68,726,959 (Human genome build GRCh37/hg19), while the distal breakpoint occurs at position chr5:68,735,005 located in intron 6 of *MARVELD2* (Fig. 2c).

To investigate the mechanisms involved in the deletion detected in family PKDF1069, the interval surrounding the breakpoints was analyzed through RepeatMasker (<http://www.repeatmasker.org/>). In addition, significantly over-represented motifs within ± 15 bp of Gross Rearrangement Breakpoint Database (G RaBD) translocations breakpoints were also sought (Abeyasinghe et al. 2003). Bioinformatic analysis revealed that the 5' breakpoint lies within an AluSg repeat located in intron 4, whereas the 3' breakpoint lies within an AluSx1 repeat located in intron 6. Alignment of 308 bp AluSg repeat and the 284 bp AluSx1 repeat showed their sequence identity to be 86% (243 of 283 nucleotides). Notably, an 18 bp sequence with 100% nucleotide identity and oriented in the same direction flanks the deletion on both sides (Fig. 2c), suggesting that this short repetitive element within highly similar Alu short interspersed nuclear elements (SINEs) may have served as a substrate for non-allelic homologous recombination. The loss of exons 4 and 5 would lead to an in-frame deletion of 107 amino acids at the protein level (p.Cys395-Gln501del; GenBank accession # NP_001033692), which comprises part of the occludin-ELL domain at the carboxy-end of tricellulin (Riazuddin et al. 2006).

Heterologous expression and targeting of mutant tricellulin deleted for exons 4–5

We concluded from previous studies of over-expression of tricellulin in stably-transfected MDCKII cells that the carboxy terminus of the protein is required for efficient transport and targeting to the basolateral membrane (Westphal et al. 2010). To test the effect of the *MARVELD2* allele segregating in family PKDF1069, which is predicted to delete 107 amino acid in-frame from the cytoplasmic tail of tricellulin, MDCK cells were transfected with the plasmid constructs encoding the full-length wild-type (TRIC^{WT}), p.Arg500* (TRIC^{R500X}, as positive control) and p.Cys395-Gln501del (TRIC^{C395-Q501del}) mutant tricellulin, all fused to GFP at the N-terminus (Fig. 2D–G). In cells with higher expression levels of TRIC^{WT}, the protein was found at the basolateral plasma membrane and weakly at bicellular tight junctions but was enriched at tricellular tight junctions (Fig. 2D). TRIC^{R500X} localized to the basolateral membrane and infrequently to the bicellular tight junctions as discrete puncti (Nayak et al. 2013) but it was not observed at tricellular junctions (Fig. 2F). Similarly, TRIC^{C395-Q501del} localized to the basolateral membrane and was almost uniformly distributed along with ZO-1 but was not enriched at the tricellular junctions (Fig. 2E). The mislocalization of the tricellulin mutants is apparent when the ratio of the fluorescence intensities of GFP at the tricellular to bicellular junctions is calculated (Fig. 2G). TRIC^{WT} shows tricellular to bicellular junction localization ratio of over 2.5, indicating that wild type full-length tricellulin preferentially, but not exclusively, localizes to tricellular junctions in

comparison to bicellular junctions. The mutant proteins had near equal fluorescence intensities at both types of tight junctions resulting in significantly lower ratios compared to TRIC^{WT} protein ($p < 0.001$), which suggests that the mutant proteins had lost their tricellular tight junction localization signal. Thus, our *in vitro* studies suggest that tricellulin with an in-frame deletion of 107 amino acids from the cytoplasmic tail is unlikely to target to tricellular tight junctions. Future work will focus on identifying the domain of tricellulin involved with tricellular tight junction targeting and the proteins, such as ILDR1 (Morozko et al. 2014), with which it interacts at this location in the cell.

Clinical characteristics of DFNB49 families

We performed several screening clinical tests to evaluate the physical, audiometric, biochemical, hematological and physiologic function of some of the affected individuals from DFNB49 families. The affected individuals of Pakistani family PKDF1069 had bilateral, moderate to profound sloping sensorineural hearing loss (Fig. 3). There is intra-familial variability seen in the severity of the hearing loss among the affected individuals of family PKDF1069 (Fig. 3). In addition to hearing loss, the affected individuals of the PKDF1069 family did not show signs or symptoms of other pathology that could constitute a syndromic, rather than a non-syndromic deafness. The affected members of the PKDF1069 were born from full-term pregnancies with reportedly normal birth weight and length. Serum biochemical analyses of these individuals, revealed normal liver, kidney and cardiac function (Tables 1 and 2). The affected individuals also did not display problems with polydipsia, polyuria, muscle weakness and fatigue, growth or mental retardation or any evidence of blood disorder. Brain Computed Tomography (CT) scans or Magnetic Resonance Imaging (MRI) of three affected individuals revealed normal brain, sinuses and orbits with no signs of bone destruction or structural abnormality, space occupying lesions or peripheral nervous system abnormalities (Table 3). Collectively, these clinical studies suggest that the deafness segregating in family PKDF1069 is non-syndromic.

To determine if other non-Pakistani DFNB49 families also present with non-syndromic hearing loss, we performed a comprehensive clinical investigation of the Slovak Roma family (Tables 4–7), segregating a splice site variant allele of *MARVELD2* (Fig. 1). Similar to family PKDF1069, the affected individual of the Slovak family also did not show any other abnormality besides hearing loss and also had normal vestibular function. However, based on the nonspecific abnormalities from the electrocardiogram and echocardiography results of this proband (the orientation of the electrical axis, increased trabeculation in the apical half of the left ventricle and the aortic valve anomalies noted) there could be syndromic cardio-pathology associated with the proband's deafness (Table 6). But these specific cardiovascular changes were not seen in any of the evaluated controls or any of the other affected individuals (Table 1) suggesting that these borderline nonspecific abnormal findings, observed in the proband from the Slovak family, are likely not associated with the DFNB49 allele. The other abnormalities (microtia, microdontia) found in several members of the Slovak Roma family did not co-segregate with hearing loss and the *MARVELD2* genotype, so they likely have different but probably still genetic causes.

Discussion

Taken together with our previous study, mutations of *MARVELD2* account for 1.5% (12 of 800 families; 95% CI: 0.8 – 2.6) of recessive hearing loss in the NCEMB Pakistani study cohort (Riazuddin et al. 2006). To date, eight variants of *MARVELD2* have been identified and all of them are clustered in the cytoplasmic tail of tricellulin (Chishti et al. 2008; Riazuddin et al. 2006; Safka Brozkova et al. 2012). Among the known alleles, c.1331+2T>C is reoccurring having been identified in eight Pakistani and three Czech Roma families (Chishti et al. 2008; Riazuddin et al. 2006; Safka Brozkova et al. 2012). It accounts for ~53% of the DFNB49 hearing loss phenotype segregating in Pakistani families (Chishti et al. 2008; Riazuddin et al. 2006), and is the only allele reported so far in the Czech Roma population (Safka Brozkova et al. 2012). Thus hierarchical mutation screening examining the c.1331+2T>C in deaf individuals would be a cost-effective approach to molecular diagnosis in both populations.

Recently, comprehensive phenotypic analysis of *Marveld2* knock-in mice revealed a more widespread pathology in various body organs besides inner ear defects (Nayak et al. 2013). In contrast to mice, in the previous studies, the affected members of the DFNB49 families did not appear to have any other obvious relevant deficit besides prelingual, sensorineural hearing loss (Riazuddin et al. 2006; Chishti et al. 2008; Safka Brozkova et al. 2012). However, these human families were not assessed to the same extent as the phenotypic evaluation of the tricellulin mutant mice. We considered the possibility that the affected members of the DFNB49 families might have other, as yet unreported disorders (Nayak et al. 2013). Therefore, we performed comprehensive clinical investigations of affected individuals of a new DFNB49 family, PKDF1069. However, analysis of liver, kidney, heart, brain, pancreas and hematological functional analyses did not reveal any functional abnormalities in these organs co-segregating with hearing loss among the affected individuals. We cannot rule out the possibility of late onset of other tissue abnormalities in affected individuals homozygous for pathogenic *MARVELD2* alleles, as the oldest affected individual evaluated in this study is 22 years old.

There are several possibilities that might explain the discrepancy between the phenotype observed in humans and mice due to tricellulin deficiency. With the exception of the inner ear, there may be redundant tight junction complexes in humans that bypass the defects observed in *Marveld2* mutant mice. Defects in tricellulin may be compensated by other tight junction proteins in humans but not rodents. Alternatively, the broader phenotypic changes in *Marveld2* mutant mice may reflect signaling defects due to tricellulin deficiency, which have no functional consequence in humans.

An inter- and intrafamilial variability in the severity of hearing loss due to recessive mutations of *MARVELD2* has been reported in Pakistani families (Riazuddin et al. 2006) including this study. Severity of hearing thresholds in our DFNB49 families does not show any correlation with age of the affected individual (Fig. 3) (Riazuddin et al. 2006). It is possible that an environmental factor (for example, infections, drugs or loud noise) may have contributed to this inter- and intra-familial phenotypic variability in hearing thresholds. However, we cannot rule out the possibility of genetic modifiers modulating the severity of

hearing loss phenotype, especially in individuals harboring the same *MARVELD2* mutation (Riazuddin et al. 2006). Consistent with a genetic modifier as a possible explanation, we have observed that the *Marvel2* (accession numbers DQ682659) knock-in allele (p.Arg497*) produces varying degrees of hearing loss when this allele is present in different congenic mouse strains (unpublished data).

Tricellulin is necessary to maintain fluid barriers in the inner ear and loss of this barrier in the cochlea leads to hearing loss (Nayak et al. 2013). Loss of tricellulin protein from the sensory epithelia in the inner ear resulted in ultrastructural abnormalities in the tricellular tight junctions, where defects were seen in the formation of the central element of the junctions as well as with the convergence of bicellular tight junction strands. It was proposed that tricellulin is necessary to connect the bicellular junctions with the tricellular tight junctions (Nayak et al. 2013). In this regard, it is important to note that the C-terminal region of tricellulin was shown to bind ZO-1, a bicellular tight junction protein (Riazuddin et al. 2006). It has been proposed that this interaction may allow proper incorporation of tricellulin in the tight junction assembly (Riazuddin et al. 2006). Accordingly, loss of the C-terminal region in the *TRIC^{R500X}* and *TRIC^{C395-Q501del}* mutants results in mislocalization of the proteins from the tricellular junctions and non-specific targeting of the mutant proteins to basolateral domains of transfected cells (Fig. 2E–F).

In summary, the hearing loss segregating in DFNB49 families due to recessive mutations of *MARVELD2* is nonsyndromic in nature, which is in contrast to the phenotype observed in *Marvel2* knock-in mice (Nayak et al. 2013). This may reflect functional redundancy with other tight junction proteins in humans or differences in other genetic, stochastic and environmental factors.

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Fig. 1.

Four families from Pakistan and Slovakia with known *DFNB49* mutations. Hearing loss in these families co-segregates with known *DFNB49* mutations, c.1331+2T>C and c.1183-1G>A, except for the grey individual in family PKDF895, who also has prelingual profound hearing but is heterozygous for the *MARVELD2* mutation, c.1331+2T>C, and does not share the disease haplotype with the other affected members of the family. Filled symbols indicate affected individuals, circles represent females and squares represent males.

Fig. 2. Non-syndromic profound deafness co-segregates with a novel *MARVELD2* deletion in family PKDF1069. **(a)** Genotypes of the participating family members are shown below each symbol. **(b)** Long range PCR used to determine the presence of the allele with the deletion versus the wild-type allele. The wild-type allele gives a 8.7 kb band, while the allele with the in-frame deletion gives a 681 bp band. The labeling of the lanes corresponds to the participating individuals shown in a. **(c)** The genomic position of the deletion breakpoints. Underlined is the 18 bp sequence with 100% nucleotide identity that flanks the deletion on both sides and is oriented in the same direction. **(d–g)** Confocal images of MDCK cells transfected with tricellulin wild-type and mutant constructs. **(d)** TRIC^{WT} is enriched at tricellular junctions in transfected MDCK cells. **(e)** TRIC^{C395-A501del} is found at both tricellular and bicellular junction regions and is no longer enriched at the former. It is also found in the basolateral domain of transfected MDCK cells. The intracellular GFP signal may represent misfolded TRIC^{C395-Q501del} protein that may be retained in the endoplasmic reticulum. **(f)** TRIC^{R500X} is also mislocalized and is no longer enriched at tricellular junctions. Scale bar: 5 μ m. **(g)** The fluorescence intensities of the GFP signal at bicellular and tricellular junctions were measured and the ratio of TJ/BJ was calculated for each construct. The wild-type protein is enriched at tricellular junction and this enrichment is lost or significantly reduced in the mutant proteins. ***, $p < 0.001$.

Fig 3.

Audiograms of the PKDF1069 members. (a) A non-carrier member (s) of the family shows completely normal hearing. (b–d) Affected members p, n and o that are homozygous for the deletion. These members show moderate-to-profound, bilateral sensorineural hearing loss.

Table 1

Clinical finding of PKDF1069 family

Features	Reference Ranges	Units	Family Members									
			Individual s	Individual l	Individual q	Individual r	Individual p	Individual n	Individual o			
Sex			Male	Male	Male	Male	Male	Male	Male	Male	Male	Male
Genotypes			+/+	+/+	+/del ^a	+/del	del/del	del/del	del/del	del/del	del/del	del/del
Present age		year	12	18	07	09	17	20	22			
Hearing loss			Normal	Normal	Normal	Normal	Affected	Affected	Affected			
S ^b Sodium	133 – 145	mmol/L	142	141	140	143	142	139	142			
S Potassium	3.3 – 5.1	mmol/L	4.22	4.53	3.8	3.92	4.49	4.43	4.13			
S Chloride	95 – 108	mmol/L	107	105	106	109	106	105	107			
S Bicarbonate	22 – 29	mmol/L	26.4	26.8	23.3	23.6	26.9	25.9	26			
S Urea Nitrogen	2.14 – 7.14	mmol/L	3.23	5.63	4.87	3.89	3.27	3.53	3.52			
S Creatinine	46.85 – 69.83	µmol/L	30.94	66.30	26.52	26.52	58.34	79.56	48.62			
S Glucose ^c	2.5 – 5.49	mmol/L	6.60	4.99	5.22	5.49	5.05	4.61	NA			
S Calcium	2.1 – 2.55	mmol/L	2.23	2.31	2.38	2.24	2.32	2.32	2.16			
S Magnesium	0.62 – 1.03	mmol/L	0.96	0.87	0.81	0.83	0.77	0.76	0.77			
S Phosphorus	0.97 – 1.94	mmol/L	1.55	1.26	1.47	1.70	1.01	1.2	1.4			
S AST ^d	10 – 50	U/L	51	39	24	80	35	30	48			
S Alkaline Phosphatase	<300	U/L	459	126	231	281	105	105	151			
S GGT ^e	8 – 61	U/L	15	16	14	9	13	24	14			
S Triglycerides	<2.26	mmol/L	1.04	0.78	0.86	0.63	1.16	0.79	0.82			
S Cholesterol	<5.2	mmol/L	3.1032	3.414	2.95	1.84	3.46	4.4	3.47			
S VLDL ^f	0 – 1.03	mmol/L	0.47	0.36	0.39	0.28	0.54	0.36	0.39			
S LDL ^g Cholesterol	<3.36	mmol/L	1.68	1.89	1.44	0.80	1.24	2.77	2.04			
S HDL ^h Cholesterol	0.90–1.68	mmol/L	1.16	1.22	1.28	0.85	1.16	1.32	1.16			
S Creatine Kinase	0 – 189	U/L	380	818	166	126	315	488	368			
Electrocardiogram			Normal ⁱ	Normal	Normal	Normal	Normal	Normal	Normal			

Features	Reference Ranges	Units	Family Members					
			Individual s	Individual l	Individual q	Individual r	Individual p	Individual n
Echocardiogram			Normal	Normal	Normal	Normal	Normal	Normal

^a del: exon 4–5 deletion;

^b „S“ denotes serum;

^c Fasting glucose level;

^d AST: Aspartate Aminotransferase;

^e GGT: Gamma-glutamyl transpeptidase;

^f VLDL: Very Low Density Lipoprotein;

^g LDL: Very Low Density Lipoprotein;

^h HDL: High Density Lipoprotein;

ⁱ Normal: Structurally and functionally normal heart with normal size left ventricle, normal left ventricle systolic function, no appreciable regional wall motion abnormality at rest and normal diastolic function. In bold are given values, that are outside the normal range.

Table 2

Clinical findings of PKDF1069 family

Features	Reference Ranges	Units	Family Members					
			Individual s	Individual l	Individual q	Individual r	Individual p	Individual o
			Normal	Normal	Normal	Normal	Affected	Affected
Complete Blood Count								
White blood cells	4 – 11	x10 ³ /ul	9.24	6.53	13.97	7.81	12.98	7.61
Red blood cells	4 – 6	x10 ⁶ /ul	4.84	5.75	5.2	4.8	4.87	6.55
Hemoglobin	115 – 175	g/L	122	142	126	122	148	143
Hematocrit	36 – 54	%	38.7	43.7	39.3	37	44.5	45.9
Mean cell volume	76 – 96	fL	80	76	75.6	77.1	91.4	70.1
Mean corpuscular hemoglobin	27 – 33	pg	25.2	24.7	24.2	25.4	30.4	21.8
Mean corpuscular hemoglobin concentration	330 – 350	g/L	315	325	321	330	333	312
%Red cell distribution width	11.5 – 14.5	%	14.4	13.1	13.7	14.1	11.9	13.9
Platelets	150 – 400	x10 ³ /ul	199	216	235	127	223	165
Mean platelet volume	7.2 – 11	fL	12.4	10.5	11.1	12.9	12.2	11.2
% Neutrophils	40 – 75	%	85.8	43.4	71.9	72.9	86.2	57.9
% Lymphocytes	20 – 45	%	7.8	41	12.5	13.4	8.6	27.9
% Monocytes	2 – 10	%	4.5	9.5	4.6	8.7	4	8.3
% Eosinophils	0 – 6	%	1.8	5.5	10.8	4.9	1	5.8
% Basophils	0 – 1.5	%	0.1	0.6	0.2	0.1	0.2	0.1
Neutrophils	1.9 – 8	x10 ³ /ul	7.92	2.83	10.05	5.69	11.2	4.41
Lymphocytes	0.9 – 5.2	x10 ³ /ul	0.72	2.68	1.74	1.05	1.11	2.12
Monocytes	0.16 – 1	x10 ³ /ul	0.42	0.62	0.64	0.68	0.52	0.63
Eosinophils	0 – 0.8	x10 ³ /ul	0.17	0.36	1.51	0.38	0.13	0.44
Basophils	0 – 0.2	x10 ³ /ul	0.1	0.04	0.03	0.01	0.02	0.01
Urine Analysis								
Glucose	Negative		Negative	Negative	Negative	Negative	Trace	Negative
Bilirubin	Negative		Negative	Negative	Negative	Negative	Negative	Negative

			Family Members						
Features	Reference Ranges	Units	Individual s	Individual l	Individual q	Individual r	Individual p	Individual n	Individual o
			Normal	Normal	Normal	Normal	Affected	Affected	Affected
Ketone	Negative		Negative	Negative	Negative	Negative	Negative	Negative	Negative
Specific gravity	1.003 – 1.030		1.015	1.010	1.020	1.020	1.020	1.020	1.010
Blood	Negative		++	+	Negative	+	Trace	Negative	Negative
pH	4.5 – 8.0		6.0	5.0	5.0	5.0	5.0	5.0	5.0
Protein	Negative		Negative	Negative	Negative	Negative	Negative	Negative	Negative
Urobilinogen	< 2.0	EU/dL	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Nitrite	Negative		Negative	Positive	Negative	Negative	Negative	Negative	Negative
Leucocyte	Negative		Negative	++	Negative	Negative	Negative	Negative	Negative
White blood cells	0 – 5	HPF ^a	Occasional	10–20	Occasional	Occasional	Occasional	Occasional	Occasional
Red blood cells	0 – 5	HPF	0–5	Occasional	Nil	Occasional	Occasional	Nil	Nil
Epithelial cells	0 – 10	HPF	Occasional	Occasional	Occasional	Occasional	Occasional	Occasional	Occasional
Hyaline cast	0 – 4	LPF ^b	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Cellular cast	0	LPF	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Granular cast	0	LPF	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Bacteria	Negative		Negative	++	Negative	Negative	Negative	Negative	Negative
Mucous	Nil		Nil	Nil	Nil	Nil	Nil	Nil	Nil
Crystal	Nil		Nil	Nil	Few	Few	Few	Few	Nil

^a High power field;^b Low power field. In bold are given values, that are outside the normal range.

Table 3

Brain Computed Tomography (CT) scan of PKDF1069 family members

	Age (y)	Genotype	Brain CT scan (Plain & Contrast study)
Individual s	12	+/+	<ul style="list-style-type: none"> Bilateral almost complete loss of normal pneumatization of mastoid processes with normal symmetrical appearance of IAM^a. Possibility of chronic mastoid disease-effects off. No other abnormality noted.
Individual l	18	+/+	<ul style="list-style-type: none"> Bilateral decreased pneumonization of mastoid processes; consistent with possible chronic mastoditis-effects off. No other abnormality noted.
Individual q	7	+del	<ul style="list-style-type: none"> Normal CT scan. No abnormal meningeal enhancement or any enhancing SOL^b seen.
Individual r	9	+del	<ul style="list-style-type: none"> Normal CT scan. No abnormal meningeal enhancement or any enhancing SOL seen.
Individual p	17	del/del	<ul style="list-style-type: none"> No significant abnormality seen in orbits/brain/PNS^c/mastoid region. IAM appear bilaterally symmetrical/normal with no evidence of sellar/supra-sellar SOL or any abnormality in the petrous-temporal bone.
Individual n	20	del/del	<ul style="list-style-type: none"> Normal CT brain/sinuses and orbits/retrobulbar areas with evidence of possible right sided chronic mastoditis-effects off. No area of bone destruction or any enhancing intra/extra-axial SOL seen.
Individual o	22	del/del	<ul style="list-style-type: none"> Slight asymmetry in the size of lateral ventricles seen with no gross intracranial pathology including infarction/hemorrhage/SOL. Normal appearance of mastoid regions and petrous-temporal bone areas/IAM. No significant abnormality in the orbits/PNS.

^a IAM: Internal Auditory Meatus;^b SOL: space occupying lesion;^c PNS: peripheral nervous system.

Table 4

Clinical finding of Slovak Family

Features	Units	Family Members									
		Subject 1 proband	Subject 2 brother		Subject 3 sister		Subject 4 mother		Subject 5 father		
Sex		Male	Male		Female		Female		Male		
Genotypes		c.1331+2T>C/c.1331+ 2T>C	c.1331+2T>C/WT		c.1331+2T>C/WT		c.1331+2T>C/WT		c.1331+2T>C/WT		
Present age	year	6	10		4		29		32		
Hearing		Affected	Normal		Normal		Normal		Normal		
		Ref Ranges ^g	Values	Ref Ranges	Values	Ref Ranges	Values	Ref Ranges	Values	Ref Ranges	Values
S ^g Sodium	mmol/L	135.0–145.0	139.3	135.0–145.0	139.6	135.0–145.0	138.7	135.0–145.0	138.5	135.0–145.0	141.2
S Potassium	mmol/L	3.40–4.70	3.89	3.40–4.70	3.86	3.40–4.70	4.08	3.50–5.10	4.06	3.50–5.10	4.54
S Chloride	mmol/L	95–105	104	95–105	103	95–105	106	95–105	104	95–105	106
S Bicarbonate	mmol/L	22.0–26.0	24.1	22.0–26.0	23.6	22.0–26.0	20.8	22.0–26.0	27.9	22.0–26.0	25.6
S Urea Nitrogen	mmol/L	<8.3	3.1	<8.3	3.5	<8.3	3.2	<8.3	4.1	<8.3	6.8
S Creatinine	μmol/L	25–42	39	25–42	4.3	23–37	29	45–84	51	59–104	81
S Glucose ^b	mmol/L	3.3–5.6	4.3	3.3–5.6	4.7	3.3–5.6	4.7	4.1–5.9	5.0	4.1–5.9	5.5
S Calcium	mmol/L	2.20–2.70	2.42	2.20–2.70	2.44	2.20–2.70	2.48	2.15–2.50	2.33	2.15–2.50	2.39
S Magnesium	mmol/L	0.70–0.95	0.83	0.70–0.86	0.87	0.70–0.95	0.87	0.66–1.07	0.86	0.66–1.07	0.93
S Phosphorus	mmol/L	1.05–1.80	1.65	1.05–1.85	1.51	1.05–1.80	1.61	0.81–1.45	0.95	0.81–1.45	1.10
S AST ^c	U/L	<40.12	26.95	<40.12	22.35	<40.12	29.94	<31.74	16.77	<40.12	17.65
S Alkaline Phosphatase	U/L	0.00–268.26	239.52	0.00–294.12	285.29	0.00–268.26	372.46	34.73–104.19	59.28	39.41–126.47	67.06
S GGT ^d	U/L	<22.75	8.38	<16.80	8.40	<22.75	9.58	<40.12	8.38	<59.99	16.20
S Triglycerides	mmol/L	0.10–1.70	0.81	0.10–1.70	0.65	0.10–1.70	0.44	0.10–1.70	0.62	0.10–1.70	0.54
S Cholesterol	mmol/L	2.10–4.30	4.51	2.10–4.30	4.37	2.10–4.30	3.34	2.90–5.00	3.99	2.90–5.00	5.49
S LDL ^e Cholesterol	mmol/L	0.00–2.59	2.76	0.00–2.59	2.66	0.00–2.59	1.96	0.00–2.59	2.05	0.00–2.59	4.29
S HDL ^f Cholesterol	mmol/L	0.90–1.45	1.68	0.90–1.45	1.77	1.15–1.68	1.40	1.15–1.168	1.99	0.90–1.45	1.27
S Creatine Kinase	U/L	<148.50	177.25	<264.71	184.71	<148.50	252.69	<170.66	95.80	<188.24	281.76
Electrocardiogram			Normal ^h		Normal		Normal		Normal		Normal

Family Members									
Features	Units	Subject 1 proband		Subject 2 brother		Subject 3 sister		Subject 4 mother	
Echocardiogram			Normal		Normal		Normal		Normal

^a „S” denotes serum;

^b Fasting glucose level;

^c AST: Aspartate Aminotransferase;

^d GGT: Gamma-glutamyl transpeptidase;

^e LDL: Very Low Density Lipoprotein;

^f HDL: High Density Lipoprotein;

^g Ref Ranges: Reference Ranges;

^h Normal: Structurally and functionally normal heart with normal size left ventricle, normal left ventricle systolic function, no appreciable regional wall motion abnormality at rest and normal diastolic function. In bold are values that are outside the normal range.

Clinical finding of Slovak Family

Table 5

Features	Units	Family Members									
		Subject 1 Proband		Subject 2 Brother		Subject 3 Sister		Subject 4 Mother		Subject 5 Father	
Complete Blood Count		Reference Ranges	Values	Reference Ranges	Values	Reference Ranges	Values	Reference Ranges	Values	Reference Ranges	Values
White blood cells	x10 ³ /ul	5.00–14.00	5.14	4.50–13.50	6.67	5.00–14.00	6.18	3.6–11.00	4.41	3.80–10.60	7.45
Red blood cells	x10 ⁶ /ul	3.90–5.30	4.05	4.00–5.20	4.36	3.90–5.30	4.41	3.8–5.2	3.91	4.40–5.90	4.88
Hemoglobin	g/L	115–135	116	115–155	123	115–135	123	120–160	115	130–180	150
Hematocrit	%	34–41	34	35–45	35	34–41	35	36–47	34	40–52	44
Mean cell volume	fL	75.0–87.0	83.7	77.0–95.0	80.0	75.0–87.0	79.8	80.0–100	87.7	80.0–100	90.7
Mean corpuscular hemoglobin	pg	24.0–30.0	28.6	25.0–33.0	28.1	24.0–30.0	27.9	26–34	29.3	26.0–34.0	30.6
Mean corpuscular hemoglobin concentration	g/L	310–370	341	310–370	352	310–370	350	320–360	334	320–360	338
%Red cell distribution width	%										
Platelets	x10 ³ /ul	150–400	308	150–400	233	150–400	331	150–400	241	150–400	272
Mean platelet volume	fL	7.0–13.0	6.9	7.0–13.0	7.9	7.0–13.0	6.6	7.0–13.0	7.5	7.0–13.0	8
% Neutrophils	%	42–85	45.8	42–85	49.5	42–85	40.7	42–85	61.4	42–85	60.4
% Lymphocytes	%	18–45	40.9	18–45	33.9	18–45	50.5	18–45	30.2	18–45	32.7
% Monocytes	%	2.0–9.0	4.7	2.0–9.0	5.4	2.0–9.0	4.2	2.0–9.0	5.6	2.0–9.0	3.6
% Eosinophils	%	0.0–4.0	4.9	0.0–4.0	8.3	0.0–4.0	1.6	0.0–4.0	0.3	0.0–4.0	1.3
% Basophils	%	0.0–1.0	0.4	0.0–1.0	0.1	0.0–1.0	0.2	0.0–1.0	0.2	0.0–1.0	0.3
Neutrophils	x10 ³ /ul	1.5–8.0	2.35	1.8–8.00	3.30	1.5–8.0	2.52	42–85	61.4	2.00–7.70	4.49
Lymphocytes	x10 ³ /ul	1.50–7.0	2.10	1.50–6.50	2.26	1.50–7.0	3.12	18–45	30.2	0.70–4.70	2.43
Monocytes	x10 ³ /ul	0.05–1.00	0.24	0.05–1.00	0.36	0.05–1.00	0.26	2.0–9.0	5.6	0.05–1.00	0.26
Eosinophils	x10 ³ /ul	0.00–0.50	0.25	0.00–0.50	0.56	0.00–0.50	0.10	0.0–4.0	0.3	0.00–0.30	0.10
Basophils	x10 ³ /ul	0.00–0.20	0.02	0.00–0.20	0.01	0.00–0.20	0.01	0.0–1.0	0.2	0.00–0.10	0.02
Urine Analysis											
Glucose	mmol/l	0.00–3.00	Negative	0.00–3.00	Negative	0.00–3.00	Negative	0.00–3.00	Negative	0.00–3.00	Negative
Bilirubin	µmol/l	0.0–8.5	Negative	0.0–8.5	Negative	0.0–8.5	Negative	0.0–8.5	Negative	0.0–8.5	Negative

Features	Units	Family Members									
		Subject 1 Proband		Subject 2 Brother		Subject 3 Sister		Subject 4 Mother		Subject 5 Father	
		Reference Ranges	Values	Reference Ranges	Values	Reference Ranges	Values	Reference Ranges	Values	Reference Ranges	Values
Complete Blood Count											
Ketone	mmol/l	0.00–0.50	Negative	0.00–0.50	Negative	0.00–0.50	Negative	0.00–0.50	Negative	0.00–0.50	Negative
Specific gravity		1.00–1.030	1.019	1.00–1.030	1.022	1.00–1.030	1.025	1.00–1.030	1.020	1.00–1.030	1.025
Blood	mg/l	0.00–0.3	Negative	0.00–0.3	Negative	0.00–0.3	Negative	0.00–0.3	Negative	0.00–0.3	Negative
pH		5.0–7.0	6.0	5.0–7.0	5.5	5.0–7.0	6.0	5.0–7.0	6.0	5.0–7.0	5.5
Protein	g/l	0.00–0.15	Negative	0.00–0.15	Negative	0.00–0.15	Negative	0.00–0.15	Negative	0.00–0.15	Negative
Urobilinogen	µmol/l	0–35	Negative	0–35	Negative	0–35	Negative	0–35	Negative	0–35	Negative
Nitrite			Negative		Negative		Negative		Negative		Negative
Leukocyte	Number/µl	0–25	Negative	0–25	Negative	0–25	Positive	0–25	Negative	0–25	Negative
White blood cells	Number/µl	0–14	1	0–14	1	0–14	53	0–14	1	0–14	1
Red blood cells	Number/µl	0–10	1	0–10	1	0–10	10	0–10	2	0–10	1
Epithelial cells	Number/µl		Negative		Negative		Negative	0–20	Present		Negative
Mucous			Present		Lot of		Present		Present		Present

Table 6

Echocardiogram and electrocardiogram findings of the Slovak family

Subject	Cardiology Doppler											ECG						
	Ao valve		TV Regurg				PV					Rhythm	QRS	PR	QTc	Transition zone	AV conduction	Repolarization
	Peak Grad	Peak Vel	Peak Grad	RVSP	Peak Vel	RA pressure	Peak Grad	Peak Vel	Mean Vel	Mean Grad	Accel time	VTI						
	mmHg	m/s	mmHg	mmHg	m/s	mmHg	mmHg	cm/s	cm/s	mmHg	ms	cm						
Subject 1 proband	6.8	1.3	25	35	2.5	10	2.7	82	46.6	1.2	143	18	sinus	-31°		V3-4	normal	normal
Subject 2 brother	6.8	1.3	27	37	2.6	10	2.4	78.2	51.1	1.2	140	15.8	sinus	75°		V3-4	normal	normal
Subject 3 sister	6.8	1.3	23	33	2.4	10	3	85.9	49.4	1.1	90	15.6	sinus	64°		V2	normal	normal
Subject 4 mother	10.2	1.6	29.2	39.2	2.7	10	4.8	1.1	72.7	2.3	130	22.5	sinus	36°		V3-4	normal	normal
Subject 5 father	9	1.5	27	37	2.6	10	4.1	1.1	65.2	2	115	17.9	sinus	50°		V2-3	normal	normal

Cardiology M Mode																			
	LV dias vol	LV sys vol	EF	FS	IVS/LVPW	SV	SV index	Cardiac output	LV mass	LV mass index	RVD (D)	IVS (D)	LVD (D)	LVPW (D)	LVD (S)	Heart rate	LA/Ao	ARD	LAD
	ml	ml	%	%		ml	ml/m ²	l/min	g	g/m ²	cm	cm	cm	cm	cm	bpm	cm	cm	cm
Subject 1 proband	66	18	73	41	1	48	52	3.6	62	67	1	0.6	3.9	0.6	2.6	76	1.1	2.2	2.4
Subject 2 brother	79	22	72	40	1	57	49	4.9	70	60	1.4	0.6	4.2	0.6	2.5	86	1.2	2.3	2.8
Subject 3 sister	58	16	72	41	1	42	51	4.1	56	68	1.4	0.6	3.7	0.6	2.2	97	1.1	2.1	2.4
Subject 4 mother	147	41	72	42	1.1	106	64	7.8	148	90	1.4	0.8	5.5	0.7	3.2	74	1.1	3	3.2
Subject 5 father	167	47	72	41	1.4	120	61	N/A	218	111	2.2	1.1	5.8	0.8	3.4	97	1	3.5	3.6

Ao valve: Aortic valve; TV Regurg: Tricuspid valve regurgitation; PV: pressure volume; ECG: electrocardiogram; RVSP: right ventricle systolic pressure; QTc: corrected QT; AV conduction: atrioventricular conduction; LV dias vol: left ventricular diastolic volume; LV sys vol: left ventricular systolic volume; EF: ejection fraction; FS: fractional shortening; IVS: interventricular septum; LVPW: left ventricular posterior wall, SV: stroke volume, RVD: right ventricular dimensions; LVD: left ventricular dimensions, LA: left atrium; ARD: aortic root diameter; LAD: left atrium diameter; N/A: not available.

Table 7**General Physical findings of the Slovak Family**

	Hearing	Genotype	Sex	Age (years)	Height (cm)	Weight (kg)	Dental exam	General physical	Skeletal survey	MRI
Subject 1 Proband	SNHL	c.1331+2T>C/c.1331+2T>C	male	6	122	25	Complete deciduous dentition, slight generalized microdontia with distinct diastemata	Microtia grade I, otherwise healthy	normal, no apparent malformations	normal inner ear and brain morphology according to visual inspection
Subject 2 Brother	normal	c.1331+2T>C/WT	male	10	147	32	Slight generalized microdontia with distinct diastemata	Microtia grade I, otherwise healthy	normal, no apparent malformations	N/A
Subject 3 Sister	normal	c.1331+2T>C/WT	female	4	110	23	Complete deciduous dentition	healthy	normal, no apparent malformations	N/A
Subject 4 Mother	normal	c.1331+2T>C/WT	female	29	164	60	Caries teeth, other wise normal	healthy	normal, no apparent malformations	N/A
Subject 5 Father	normal	c.1331+2T>C/WT	male	33	176	79	Incomplete dentition-after extractions, caries teeth, no microdontia or diastemata between remaining teeth	Microtia grade I, otherwise healthy	normal, no apparent malformations	N/A