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Gene structure and mutant alleles of *PCDH15*: nonsyndromic deafness DFNB23 and type 1 Usher syndrome

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Abstract

Mutations of *PCDH15*, encoding protocadherin 15, can cause either combined hearing and vision impairment (type 1 Usher syndrome; USH1F) or nonsyndromic deafness (DFNB23). Human *PCDH15* is reported to be comprised of 35 exons and encodes a variety of isoforms with 3 to 11 ectodomains (EC), a transmembrane domain and a carboxy-terminal cytoplasmic domain (CD). Building on these observations we describe an updated gene structure that has four additional exons of *PCDH15* and isoforms that can be subdivided into four classes. Human *PCDH15* encodes three alternative, evolutionarily conserved unique cytoplasmic domains (CD1, CD2 or CD3). Families ascertained on the basis of prelingual hearing loss were screened for linkage of this phenotype to markers for *PCDH15* on chromosome 10q21.1. In seven of twelve families segregating USH1 we identified homozygous mutant alleles (1 missense, 1 splice site, 3 nonsense and 2 deletion mutations) of which six are novel. One family was segregating nonsyndromic deafness DFNB23 due to a homozygous missense mutation. To date in our cohort of 557 Pakistani families, we have found 11 different *PCDH15* mutations that account for deafness in 13 families. Molecular modeling provided mechanistic insight into the phenotypic variation in severity of the *PCDH15* missense mutations. We did not find pathogenic mutations in five of the twelve USH1 families linked to markers for *USH1F*, which suggest either the presence of mutations of yet additional undiscovered exons of *PCDH15*, mutations in the introns or regulatory elements of *PCDH15*, or an additional locus for type I USH at chromosome 10q21.1.

Keywords

DFNB23; Usher syndrome; protocadherin 15; *PCDH15*; deafness; retinitis pigmentosa

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Electronic database information
GENSCAN, http://genes.mit.edu/GENSCAN.html
Protocadherin 15 Molecular Modeling data, http://www.cmbi.ru.nl/~hvensela/PCDH15
WHAT IF server, http://swift.cmbi.ru.nl
YASARA, www.yasara.org

Introduction

Causes of profound childhood deafness include environmental and genetic factors. In North American and European populations hereditary deafness is estimated to occur in 1 in every 2,000 newborns (Morton 1991). Approximately 30% of this hearing impairment co-occurs with other clinical features such as loss of vision (Bergstrom et al. 1971). Usher syndrome (USH) is characterized by a loss of vision due to retinitis pigmentosa (RP) and bilateral sensorineural deafness (Smith et al. 1994). From studies in Scandinavia, Colombia, United Kingdom and the United States the prevalence of USH is between 1/16,000 and 1/50,000 (Petit 2001).

USH is classified into three clinical subtypes. Type 1 USH (USH1) is the most genetically heterogeneous. To date, there are seven USH1 loci (USH1B, USH1C, USH1D, USH1E, USH1F, USH1G, and USH1H) and five USH1 genes have been identified (Ahmed et al. 2008; Petit 2001; Weil et al. 2003). Particular mutations of four of these USH1 genes, *MYO7A*, *USH1C*, *CDH23*, and *PCDH15* can also cause nonsydromic hearing loss, DFNB2, DFNB18, DFNB12 and DFNB23, respectively (Ahmed et al. 2003; Ahmed et al. 2002; Bork et al. 2001; Riazuddin et al. 2008).

Mouse models have been instrumental in identifying the genes for human USH1 (Ahmed et al. 2001; Alagramam et al. 2001b; Bolz et al. 2001; Weil et al. 1995; Weil et al. 2003). For example, the Ames waltzer (*av*) phenotype is due to recessive mutations of *Pcdh15* (Alagramam et al. 2001a). Homozygous *av* mice show degeneration of inner ear neuroepithelia associated with deafness and vestibular dysfunction but no RP (Ball et al. 2003; Haywood-Watson et al. 2006), and are only a model for DFNB23 nonsyndromic deafness (Ahmed et al. 2003).

We previously reported a large number of wild type alternatively spliced transcripts of mouse *Pcdh15*, which utilize a subset of the 39 exons, and can encode a signal sequence, an extracellular domain with 3 to 11 ECs, a single-pass transmembrane domain and one of three different carboxy-terminus cytoplasmic domains (Ahmed et al. 2006) referred to as CD1, CD2 or CD3. Here we report mutations of *PCDH15* in seven families segregating USH1F, and one family segregating nonsyndromic deafness DFNB23. We found six novel mutant alleles of *PCDH15*. We also provide a more complete gene structure for *PCDH15* that includes four additional exons encoding three alternative cytoplasmic domains.

Materials and methods

Subject enrollment

This study was approved by the Institutional Review Board (IRB) at the National Centre of Excellence in Molecular Biology, Lahore, Pakistan (FWA00001758), and the Central NeuroScience IRB at the National Institutes of Health, USA (OH-93-N-016). Subjects were ascertained in rural areas of the Punjab and Sindh provinces of Pakistan. Written informed consent was obtained from all adults and parents of minors under the age of 18 years.

Clinical evaluation

We performed medical history interviews to identify possible clinical features of syndromic hearing loss and rule out potential environmental causes. Affected and some unaffected subjects underwent a general otologic examination. Hearing was evaluated in some affected and unaffected subjects by pure-tone air- and bone-conduction audiometry with or without tympanometry. No air-bone gaps were observed in any tested individuals. Vestibular function was assessed by tandem gait, Romberg testing and electronystagmography (ENG) with caloric stimulation. Funduscopic and electroretinography (ERG) examinations were performed by an

ophthalmologist to confirm the absence or presence of RP. The ages of the affected individuals at the time of examination ranged from 8 to 27 years.

DNA isolation, genotyping and mutational analyses

Genomic DNA was extracted from peripheral blood samples using a standard protocol. We first screened for linkage of the deafness phenotype to STR (short tandem repeat) markers for all of the reported DFNB/USH loci using genomic DNA from affected and unaffected members of 557 families segregating autosomal recessive hearing loss. The *USH1F/DFNB23* linked markers used are *D10S1643*, *D10S546*, and *D10S2522*.

All of the exons of *PCDH15* (accession numbers AY029237; EU718480; EU718481; EU718482) and approximately 100 base pairs of intronic sequences flanking each exon (Ahmed et al. 2003; Ahmed et al. 2001) were PCR-amplified and sequenced using genomic DNA from an affected member of each USH1F/DFNB23 family. In the eight families where mutations in *PCDH15* were found, DNA from all affected and unaffected members were then examined for the mutation.

cDNA cloning and sequence analysis of *PCDH15*

Downstream of the exon encoding the reported carboxy-terminus of the cytoplasmic domain (CD1) of human *PCDH15* (Ahmed et al. 2003) there are regions of sequence conservation with predicted open reading frames. RT-PCR analyses revealed transcripts of *PCDH15* in human retina cDNA (GEtRare™, Genemed) that have a subset of additional alternatively spliced exons that encode two novel cytoplasmic domains designated CD2 and CD3. All PCR products were subcloned and both strands were sequenced. PCR primers used for the amplification of *PCDH15* are reported in Ahmed et al. 2001. The primers used to PCR amplify the 4 novel exons of *PCDH15* described here are provided in Supplemental Table 1.

Molecular Modeling

Molecular modeling was performed using the C-cadherin crystal structure as a template (PDB-identifier 1q55) (He et al. 2003). Sequence identity between the EC repeat 2 and 3 of protocadherin 15 and C-cadherin was 26% over 252 amino acids. Modeling was done with the WHAT IF server (http://swift.cmbi.ru.nl) and YASARA (www.yasara.org) using standard parameter settings (Krieger et al. 2002; Vriend 1990).

Results

Linkage and Mutational Analyses

In 13 unreported Pakistani families (Fig. 1 and data not shown), we observed co-segregation of deafness with homozygosity for *USH1F/DFNB23*-linked STR marker genotypes. We identified homozygous mutant alleles of *PCDH15* in affected individuals from eight of these 13 families (Fig. 1 and Table 1). These variants co-segregated with deafness in each of the eight families (Fig. 1) and were not found in 100 ethnically matched hearing control individuals (Table 1).

Missense mutations—In family PKDF756 we found a transversion mutation (c.400C→G) in exon 5, resulting in a substitution of glycine for arginine at position 134 (p.R134G). This residue is located in the first ectodomain (EC1; Table 1). Amino acid alignments of several protocadherin 15 orthologues indicate conservation of arginine-134 (Fig. 2). A ClustalW amino acid sequence alignment of the eleven EC domains of protocadherin 15 reveals that the p.R134G mutation does not disrupt a conserved calcium-binding motif (Fig. 3).

All affected individuals of family PKDF756 were said to have begun walking at or before 12 months of age, and subsequent examinations show normal vestibular function (Table 1). Fundus examinations of two older individuals (12 and 16 years) show no evidence of RP, which was further confirmed by normal ERG waves amplitudes. However, these affected individuals are not old enough to make a conclusive diagnosis of nonsyndromic hearing loss DFNB23. The onset of RP may be delayed and appear in the second or third decade of life. However, the oldest affected individual in family PKDF70 segregating p.R134G was 44 years old at the time of examination and had normal ERG wave amplitudes (Ahmed et al. 2003). These data indicate that p.R134G is associated with nonsyndromic hearing loss DFNB23 (Ahmed et al. 2003).

The subjects from the remaining seven Pakistani families segregating *PCDH15* mutations are profoundly deaf and started walking independently between 12-28 months (Table 1). Balance function of the 13 affected individuals from these seven families was evaluated by tandem gait and Romberg test. None of these affected individuals had a normal tandem gait or a normal Romberg test (Table 1). Fundus examination of the 13 affected individuals revealed a varied occular phenotype (Table 1) that ranged from slight pigmentary changes to complete retinal degeneration.

Sequence analysis of all *PCDH15* exons (Table 1) in affected members of USH1 family PKDF875 demonstrated homozygosity for a missense change at a conserved residue in EC2 (c.533A→G, p.D178G; Fig. 2) encoded by exon 6. Alignments of protocadherin 15 EC domains shows that p.D178G does disrupt a conserved DXD calcium-binding motif of EC2 (Fig. 3).

Splice-site mutation—Family PKDF248 is segregating a splice-donor-site mutation (c. 3717+1G→T) in intron 27 that alters the consensus "G" of the "GT" donor splice site of exon 27 (Table 1). The GENSCAN (http://genes.mit.edu/GENSCAN.html) predicted effect of c. 3717+1G→T, when included in the otherwise wild type genomic sequence, is the use of a cryptic splice donor site in intron 27, a shift in the translation reading frame followed by a premature stop codon (p.V1240LfsX2). If mutant mRNA is not entirely degraded by the nonsense mediated decay pathway, the predicted protein could encode all of the 11 EC domains, but would lack the transmembrane and the cytoplasmic domain.

Protein truncating mutations—Three mutations causing premature stop codons were identified (Table 1). Affected individuals of family PKDF338 are homozygous for a transition mutation (c.7C→T) resulting in a stop codon at position 3 (p.R3X). This mutation was previously reported segregating with USH1 in two families (Ahmed et al. 2001;Alagramam et al. 2001b). In family PKDF809, we found a transversion mutation (c.1940C→G), converting a serine codon at position 647 to a stop codon (p.S647X). This mutation is predicted to truncate the protein in EC6 domain. Another nonsense mutation (c.2052C→A) causes a truncation in exon 17 encoding the EC6 domain (p.Y684X; Table 1) in family PKDF801. Affected members of families PKDF891 and PKDF770 (Fig. 1) are segregating a homozygous c.2483delT, which is predicted to truncate the protein before EC8, and c.4257delA located in exon 32 encoding cytoplasmic domain 1 (CD1), respectively (Table 1).

Novel exons of *PCDH15*

For five families segregating deafness linked to markers for *PCDH15* we did not find pathogenic variants of *PCDH15* after sequencing the 35 reported exons of this gene. Four of these five families have significant simulated LOD scores of 3.8, 5.2, 3.3 and 3.6. These data suggest either the presence of mutations of yet additional undiscovered exons of *PCDH15*, mutations in the introns or regulatory elements of *PCDH15* (Alagramam et al. 2007), or an additional locus for type I USH at chromosome 10q21.1. Conserved synteny between mouse

and human allowed us to identify four new exons (previously annotated as ESTs in the NCBI database), which encode two novel, conserved, cytoplasmic domains (CD2 and CD3) of protocadherin 15 (Fig. 4A). The amino acid sequences of CD1, CD2 and CD3 are entirely different from one another. Consistent with the nomenclature for the isoforms of *Pcdh15* we reported for the mouse (Ahmed et al. 2006), there are also four isoform classes of human isoforms of *PCDH15* (Fig. 4A). In human NK/T lymphoma cells, Rouget-Quermalet and co-authors reported a secreted isoform of protocadherin 15 (SI), which contains the first 21 exons of *PCDH15* encoding the signal peptide and six EC (Rouget-Quermalet et al. 2006).

The amino acid sequences of human and mouse CD1, CD2 and CD3 cytoplasmic domains of *PCDH15* have 55% (67%), 83% (91%) and 77% (84%) identity (similarity), respectively (Supplemental Figs. 1-3). In addition to characterizing the isoforms of human *PCDH15*, we also determined the expression pattern of CD1, CD2 and CD3 isoforms in various tissues. *PCDH15-CD1* has a limited pattern of expression and was detected in human testis, retina and cochlear cDNAs (Fig. 4B). *PCDH15-CD2* expression was present in the human heart, kidney, thymus, spleen, testis, retina and cochlear cDNAs (Fig. 4B), whereas *PCDH15-CD3* is a widely transcribed isoform and was detected in almost all of the cDNAs from 18 tissues that we tested (Fig. 4B). However, mutational screening of the novel exons of *PCDH15* encoding CD2 and CD3 did not reveal additional pathogenic variants.

The USH1 linkage interval for the five presumably *PCDH15*-mutation-negative families include about 42 genes. After excluding putative regulatory elements and genomic deletions encompassing *PCDH15*, the 42 genes would then be screened for mutations, one of which may prove to be a third USH1 gene on chromosome 10q11.2-q21.1.

Molecular Modeling

The aspartic acid (p.D178) mutated in family PKDF875 segregating USH1 resides in the calcium binding motif in the loops between EC2 and EC3 (http://www.cmbi.ru.nl/~hvensela/PCDH15/). Glycine does not have a side chain in contrast to aspartic acid and therefore cannot interact with the calcium atoms. As a result the p.D178G allele is predicted to show decreased calcium affinity. The previously reported p.G262D mutation associated with DFNB23 is located close to the calcium-binding residues in the loops between EC2 and EC3 (Ahmed et al. 2003). Even though glycine-262 is not directly involved in binding calcium, it is predicted to help maintain the correct shape of the calcium-binding domain. Aspartic acid has a large side chain, while glycine does not have a side chain, so the aspartic acid at this position will disturb the local structure that is needed to bind calcium. R134G is also associated with nonsyndrome deafness DFNB23 (Table 1). This mutation is located in the middle of EC1 with its side chain pointing outwards. This domain is known to be important for interaction with a partnering cadherin or protocadherin monomer (Prakasam et al. 2006). Mutation from the hydrophilic and positively charged arginine into a glycine might disturb these interactions. Indeed p.R134G was shown *in vitro* to result in the loss of interaction between protocadherin 15 and cadherin 23 (Kazmierczak et al. 2007).

Discussion

To date, we have identified 18 families co-segregating deafness linked to markers for *USH1F/DFNB23* (Ahmed et al. 2003; Ahmed et al. 2001). Mutations of *PCDH15* were found in 13 of these 18 families. We did not find a pathogenic mutation in five of the twelve USH1 families. *CDH23* in humans is located approximately 17.8 cM from *PCDH15* on chromosome 10q22.1 (Bolz et al. 2001; Bork et al. 2001). Mutations of *CDH23* can cause either type 1 USH or DFNB12 nonsyndromic hearing loss (Astuto et al. 2002; Bork et al. 2001). On the basis of meiotic recombinations in affected individuals, we can exclude mutations of *CDH23* as the

cause of USH1 segregating in four of these five *PCDH15*-linked but *PCDH15* mutation-negative families. However, our genetic analyses do not exclude *PCDH15*.

To date, 21 recessive alleles of *PCDH15* at the *USH1F* locus and two mutant *DFNB23* alleles have been reported (Ahmed et al. 2003; Cremers et al. 2007). In addition, digenic inheritance was suggested as an explanation for three USH1 singeltons (Zheng et al. 2005). Each affected individual was found to be carrying one recessive mutant allele of *PCDH15* and one recessive mutant allele of *CDH23* (Zheng et al. 2005), and it is the combination of these two non-allelic mutations that was presumed to cause type 1 USH (Zheng et al. 2005). However, not all the exons of *PCDH15* could have been checked for mutations and mutations were screened by SSCP analyses, not by direct sequencing of genomic DNA. Moreover, one of the three USH1 "digenic subjects" (family 1677) was reported to be homozygous for a pathogenic mutation T1209A of *CDH23,* while also a carrier of 5601delAAC in *PCDH15* (Zheng et al. 2005) and is thus not an example of digenic inheritance. In our opinion, the evidence for digenic inheritance responsible for type 1 Usher syndrome is not robust.

In our cohort of 557 Pakistani families ascertained on the basis of severe to profound deafness, 2.3% have mutations of *PCDH15*. The majority of mutations of *PCDH15* occur within the region encoding the EC domains or in CD1 (Supplementary Table 2). Mutations associated with nonsyndromic deafness DFNB23 or type 1 USH have not been found in the exons encoding the transmembrane domain or in exons encoding the alternative cytoplasmic domains CD2 and CD3 (Supplementary Table 2). Protocadherin-15-CD2 and protocadherin-15-CD3 are expressed in many cell types outside of the auditory system, and perhaps have other necessary functions. Although our sample size of mutations of *PCDH15* is still small, it is tempting to speculate that mutations of the exons encoding CD2 and CD3 may not result in Usher syndrome but some other pleiotropic disorder.

The three missense mutations (p.R134G, p.D178G, p.G262D) of *PCDH15* are located within exons 5 to 8 encoding the extracellular N-terminus of protocadherin 15. R134G and G262D are associated with nonsyndromic deafness DFNB23 and do not changes the conserved sequence of the DXD, LDRE and DXNDN motifs of the EC domains. These motifs are responsible for calcium-binding and rigidification of the EC domain (Alattia et al. 1997). So far only one homozygous missense mutation (p.D178G) is associated with USH1F and it does alter the canonical DXD motif of EC2 (Fig. 3), which is predicted to result in a reduced affinity for calcium. By comparison, all truncating mutant alleles of *PCDH15* are associated with type 1 USH. Similarly, all of the 31 reported truncating mutant alleles of *CDH23* cause USH1D (Astuto et al. 2002; Bolz et al. 2001; Bork et al. 2001; Ouyang et al. 2005; Roux et al. 2006). A better understanding as to how the retina is spared in deaf individuals homozygous for presumably hypomorphic mutations of *PCDH15*, *CDH23*, *MYO7A* or *USH1C* (Ahmed et al. 2003; Ahmed et al. 2002; Bork et al. 2001; Riazuddin et al. 2008) should be helpful in developing interventions that delay the age of onset and/or slow the progression of the RP component of Usher syndrome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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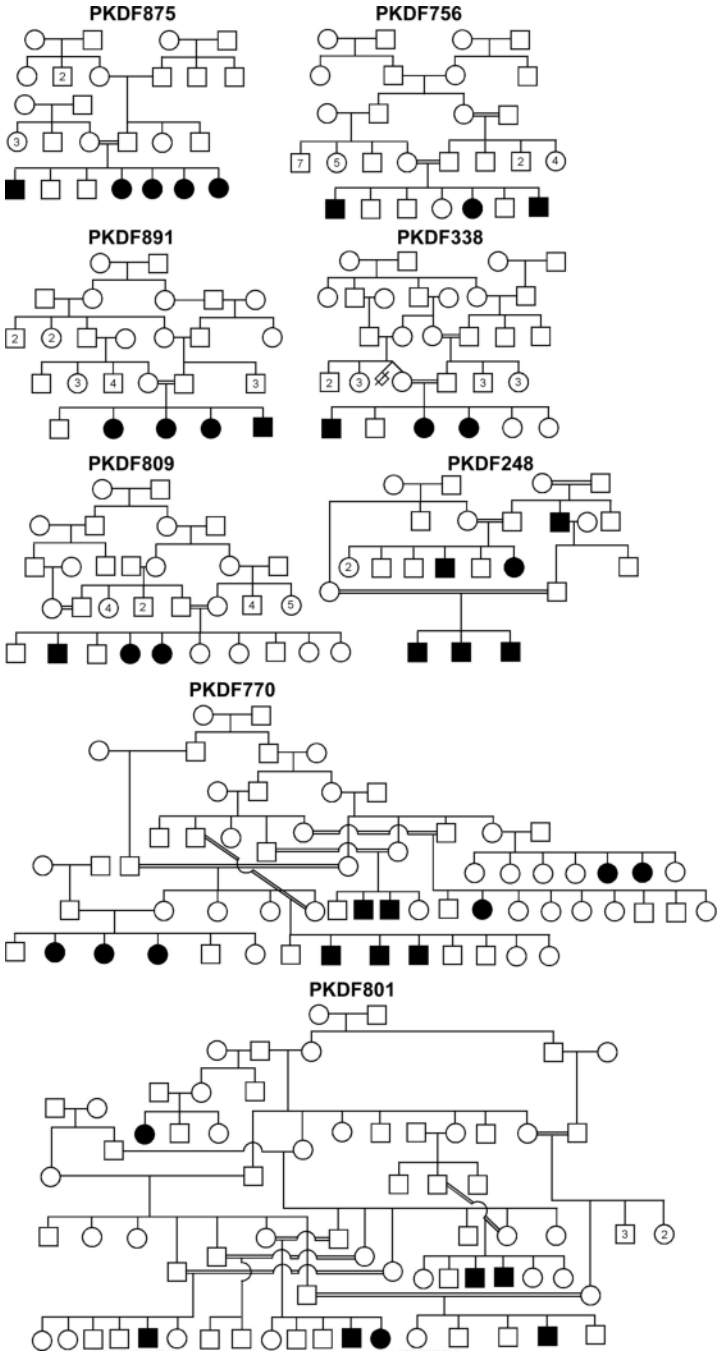

Fig. 1.
Pedigrees of eight Pakistani families segregating recessive sensorineural hearing loss linked to markers for *PCDH15*.

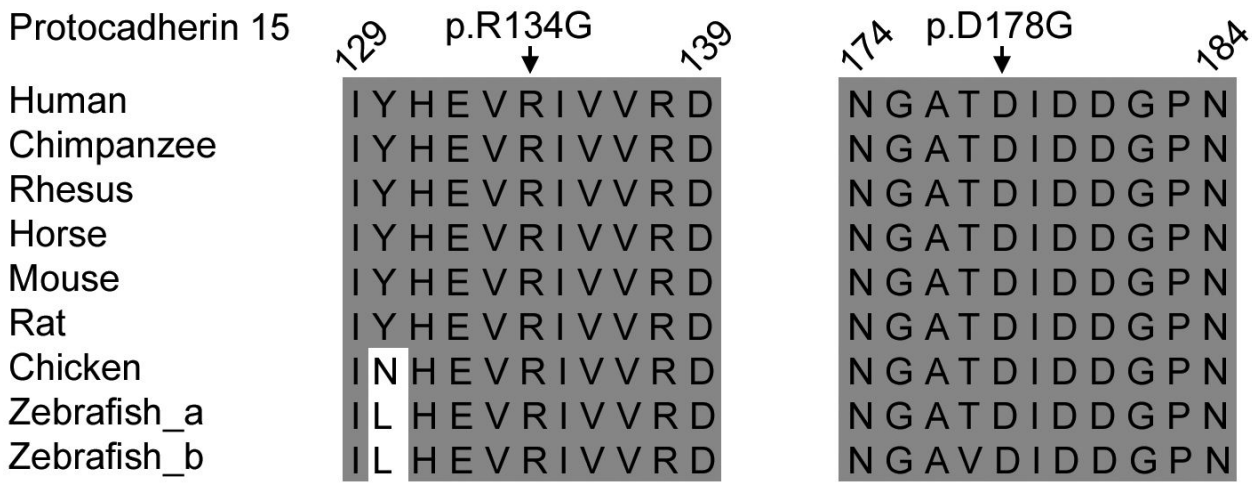

Fig. 2.
ClustalW multiple amino acid sequence alignment of protocadherin 15 orthologs in two noncontiguous regions show that p.R134 and p.D178 are conserved across species (shaded background, same amino acids; light background, non-conserved amino acids).

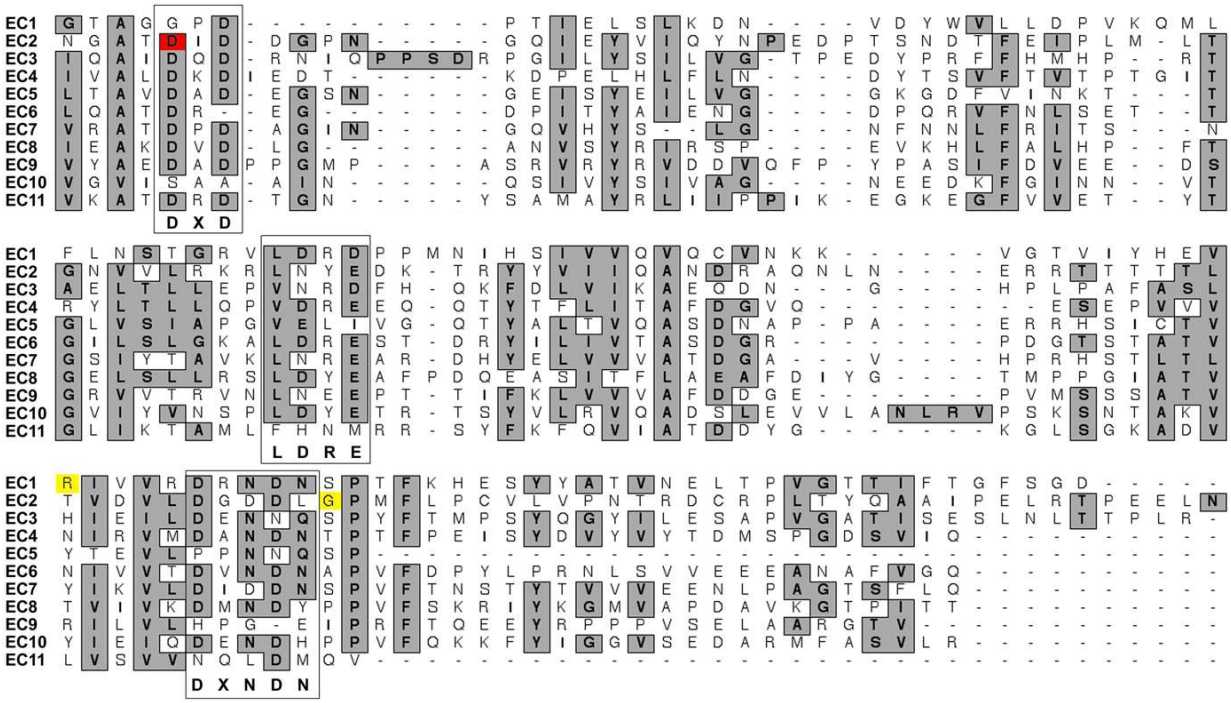

Fig. 3.

Alignment of the eleven protocadherin 15 EC domains with missense mutations causing USH1F (red) and DFNB23 (yellow). A gray background indicates highly conserved or similar residues. The DXD, LDRE and DXNDN calcium-binding motifs are boxed.

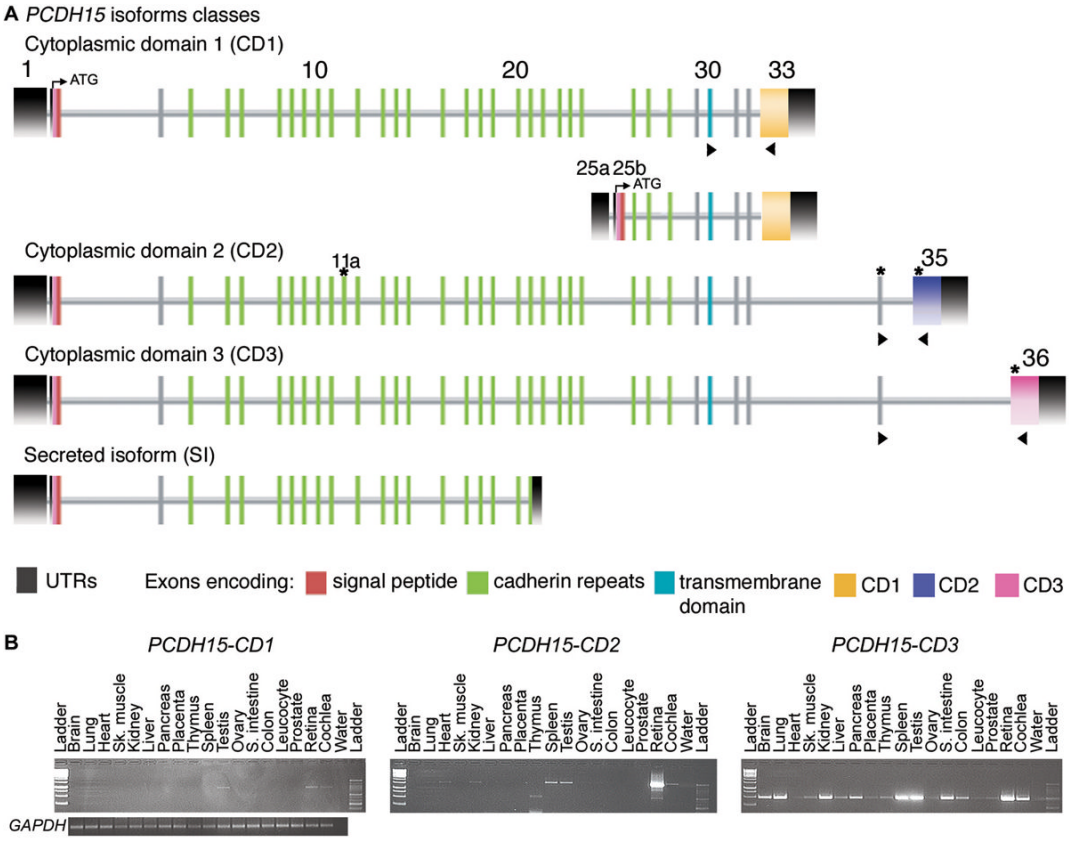

Fig. 4.

Structure and expression profile of *PCDH15* isoforms. **a** Four isoform classes of *PCDH15* defined by the presence or absence of one of three alternative, unique cytoplasmic domains (CD1, CD2, or CD3). Additional exons discovered after the initial report of *PCDH15* structure (Ahmed et al. 2001) are designated with a letter-suffix (11a, 25a, 25b) since they are located among the previously reported 33 exons (Ahmed et al. 2003). Black shaded boxes designate the 5′UTR and 3′UTR. A signal peptide (red) is encoded by exon 2 and a transmembrane domain (blue) is encoded by exon 30. Four newly discovered exons identified in this study are marked with asterisks. Exons encoding the cadherin repeats are represented by green boxes and the exons 33 (yellow), 35 (purple) and 36 (pink) encode three alternative cytoplasmic domains (CD1, CD2, CD3), respectively. **b** Expression profiles of the three isoform classes of *PCDH15* that have different cytoplasmic domains (CD1, CD2 and CD3) are shown. PCR primers (arrowheads in panel a) were designed to amplify CD1, CD2 and CD3 from cDNA prepared from different human tissues. Left expression profile, *PCDH15-CD1* mRNA is present in testis, retina, and cochlear cDNA. Middle profile, *PCDH15-CD2* is found in human heart, kidney, thymus, spleen, testis, retina, and cochlea. Right profile, *PCDH15-CD3* is widely expressed. PCR amplification of *GAPDH* cDNA was used as a control for the quality and quantity of RT-PCR template.

Table 1

Mutations and clinical data of affected members of eight families segregating mutations of *PCDH15*

Nucleotide change[a]	Exon	Effect on Protein	Domain	Allele frequency	Family	Ethnicity	Auditory phenotype (sensorineural)[d]	Visual phenotype[e]	Age at ambulation (months)	Vestibular function[f]
Missense mutations:										
c.400C→G	5	p.R134G	EC1	0/200	PKDF756	Punjabi	severe to profound	+ERG at age 12 years, +FE +ERG at age 16 years, +FE	12-13	+TG, +RM +TG, +RM
c.533A→G	6	p.D178G	EC2	0/200	PKDF875	Punjabi	profound	−FE at age 24 years −FE at age 27 years	12-15	−TG, −RM −TG, −RM
Splicing variants:										
c.3717+1G→T	27	splicing error	*b*	0/200	PKDF248	Sindhi	profound	±FE at age 15 years ±FE at age 16 years	12-15	−TG, −RM −TG, −RM
Nonsense mutations:										
c.7C→T	2	p.R3X	SP	0/200	PKDF338	Punjabi	profound	±FE at age 14 years ±FE at age 16 years	24-28	−TG, −RM −TG, −RM
c.1940C→G	16	p.S647X	EC6	0/200	PKDF809	Punjabi	profound	±FE at age 17 years −FE at age 27 years	12-15	−TG, −RM −TG, −RM
c.2052C→A	17	p.Y684X	EC6	0/200	PKDF801	Punjabi	profound	±FE at age 12 years −FE at age 21 years	12-15	−TG, −RM −TG, −RM
Deletions:										
c.2483delT	19	p.E829KfsX12	*c*	0/200	PKDF891	Punjabi	profound	±FE at age 8 years −FE at age 10 years −FE at age 12 years	12-15	−TG, −RM −TG, −RM −TG, −RM
c.4257delA	32	p.L1419FfsX99	CD1	0/200	PKDF770	Saraiki	profound	−FE at age 19 years −FE at age 23 years	12-15	−TG, −RM −TG, −RM

SP, signal peptide; EC, ectodomain; CD1, cytoplasmic domain 1.

[a] Codon numbering is based on cDNA and starts with the first in-frame methionine (accession no. AY029237). DNA numbering based on +1 as the A of the initiation codon.

[b] Located in sequence between EC11 and transmembrane domain.

[c] Located in sequence between EC7 and EC8.

[d] evaluated by pure-tone audiometry.

[e] eletroretinography (ERG) response: −, extinguished; ±, subnormal; +, normal; for fundus examination (FE) results: −, typical RP; ±, subnormal RP-like findings; +, normal.

[f] +, normal vestibular function; −, vestibular dysfunction as determined by Tandem Gait (TG) and Romberg tests (RM).