PCDH15 is expressed in the neurosensory epithelium of the eye and ear and mutant alleles are responsible for both USH1F and DFNB23

Zubair M. Ahmed1,4, Saima Riazuddin1, Jamil Ahmad4, Steve L. Bernstein5, Yan Guo5, Muhammad F. Sabar4, Paul Sieving6, Sheikh Riazuddin4, Andrew J. Griffith2,3, Thomas B. Friedman1, Inna A. Belyantseva1 and Edward R. Wilcox1,*

1Section on Human Genetics, 2Section on Gene Structure and Function, Laboratory of Molecular Genetics, and 3Hearing Section, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Rockville, MD, USA, 4National Center of Excellence in Molecular Biology, Punjab University, Lahore, Pakistan, 5Department of Ophthalmology, University of Maryland School of Medicine, Baltimore, MD, USA and 6National Eye Institute, National Institutes of Health, Bethesda, MD, USA

Received June 27, 2003; Revised and Accepted October 17, 2003 DDBJ/EMBL/GenBank accession no. *

Recessive splice site and nonsense mutations of PCDH15, encoding protocadherin 15, are known to cause deafness and retinitis pigmentosa in Usher syndrome type 1F (USH1F). Here we report that non-syndromic recessive hearing loss (DFNB23) is caused by missense mutations of PCDH15. This suggests a genotype–phenotype correlation in which hypomorphic alleles cause non-syndromic hearing loss, while more severe mutations of this gene result in USH1F. We localized protocadherin 15 to inner ear hair cell stereocilia, and to retinal photoreceptors by immunocytochemistry. Our results further strengthen the importance of protocadherin 15 in the morphogenesis and cohesion of stereocilia bundles and retinal photoreceptor cell maintenance or function.

INTRODUCTION

Usher syndrome type 1 (USH1; OMIM 602083) is a hereditary neurosensory disorder characterized by profound congenital deafness, vestibular areflexia and retinitis pigmentosa (1,2). Seven USH1 loci have been mapped and genes for five of them have been identified: USH1B (MYO7A), USH1C (USH1C), USH1D (CDH23), USH1F (PCDH15) and USH1G (SANS) (3–10). These genes encode unconventional myosin VIIA, harmonin, cadherin 23, protocadherin 15 and SANS, respectively (3–10). Although most of the mutant alleles of these genes are associated with Usher syndrome, some missense mutations of USH1C and CDH23 are associated with non-syndromic hearing loss (6,11,12).

To date, all of the reported mutations of PCDH15 on chromosome 10q21.1 cause Usher syndrome (3,4,13). PCDH15 belongs to the cadherin superfamily of calcium-dependent cell–cell adhesion molecules (3). Protocadherins represent a large family of non-classical cadherins that are structurally and functionally divergent from the classic cadherins (14). In higher vertebrates, protocadherins are thought to be involved in a variety of functions, including neural circuit formation and synapse formation (15). In Ames waltzer (av) mice, recessive mutations of Pcdh15 cause deafness with disorganized stereocilia bundles and degeneration of inner ear neuroepithelia (16–19). This phenotype is shared with all of the known mouse strains segregating mutant alleles of USH1 orthologs: shaker-1 (sh1), waltzer (v) and jackson shaker (js), the mouse models for Usher syndrome type 1B, 1D and 1G, respectively (20–22). Recently, it has been shown that myosin VIIa, harmonin and cadherin 23 co-localize in the stereocilia of hair cells, and can interact with each other in vitro and in heterologous expression systems (23,24). It has been proposed that these proteins may form a complex essential for cohesiveness of the stereocilia bundle (23,24), raising the possibility that similar interactions may also occur in the eye. Myosin VIIa, harmonin and cadherin 23 are expressed in photoreceptor cells of mouse retina, whereas myosin VIIa is

*To whom correspondence should be addressed at: 5 Research Court, 2A-19, Rockville, MD 20850, USA. Tel: +1 3014024162; Fax: +1 3014808019; Email: wilcoxe@nidcd.nih.gov

1PCDH15 isoform B reported in this manuscript has been deposited in NCBI GenBank and the accession number is AY388963.
also expressed in the apical processes of retinal pigmented epithelium (RPE) (7,24–27). Although myosin VIIa appears to be present in connecting cilium of photoreceptors, the subcellular locations of harmonin, cadherin 23 and SANS in photoreceptors are unknown (24,26,27). Interestingly, the mouse models of cadherin 23, protocadherin 15 and SANS mutations do not have any reported retinal phenotype, which may reflect interspecies differences in the functional requirements for these proteins or functional redundancy with other proteins in the retina. We thus sought to address the molecular genetic and cell biological bases for these observations of protocadherin 15 function and its associated mutant pathologies in the eye and ear.

RESULTS

Mutant alleles of PCDH15 are associated with nonsyndromic recessive deafness

We screened DNA samples from ~400 families with recessive prelingual hearing loss with short tandem repeat (STR) markers closely linked to PCDH15 (3) to test the hypothesis that there are mutant alleles of PCDH15 associated with non-syndromic deafness. Recessive deafness in three families (PKSR54a, PKDF70 and PKDF139; Fig. 1A) co-segregated with STR markers linked to PCDH15 (Table 1). Pure-tone air conduction audiometry showed severe to profound sensorineural hearing loss segregating in family PKSR54a and profound deafness in families PKDF70 and PKDF139. Families PKSR54a and PKDF70 have no history of nyctalopia, and the funduscopy and electroretinograms (ERGs) were normal in two older affected individuals (Fig. 2) from each family (age range 13–44 years). Electronystagmography (ENG) with caloric stimulation

![Figure 1. DFNB23 and Usher families with PCDH15 mutations. (A) Pedigrees of families segregating non-syndromic recessive deafness DFNB23 and Usher syndrome type 1F with their haplotypes for markers on chromosome 10. Each family has a different PCDH15-linked haplotype (B) PKSR54a-electropherograms of amplimers from genomic DNA templates illustrate homozygosity for a 785G > A transition mutation (G262D, exon 8) in an affected individual and homozygosity for the wild-type allele in an unaffected individual. PKDF-70-electropherograms illustrating genotypes of a homozygous wild-type allele and a person homozygous for 400C > G transversion mutation. PKDF-139-electropherograms of the transition mutation 1927C > T and the wild-type allele are shown.

Table 1. Two-point LOD scores (at $\theta = 0$)

<table>
<thead>
<tr>
<th>Marker</th>
<th>LOD scores for family</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PKSR54a</td>
</tr>
<tr>
<td>D10S1643</td>
<td>3.49</td>
</tr>
<tr>
<td>D10S546</td>
<td>3.97</td>
</tr>
<tr>
<td>D10S2529</td>
<td>2.28</td>
</tr>
<tr>
<td>D10S2522</td>
<td>4.11</td>
</tr>
</tbody>
</table>
This page contains scientific text discussing the expression and localization of protocadherin 15 (PCDH15) in the eye, as well as its role in rare forms of deafness and vision loss. The text describes the detection of protocadherin 15 in sensory epithelia, its expression in distinct domains of the eye and ear, and its role in the differentiation of auditory and vestibular hair cells. The study of PCDH15 in various species, including human, mouse, rat, zebrafish, and puffer fish, highlights its conservation and importance in these sensory systems. The text also touches on the methods used for detecting PCDH15, such as immunofluorescent confocal microscopy and Western blot analysis, and the clinical implications of mutations in PCDH15, linking it to Usher syndrome and non-syndromic hearing loss. The discussion section emphasizes the specific role of protocadherin 15 in the differentiation of stereocilia and the potential for its mutations to affect both hearing and vision.
The clinical complexity of Usher syndrome reflects the high level of genetic heterogeneity, background and stochastic effects as well as shared structural features and physiological pathways in the cochlea and retina. The expression of protocadherin 15 in the organ of Corti (Fig. 5B, C, E and F) and in the vestibular apparatus (Fig. 5H, I, K and L) is consistent with the clinical outcome of USH1F subjects and av mice. The finding that protocadherin 15 staining is present in photoreceptor cells (Fig. 4) suggests that USH1F subjects are likely to have significant defects in color vision, and may have more frequent central visual complaints, night blindness and progressive peripheral vision loss, which is typical of rod
photoreceptor dysfunction. The clinical phenotype (progressive retinitis pigmentosa and tunnel vision) of older affected individuals (aged 6 and 9 years) of PKDF139 family are consistent with this expectation.

Hearing function depends upon the mechano-electrical transduction of auditory and vestibular stimuli which occur within polarized hair cell stereocilia bundles that are arranged in rows of increasing height (29). There are many different interconnections, including tip links, horizontal top connectors, lateral or shaft links and ankle links between adjacent stereocilia that are thought to help establish and maintain the integrity of the stereocilia bundles (30–32). Ankle links are present in mouse hair cell stereocilia up to P12 and then begin to disappear and are completely absent in adult mice (33). Harmonin isoform b and cadherin 23 are found in cochlear stereocilia during hair cell development, but not at P30 (23), raising the possibility that these proteins may be important for the development of stereocilia bundle and may have a role in the formation and maintenance of some transient links, such as ankle links, but not in the long-term maintenance of stereocilia bundle morphology. In contrast, lateral links are very fine strands that connect the membranes of adjacent stereocilia and are present along the length of the stereocilia even in adult mice (31,32). The developmental pattern and the distribution of protocadherin 15 in stereocilia of inner ear hair cells of adult mice (Fig. 5), taken together with the known function of protocadherins in cell adhesion, suggests that protocadherin 15 may have a role in the development and maintenance of these lateral links between stereocilia. The two missense mutations (R134G and G262D) associated with non-syndromic deafness are present in the first and second EC domains, respectively. We hypothesize that these missense mutations impair the ability of the EC domains to interact with each other to form the lateral links, hence destabilizing stereocilia bundles and causing deafness.

Our observation of protocadherin 15 staining in photoreceptors and retinal degeneration observed in USH1F individuals suggests that PCDH15 is important for the maintenance or function of the photoreceptor cells. However, the two missense mutations that we found might not significantly impair the functional abilities of protocadherin 15 in the retina, hence DFNB23 individuals have normal vision. It is also possible that these mutations affect alternative transcripts, which are not necessary for retinal function in humans.

There is no reported retinal pathology in mice homozygous for truncating alleles of Pcdh15 (36). This may reflect functional redundancy with other proteins in the retina or differences in other genetic, stochastic, and environmental factors such as light exposure. Elucidation of the causes of this dissimilarity could reveal molecular or cellular pathways for potential interventions to prevent or retard RP in USH1.
MATERIALS AND METHODS

Linkage and sequence analyses

Institutional review board approval (OH93-N-016) and written informed consent were obtained for all subjects in this study. To determine if affected members of these families have balance or ocular abnormalities, medical histories were taken with an emphasis on the vestibular and retinal phenotypes. Pure-tone audiometry, funduscopy or electroretinography (ERG) and electronystagmography (ENG) exams were performed on selected affected individuals from each family.

DNA was extracted from peripheral blood or buccal cells. DNA samples were PCR amplified using fluorescently labeled primers flanking polymorphic STR at USH1F. PCR products were visualized by gel electrophoresis on an ABI 377 DNA sequencer and genotypes determined using Genescan and Genotyper software (PE Applied Biosystems). The 33 coding and non-coding exons of PCDH15 (Genbank accession number AJ029237) in the participating members of families PKSR54a, PKDF70 and PKDF139 were PCR amplified and sequenced as described elsewhere (3).

LOD scores were calculated using FASTLINK (34). The disease was coded as fully penetrant and the disease allele frequency was set at 0.001. Meiotic recombination frequencies were assumed to be equal for males and females. The allele frequencies of the STRP markers were calculated by genotyping 96 random normal individuals from the same population.

Antibodies

Protocadherin 15 antiserum (PB303) was raised in rabbit against a synthetic peptide (CGAEPHRHPGILRHVKNLAELK; corresponding to residues 1860–1882 of the mouse sequence, Genbank accession number AAG53891). Amino acid residues used for antiserum production are 85% identical among mouse and human protocadherin 15. Antiserum was affinity purified (Pierce Biotechnology, Rockford, IL, USA) with the synthetic peptide used as the immunogen. Fluorescein-conjugated anti-rabbit IgG secondary antibody was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL, USA).

Western blot analysis

C57BL/6 mouse brain, retina, liver and spleen were sonicated in an ice-cold protease inhibitor cocktail (Calbiochem, La Jolla, CA, USA). Proteins were extracted and denatured by boiling for 5 min in SDS–PAGE sample buffer (0.125 M Tris–HCl, 20% glycerol, 4% SDS, 0.005% Bromophenol blue). A 50 µg protein sample was separated on a 4–20% gradient Tris–glycine gel (Novex, San Diego, CA, USA) and transferred to polyvinylidenefluoride (PVDF) membranes, blocked overnight (O/N) with 5% dry milk in TBST (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20), and stained with anti-Pcdh15 antiserum (PB303; 1:2000 in blocking solution) for 2 h at room temperature. After three washes the membranes were incubated in a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit secondary antibody (Promega, Madison, WI, USA) for 30 min and developed using the ECL plus western blotting detection system (Amersham Pharmacia Biotech, Arlington Heights, IL, USA).

Expression construct

A cDNA encoding the cytoplasmic region of protocadherin 15 (AAG53891; amino acids 1403–1943) was obtained by PCR from a mouse (P0) retinal cDNA library. PCR product was subcloned into pHcRed1-C1 vector (Clontech, Palo Alto, CA, USA) for expression in lymphoblast cells, which, based on RT–PCR analysis, do not have endogenous expression of PCDH15 (data not shown). Lymphoblast cells were cultured in 10% fetal bovine serum (FBS)-supplemented Dulbecco’s modified Eagle’s medium (DMEM). Transient transfection of these cells was performed using Lipofectamine-2000 reagent (Life Technologies, Gaithersburg, MD, USA). Fluorescence immunocytochemistry was carried out on fixed cells (4% PFA for 15 min). The staining procedure used was same as described below for the inner ear tissue.

Immunocytochemistry of mouse inner ear

Immunocytochemistry was performed as described previously (35). After 2 h fixation in 4% PFA, organ of Corti and vestibular organs were dissected in PBS at room temperature using a fine needle. Organ of Corti tissue samples were permeabilized in 0.5% Triton X-100 for 30 min and then washed in PBS. Non-specific binding sites were blocked using 5% normal goat serum (Life Technologies, Gaithersburg, MD, USA) and 2% bovine serum albumin (ICN, Aurora, OH, USA) in PBS. Samples were incubated for 2 h in the anti-Pcdh15 antiserum (PB303) at a concentration of ~5 µg/ml in blocking solution. After three rinses in PBS, samples were incubated in a 1:200 dilution of the FITC conjugated anti-rabbit IgG secondary antibody for 30 min. After washing three times with PBS, samples were mounted using ProLong Antifade kit.
Laboratories, Burlingame, CA, USA). The slides were not

immunostained with alkaline phosphatase secondary antibody (Vector Laboratories, Burlingame, CA, USA). The slides were deparaffinized in xylene and rehydrated through graded ethanol and PBS. Tissue sections were reacted O/N at 4°C with primary antibody (PB303) at a concentration of ~5 μg/ml in 2% BSA. Treated sections were incubated with alkaline phosphatase secondary antibody (Vector Laboratories, Burlingame, CA, USA). Adolescent monkey eyes (3 years of age) were obtained by S.L.B. from John Cogan (Bureau of Biologics, FDA, Bethesda, MD, USA). Tissue was fixed in 4% PFA-PBS and frozen in OCT. Ten micron frozen sections were blocked for 1 h with 5% normal goat serum containing 2% bovine serum albumin. Tissue sections were reacted O/N at 4°C with primary antibody (PB303) at a concentration of ~5 μg/ml containing 2% BSA. Treated sections were incubated with alkaline phosphatase secondary antibody (Vector Laboratories, Burlingame, CA, USA). The slides were not counterstained.

ACKNOWLEDGEMENTS

We thank the families for their participation in this study, which was supported by NIDCD/NIH, Intramural Research fund Z01DC00035-06, Z01DC00039-06 and Z01DC00064-02. Part of this study in Pakistan was supported by Higher Education Commission, Islamabad, Pakistan and by the International Centre for Genetic Engineering and Biotechnology, Trieste, Italy under Project CRP/PAK02-01 (contract no. 02/013). S.L.B. is the recipient of a career development award (CDA) from Research to Prevent Blindness (RPB-USA), and funded by the V. Kann Rasmussen Foundation (Denmark). We thank Shaheen Khan and Barbara Ploplis for their technical help. We also thank Tomoko Makishima, Rob Morell, Julie Schultz, Tamar Ben-Yosuf, Kiyoto Kurima, Ayala Lagziel, Doris Wu and Dennis Drayna for advice and comments in preparing the manuscript.

REFERENCES


