

CDH23 Mutation and Phenotype Heterogeneity: A Profile of 107 Diverse Families with Usher Syndrome and Nonsyndromic Deafness

L. M. Astuto,¹ J. M. Bork,³ M. D. Weston,¹ J. W. Askew,¹ R. R. Fields,¹ D. J. Orten,¹ S. J. Ohliger,³ S. Riazuddin,³ R. J. Morell,³ S. Khan,⁴ S. Riazuddin,⁴ H. Kremer,⁵ P. van Hauwe,⁶ C. G. Moller,⁷ C. W. R. J. Cremers,⁵ C. Ayuso,⁸ J. R. Heckenlively,⁹ K. Rohrschneider,¹⁰ U. Spandau,¹⁰ J. Greenberg,¹¹ R. Ramesar,¹¹ W. Reardon,¹² P. Bitoun,¹³ J. Millan,¹⁴ R. Legge,² T. B. Friedman,³ and W. J. Kimberling¹

¹Center for the Study and Treatment of Usher Syndrome, Boys Town National Research Hospital, and ²Department of Ophthalmology, University of Nebraska Medical Center, Omaha; ³Laboratory of Molecular Genetics, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Rockville, MD; ⁴National Center of Excellence in Molecular Biology, Punjab University, Lahore, Pakistan; Departments of ⁵Otorhinolaryngology and ⁶Human Genetics and Otorhinolaryngology, University Medical Center Nijmegen, Nijmegen, The Netherlands; ⁷Department of Audiology, Sahlgrenska University Hospital, Göteborg, Sweden; ⁸Fundacion Jimenez Diaz, Madrid; ⁹Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles; ¹⁰Department of Ophthalmology, University of Heidelberg, Heidelberg; ¹¹Department of Human Genetics, University of Cape Town Medical School, Cape Town; ¹²Institute of Child Health, University of London, London; ¹³Génétique Médicale, Centre Hospitalier Universitaire, Paris; and ¹⁴Unit of Genetics, Hospital Universitari La Fe, Valencia, Spain

Usher syndrome type I is characterized by congenital hearing loss, retinitis pigmentosa (RP), and variable vestibular areflexia. Usher syndrome type ID, one of seven Usher syndrome type I genetic localizations, have been mapped to a chromosomal interval that overlaps with a nonsyndromic-deafness localization, *DFNB12*. Mutations in *CDH23*, a gene that encodes a putative cell-adhesion protein with multiple cadherin-like domains, are responsible for both Usher syndrome and *DFNB12* nonsyndromic deafness. Specific *CDH23* mutational defects have been identified that differentiate these two phenotypes. Only missense mutations of *CDH23* have been observed in families with nonsyndromic deafness, whereas nonsense, frameshift, splice-site, and missense mutations have been identified in families with Usher syndrome. In the present study, a panel of 69 probands with Usher syndrome and 38 probands with recessive nonsyndromic deafness were screened for the presence of mutations in the entire coding region of *CDH23*, by heteroduplex, single-strand conformation polymorphism, and direct sequence analyses. A total of 36 different *CDH23* mutations were detected in 45 families; 33 of these mutations were novel, including 18 missense, 3 nonsense, 5 splicing defects, 5 microdeletions, and 2 insertions. A total of seven mutations were common to more than one family. Numerous exonic and intronic polymorphisms also were detected. Results of ophthalmologic examinations of the patients with nonsyndromic deafness have found asymptomatic RP-like manifestations, indicating that missense mutations may have a subtle effect in the retina. Furthermore, patients with mutations in *CDH23* display a wide range of hearing loss and RP phenotypes, differing in severity, age at onset, type, and the presence or absence of vestibular areflexia.

Introduction

Usher syndrome is a frequent cause of recessive syndromic deafness and is estimated to account for (a) 3%–6% of the congenitally deaf population (Vernon 1969), (b) ~18% of those with retinitis pigmentosa (RP), and (c) >50% of the deaf-blind population (Boughman et al.

1983). The frequency of Usher syndrome has been estimated to be 3.5/100,000 in Sweden and Finland (Nuutila 1970; Grondahl 1987), 3.2/100,000 in Colombia (Tamayo et al. 1991), and 4.4/100,000 in the United States (Boughman et al. 1983).

The standard classification of Usher syndrome recognizes three distinct clinical categories (Kimberling and Moller 1995). Type I is manifested by severe to profound congenital hearing impairment, vestibular dysfunction, and retinal degeneration beginning in childhood, whereas type II is manifested by moderate to severe hearing impairment, normal vestibular function, and later onset of retinal degeneration; type III, the least common form of Usher syndrome, presents with progressive hearing loss and a variable retinal and vestibular phenotype.

Received March 28, 2002; accepted for publication May 1, 2002; electronically published June 19, 2002.

Address for correspondence and reprints: Dr. William J. Kimberling, Director of the Center for the Study and Treatment of Usher Syndrome, Professor of Otolaryngology, Pathology, and Medical Sciences, Boys Town National Research Hospital, Omaha, NE 68131. E-mail: kimber@boystown.org

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At least seven different *USH1* genes are responsible for the more severe, type I syndrome. These genes have been mapped to chromosomes 10q21.1, 10q22.1, 11q13.5, 11p15.1, 14q32, 17q24-25, and 21q21 (see the Hereditary Hearing Loss Homepage). There are no studies reporting clinical differences between these seven forms, but they are differentiated on the basis of either linkage analysis in informative families or mutational analyses of the genes involved. Currently, four *USH1* genes have been identified. Defects in myosin VIIa, harmonin, cadherin 23, and protocadherin 15 are responsible for causing Usher syndrome type I subtypes B, C, D, and F, respectively (Weil et al. 1995; Bitner-Glindzicz et al. 2000; Verpy et al. 2000; Ahmed et al. 2001; Alagramam et al. 2001; Bolz et al. 2001; Bork et al. 2001).

In 1996, by homozygosity mapping of an inbred Pakistani family, Wayne et al. localized *USH1D* (MIM 601067) to the long arm of chromosome 10 (Wayne et al. 1996), a chromosomal region that also contained *DFNB12* (MIM 601386), a nonsyndromic-deafness localization (Chaib et al. 1996). Mutations in *CDH23* (MIM 605516) cause both Usher syndrome type ID and *DFNB12*. Missense mutations were found exclusively in five consanguineous families with nonsyndromic deafness (Bork et al. 2001) and in a sixth, large consanguineous family with nonsyndromic deafness (A. P. M. Brouwer, R. J. E. Pennings, M. Roeters, P. Van Hauwe, L. M. Astuto, L. H. Hoefsloot, P. L. M. Huygen, B. van den Helm, A. F. Deutman, J. M. Bork, W. J. Kimberling, F. P. M. Cremers, C. W. R. J. Cremers, and H. Kremer, unpublished data [hereafter referred to as "Brouwer et al., unpublished data"]). Splicing variants, deletions, nonsense mutations, and missense mutations of *CDH23* have been identified in patients with Usher syndrome type I (Bolz et al. 2001; Bork et al. 2001; von Brederlow et al. 2002).

CDH23 has 69 exons and encodes a predicted 3,354-amino-acid protein with 27 cadherin extracellular (EC) repeats, a transmembrane domain, and a unique cytoplasmic domain. The product of *CDH23* has similarity to the well-characterized protein E-cadherin; the EC repeats of E-cadherin are responsible for homophilic cell-cell adhesion, which is established by the formation of parallel stable EC-domain dimers at the same cell surface and at two opposing cell surfaces. This stability is due to interdomain rigidification of the EC domains, achieved by the binding of three calcium ions to highly conserved peptide sequences (LDRE, DXNDN, and DXD) (Nollet et al. 2000). The functions of cadherin 23 have not been defined, but it is hypothesized to be involved in the establishment of cell-cell contacts and in the organization of the EC matrix.

In the present study, we sought to further investigate the genotype/phenotype relationship, proposed by Bork et al. (2001), between *CDH23* mutations and the con-

sequent hearing, visual, and vestibular phenotype. Using a comprehensive mutation-detection strategy via heteroduplex, SSCP, and direct sequence analyses of all 69 exons from 107 probands, we were able to gain insight into the distribution and nature of diverse *CDH23* mutations and their resulting phenotypes.

Subjects, Material, and Methods

Subjects and Clinical Studies

A total of 107 families with Usher syndrome and recessive nonsyndromic deafness (RNSD), comprising 281 unaffected and 173 affected members, were sampled and studied; 12 families were consanguineous. A total of 68 families (48 with Usher syndrome type ID and 20 with *DFNB12*) were multiplex and compatible with linkage to the *USH1D* region; and the remaining 39 families were characterized by sporadic cases (21 with Usher syndrome type I and 18 with RNSD). The 38 families with RNSD were drawn from an original population of 157 families referred with nonsyndromic hearing loss to Boys Town National Research Hospital. In all families, mutations in *MYO7A* and *GJB2* were excluded by linkage and/or mutational analysis. Families were collected in the United States (57 families), Sweden (17 families), The Netherlands (10 families), Germany (10 families), Spain (3 families), Pakistan (3 families), South Africa (2 families), France (2 families), Italy (2 families), and Ireland (1 family). Several of these families were part of three previous studies (Kelley et al. 1998; Astuto et al. 2000; Bork et al. 2001). Not all individuals in the present study were sequenced for all 69 *CDH23* exons.

Cases were included on the basis of the following criteria: (1) both parents had normal hearing, and (2) the proband's hearing loss was moderate to profound. Patients with Usher syndrome meet the further criteria of presenting with evidence of RP, including extinguished or

Table 1

Twelve Novel Mutations in the *CDH23* Gene Observed in 9 of 38 Probands with RNSD

Nucleotide (Codon) Change	Proportion of		Chromosomes	Family
	Exon	Domain		
371A→G (D124G)	5	EC1	1/214	1846
1355A→G (N452S)	13	EC4	1/214	3055
1439T→A (L480Q)	13	EC5	1/214	2640
1745G→A (R582Q)	15	EC6	1/214	1194
3178C→T (R1060W)	26	EC10	1/214	3063
3557G→A (G1186D)	29	EC11	1/214	2640
4756G→C (A1586P)	37	EC15	2/214	PKDF030
4783G→A (E1595K)	37	EC15	1/214	1566
5536G→A (D1846N)	42	EC17	1/214	3055
6442G→A (D2148N)	47	EC20	3/214	3409, 1668
7393C→T (R2465W)	52	EC23	1/214	1846
7823G→A (R2608H)	54	EC24	1/214	1668

Table 2**Mutations in the *CDH23* Gene Observed in 35 of 69 Probands with Usher Syndrome**

Nucleotide (Codon) Change	Exon	Domain	Proportion of Chromosomes	Family
Missense mutations:				
3617C→G (P1206R)	30	EC11	1/214	1078
3625A→G (T1209A)	30	EC11/EC12 ^a	2/214	220
5237G→A (R1746Q) ^b	40	EC16/EC17 ^a	6/214	792, 809, 2790, 3308
7549A→G (S2517G)	53	EC23/EC24 ^a	1/214	1056
8230G→A (G2744S)	57	EC26	1/214	1054
8497C→G (R2833G)	58	EC26	1/214	980
9524G→A (R3175H)	67	CYTO	1/214	3051
Splicing variants:				
IVS4+1G→A	4	EC1	13/214	1185, 1197, 1285, 1288, 1289, 1291, 1321, 1324
1450G→C ^c (A484P)	14	EC5	2/214	1517
IVS20+1G→A	20	EC7	3/214	1066, 2059, 3036
3105A→C ^c (T1035T)	25	EC10	1/214	2059
4488G→C ^c (Q1496H) ^b	35	EC14	1/214	1444
IVS45-9G→A ^b	46	EC19	7/214	1071, 3036, 3150, PKSRB, PKSR13a
7872G→A ^c (E2624E)	54	EC25	1/214	2797
Nonsense:				
172C→T (Q58X)	3	EC1	1/214	1291
4504C→T (R1502X)	36	EC14	5/214	1184, 1197, 1198, 1288
6307G→T (E2103X)	47	EC20	1/214	3150
Deletions:				
193delC	3	EC1	1/214	216
1087delG	10	EC4	2/214	2738
1112delT	10	EC4	2/214	359, 792
6155delC	46	EC19	1/214	1130
6968delC	49	EC22	1/214	51
Insertions:				
3840insATGA	31	EC12	1/214	216
9626insC	68	CYTO	1/214	897

^a Mutation lies within linker region between these two EC-repeat domains.

^b Mutations reported by either Bolz et al. (2001) or von Brederlow (2002).

^c Detected at either beginning or end of the exon, predicted to decrease splicing efficiency.

subnormal electroretinography (ERG) recordings. We refer to typical families with Usher syndrome type I as those presenting with profound deafness, RP, and absent vestibular function; atypical families are categorized as having a milder affliction in one or more of the three sensory systems (hearing, balance, and vision) involved. Two families with Usher syndrome type II were also included. Computed-tomography scans of the inner ear were also obtained for several families, and the results were normal in all cases. For each family, a pedigree was drawn. Clinical data were collected from medical histories and/or examinations, either at Boys Town National Research Hospital in Omaha or by various collaborators at their local clinical facilities. The local human-subjects committees approved the present study, and informed consent was obtained from all participants.

Blood Collection and DNA Extraction

Blood samples were collected, and genomic DNA was extracted by a Puregene kit (Gentra Systems).

PCR Conditions

Oligonucleotide primers designed by Bolz et al. (2001) and Bork et al. (2001) were used for PCR and for cycle-sequencing reactions. PCR elements were as follows: 1 × reaction buffer, 200 μM each dNTP, 2.5 mM MgCl₂, 5 pmol of each forward and reverse primer, and 0.5 U of *Taq* DNA polymerase. PCR conditions were as follows: 95°C for 10 min, followed by 35–40 cycles of 95°C for 30 s, 55°–65°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 10 min.

Heteroduplex Analysis (HA)

PCR products amplified from syndromic and nonsyndromic probands and cloned control DNA were mixed, heated at 95°C for 3 min, and cooled to 25°C during a 45-min period. The reannealed reaction products were then electrophoresed through a 35-cm × 43-cm × 1-mm volume of MDE (FMC) gel, at an average of 900 V for 18–24 h, were stained with 1 μg of ethidium bromide/ml, and were visualized under UV light. RP11-472K8 BAC DNA (AC016823) was amplified for each *CDH23*

Table 3**Presumed Nonpathogenic Variants in *CDH23***

INTRONIC VARIANTS		EXONIC VARIANTS	
Nucleotide Change	Exon-Specific PCR Product	Nucleotide (Codon) Change	Exon
IVS-121C→T	1	7C→T (R3C)	1
IVS-88A→C	1	198G→A (V66V)	3
c.-1C→T	1	366T→C (V122V)	5
IVS1+12C→T	1	1038G→A (P346P)	10
IVS5+13G→A	5	1053C→T (S351S)	10
IVS5+26A→G	5	1469G→C (G490A)	14
IVS6+41C→A	6	1487G→A (S496N)	14
IVS6+64C→T	6	2316T→C (N772N)	21
IVS8+101G→A	8	2388T→C (D796D)	21
IVS9-24G→A	10	3009T→C (S1003S)	25
IVS10+13A→G	10	3231T→G (P1077P)	27
IVS11-107A→T	12	3664G→A (A1222T)	30
IVS11-89C→T	12	4051G→A (D1351N)	31
IVS14-12G→A	15	4299T→A (P1433P)	34
IVS15-47T→C	16	4310G→A (R1437Q)	34
IVS16+79A→G	16	4341T→C (D1447D)	34
IVS16-220C→T	17	4723G→A (A1575T)	37
IVS16-46T→C	17	4858G→A (V1620M)	38
IVS18-40G→T	19	5023G→A (V1675I)	38
IVS18-21C→G	19	5100C→T (Y1700Y)	39
IVS19-19G→A	20	5411G→A (R18804Q)	41
IVS20+135C→T	20	5541C→T (N1847N)	42
IVS21+26T→C	21	5660C→T (T1887I)	42
IVS23-17C→T	24	5996C→G (T1999S)	45
IVS25-60C→T	26	6130G→A (E2044K)	46
IVS29-12C→T	30	6197G→A (R2066Q)	46
IVS31-183G→C	32	6315C→T (V2105V)	47
IVS31-160C→T	32	6847G→A (V2283I)	49
IVS31-83A→G	32	6918G→A (L2306L)	49
IVS33-53T→C	34	7073G→A (R2358Q)	50
IVS33-71C→T	34	7139C→T (P2380L)	50
IVS35+32C→G	35	7467C→T (R2489R)	52
IVS37-135C→T	38	7572G→A (A2524A)	53
IVS37-79G→A	38	7762G→C (E2588Q)	54
IVS37-49T→C	38	8798T→A (V2933E)	60
IVS38-48G→C	39	8860G→A (D2954N)	60
IVS39+44C→G	39	8885A→G (N2962S)	60
IVS39+73C→T	39	8895C→T (P2965P)	60
IVS41-44T→C	42	8907C→T (R2969R)	60
IVS41-10A→G	42	9015G→A (A3005A)	61
IVS48-81G→A	49	9873G→A (T3291T)	69
IVS52+6G→A	52		
IVS58+25G→A	58		
IVS60-14C→A	61		
IVS61+8G→A	61		

exon and was used as a control. Mixing with BAC control DNA prior to heteroduplex formation allows the detection of both homozygous and heterozygous mutations. A set of 96 genetically independent control samples of mixed European ancestry also was studied, to aid in differentiation between probable pathologic mutations and polymorphisms. The controls were obtained through a reading-disability research project in which no history of

hearing or visual loss had been identified. If the mutation was found in one or more of the 96 control samples, it was considered to be nonpathologic.

SSCP Analysis

SSCP analysis was performed on several *CDH23* exons. The amplified DNA was run on 5% polyacrylamide gels, with 10% glycerol, in 1 × Tris-borate EDTA. Samples were electrophoresed either at 45 W, in a cold room, or at 40 W, with cooling electrophoresis equipment (Gibco-BRL). A set of 96 normal independent genomic control samples was also studied, to aid in the identification of probable pathologic mutations and in the differentiation between them and polymorphisms.

DNA Sequencing

PCR products showing band shifts in SSCP and/or HA were purified and concentrated by Microcon 100 filters (Millipore) and Millipore 96-well filter plates, prior to sequencing. ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kits (Perkin Elmer) were used to generate sequence from both purified PCR products and BAC DNA templates. Five microliters of PCR products were sequenced with 4 pmol of *CDH23* primers, 3 μl of ABI Ready Reaction mix, and 5 × buffer, in a total volume of 10 μl, for 35 cycles of 95°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Products of the sequencing reactions were precipitated and resuspended in a 5:1 ratio of formamide/dye loading buffer. Two microliters were loaded onto 4.8% PAGE-PLUS (Amresco) sequencing gels and were run on the ABI Prism model 377 DNA sequencer, according to ABI protocols. Sample files were generated by ABI Sequencing Analysis 3.0. Fractura 2.0 (ABI) was used to remove ambiguous sequences. Contig assembly was performed by the DNASTAR SeqMan 5.03 program. In some cases, direct sequencing could not unequivocally identify the DNA sequence change; for those cases, PCR products were cloned into a plasmid vector (TOPO TA Cloning Kit; Invitrogen), to separate individual alleles prior to sequencing.

Splicing Program

All IVS changes were analyzed by the BDGP splice-site-prediction program (available at the Berkeley Drosophila Genome Project Splice Site Prediction by Neural Network Web site). IVS sequence changes that predict a loss of splicing-acceptor or -donor activity and that are not present in control samples are considered pathologic.

The Sequence Manipulation Suite: Multiple Align
Show

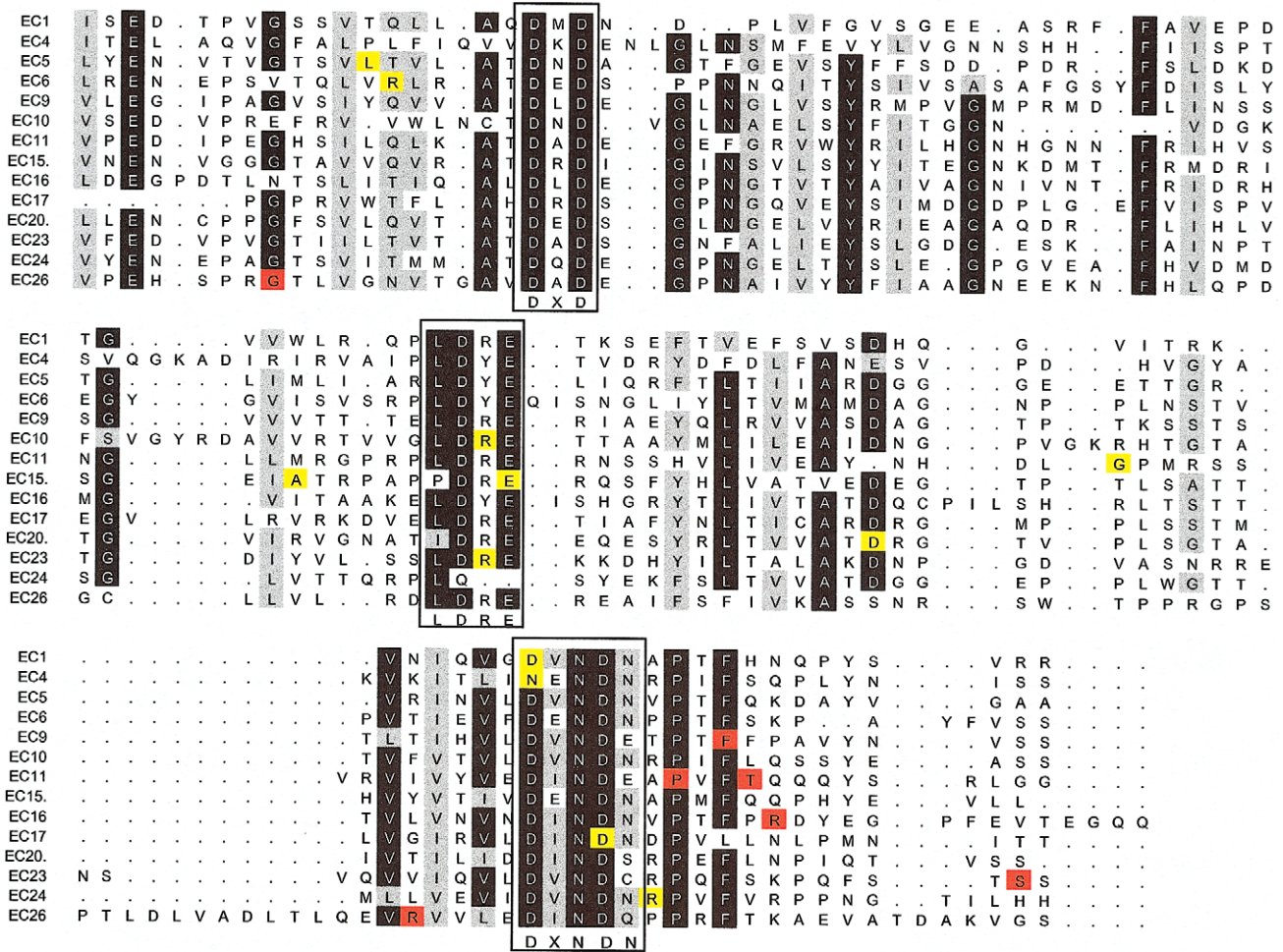


Figure 1 Alignment of cadherin 23 EC domains with missense mutations causing Usher syndrome type ID and recessive DFNB12. DNASTAR Lasergene and the program of The Sequence Manipulation Suite were used to align 14 EC domains. Corresponding EC domains are indicated to the left of the lineup. Dark-gray-shaded residues are >90% identical in all sequences; light-gray-shaded residues are 70% conserved in all aligned sequences. The LDRE, DXNDN, and DXD calcium-binding motifs are boxed. The sites of missense mutations identified in the present study (tables 1 and 2) are highlighted in yellow (in the case of DFNB12) and red (in the case of USH1D).

Results

Mutational Analyses

Two hundred fourteen chromosomes were screened for mutations in *CDH23*, by a combination of heteroduplex, SSCP, and/or sequencing analyses. A total of 36 different *CDH23* mutations were identified in 45 families. Tables 1 and 2 list the mutations observed in, respectively, the families with DFNB12 and the families with Usher syndrome type ID. Table 3 lists the presumed nonpathogenic variants in *CDH23*. Codon numbering starts with the first in-frame methionine of the human

CDH23 (GenBank accession number AF312024; Bolz et al. 2001).

Nonsense mutations.—Three mutations causing premature stop codons were identified in six probands with Usher syndrome type ID. Heterozygous nucleotide substitutions resulted in two premature stop codons—Q58X and E2103X, in exons 3 and 47, respectively. A 4504C→T transition, producing R1502X in exon 36, was identified exclusively in four Swedish families. Genealogical research revealed that all four families originated from or near the Västerbotten region in northern Sweden, suggesting a founder effect for R1502X.

Deletions/insertions.—Seven mutations that created a

Table 4**Clinical Summary of Eight RNSD Cases with CDH23 Mutations**

Ethnicity ^a and Family	Mutation	Sensorineural Hearing Loss ^b	Results of Ophthalmologic Examination ^c	Visual-Field Type ^d	Age at Ambulation (mo)	Vestibular Function ^e
African American: 2640	L480Q/G1186D	Moderate to severe, progressive				
Dutch: 1194	R582Q/?	Moderate to severe, progressive Moderate to severe	±ERG at age 11 years, ±FE ±ERG at age 9 years, ±FE	1 1	10 10	
European: ^d 1566	E1595K/?	Severe to profound Severe to profound				
1846	R2465W/D124G	Severe to profound, progressive Severe to profound, progressive	+ERG at age 11 years, ±FE +ERG at age 9 years, ±FE		12 10 11	
3055	N452S/D1846N	Severe to profound, asymmetric				
3063	R1060W/?	Moderate to severe	±ERG at age 20 years, ±FE	1	12	+
German: 1668	D2148N/R2608H	Moderate to severe, progressive Moderate to severe/right, severe to profound/left, progressive, asymmetric	±ERG at age 29 years, +FE ±ERG at age 25 years, +FE	1 1	11	+
Pakistani: PKDF030	A1586P/A1586P	Profound Profound	+ERG at age 18 years +ERG at age 22 years			+

^a "European" denotes an ancestry of mixed European origins. Clinical findings on family 3409 will be reported elsewhere.

^b Verified by pure-tone audiometry.

^c For ERG response, - = extinguished, ± = subnormal, and + = normal; for FE results, - = typical RP, ± = subnormal RP-like findings, and + = normal. FE = fundus examination.

^d 1 = Normal visual fields OU (type 1). Visual-field loss was measured by Goldmann perimeter. Rates of visual-field loss are as described by Grover et al. (1997).

^e + = Normal vestibular function, as determined by video electro-nystagmography with caloric testing and/or examination in a rotary chair.

Table 5
Clinical Summary of Families with Usher Type I and CDH23 Mutations

Ethnicity and Family ^a	Mutation	Sensorineural Hearing Loss ^b	Results of Ophthalmologic Examination ^c	Visual-Field Type ^d	Age at Ambulation (mo)	Vestibular Function ^e
Typical Usher syndrome type I:						
German:						
3150	IVS45-9G→A/E2103X	Profound			18	-
Mexican, Native American:						
2738	1087delG/1087delG	Profound	-ERG at 17 years, -FE	3	21	-
Pakistani:						
PKRB	IVS45-9G→A/IVS45-9G→A	Profound	-ERG at 35 years			-
		Profound	-ERG at 20 years			-
Spanish:						
1078	P1206R/?	Profound	-ERG at 19 years, -FE	4	18	-
		Profound	-ERG at 11 years, -FE	4	24	-
1444	Q1496H/?	Profound	-ERG at 30 years, -FE	4	18	-
		Profound	-ERG at 28 years, -FE	4	22	-
Swedish:						
1185	IVS4+1G→A/IVS4+1G→A	Profound	-ERG at 10 years	3	20	-
		Profound		5 at 80 years		
1324	IVS4+1G→A/IVS4+1G→A	Profound	-ERG at 21 years	4	24	-
		Profound		4	24	-
1291	Q58X/IVS4+1G→A	Profound		5 at 75	24	
1197	IVS4+1G→A/R1502X	Profound	-ERG at 15 years	4	18	-
1198	R1502X	Profound	-ERG at 18 years	2 at 47 years	36	-
897	9626msC/?	Profound		3	18	-
Atypical Usher syndrome type I:						
Dutch:						
1066	IVS20+1G→A/?	Profound	-ERG at 5 years, -FE	4	12	
		Profound		4	18	
1071	IVS45-9G→A/?	Profound	-ERG, -FE	4	36	
		Severe to profound	-ERG at 23 years, -FE	4		
European:						
3308	R1746Q/R1746Q	Profound	+ERG at 3.5 years		19	±
2059	IVS20+1G→A/T1035T	Severe, progressive, asymmetric	±ERG at 12 years, ±FE		14	
		Moderate to severe, progressive	-ERG at 9 years, ±FE		12	

216	193delC/3840insATGA	Profound	± ERG at 20 years, -FE	4	18	-
		Profound			18	-
792	1112delT/R1746Q	Profound	-ERG at 44 years, -FE	4	26	
		Profound	-ERG at 38 years, -FE	4	15	
359	R1746Q/WT ^f	No hearing loss	-ERG at 73 years, ±FE	4	24	-
	1112delT/?	Profound	± ERG at 21 years, -FE	4	18	-
1130	6155delC/?	Profound	± ERG at 19 years, -FE	4	10	-
		Profound	-ERG at 14	4	13	
51	6968delC/?	Profound, progressive	-ERG at 12 years, -FE	3	22	
German:						
2790	R1746Q/?	Severe to profound, asymmetric	± ERG at 29 years, -FE	4	13	
Irish:						
809	R1746Q/R1746Q	Profound	± ERG at 27 years, ±FE			
		Profound	-ERG at 16 years, -FE			“Narrowed”
Sephardic Jewish:						
1054 ^g	G2744S/?	Severe	-ERG at 21 years, -FE		22	
		No hearing loss	± ERG at 17 years, +FE		36	
Spanish:						
3036	IVS20+1G→A/IVS45-9G→A	Profound	-ERG at 16 years, -FE	4	16	±
		Profound	-ERG at 12 years, -FE	4	16	±
Swedish:						
1184	R1502X/R1502X	Profound	-ERG at 21 years	3	14	
		Profound	-ERG at 23 years	3	15	+
1288	IVS4+1G→A/R1502X	Profound	-ERG at 12 years	3	20	+
		Profound		4	24	
980	R2833G/?	Moderate to profound, progressive		3	12	+

^a Families 220, 1056, 1285, 1289, 1321, 3051, and PKSR13a have been omitted, since adequate clinical information could not be obtained on them. “European” denotes an ancestry of mixed European origins. Clinical findings on family 3409 will be reported elsewhere.

^b Verified by pure-tone audiometry.

^c For ERG response, - = extinguished, ± = subnormal, and + = normal; for FE results, - = typical RP, ± = subnormal RP-like findings, and + = normal. FE = fundus examination.

^d 1 = Normal visual fields (type 1); 2 = presence of a partial or complete ring scotoma (type 2); 3 = concentric central-field loss with a remaining peripheral island (type 3); 4 = marked concentric loss (type 4); 5 = partial peripheral restriction (type 5) OU. Visual-field loss was clinically measured by Goldmann perimeter. Rates of visual-field loss are as described by Grover et al. (1997).

^e - = Absent, ± = mild, and + = normal, as determined by video electro-nystagmography with caloric testing and/or rotary-chair examination.

^f Father is heterozygous for mutation and presented with RP without hearing loss.

^g Clinical description has been summarized by Bitoun et al. (1991).

Table 6

Summary of *CDH23* Mutations Identified in Present Study and in Patients Who Elsewhere Have Been Reported as Being Affected with Either RNSD or Usher Type I

Mutation	Domain	Phenotype ^a	Reference
Q58X	EC1	Usher syndrome type I	Present study
193delC	EC1	Atypical Usher syndrome	Present study
IVS4+1G→A	EC1	Usher syndrome type I, atypical Usher syndrome	Present study
D124G	EC1	RNSD	Present study
1087delG	EC4	Usher syndrome type I	Present study
1112delT	EC4	Atypical Usher syndrome	Present study
N452S	EC4	RNSD	Present study
L480Q	EC5	RNSD	Present study
A484P	EC5	Usher syndrome type I ^b	Present study
R582Q	EC6	RNSD	Present study
IVS20+1G→A	EC7	Atypical Usher syndrome	Present study
D990N	EC9	RNSD	Bork et al. (2001)
T1035T	EC10	Atypical Usher syndrome	Present study
R1060W	EC10	RNSD	Present study
G1186D	EC11	RNSD	Present study
P1206R	EC11	Usher syndrome type I	Present study
T1209A	EC11/EC12 ^c	Usher syndrome type I ^b	Present study
3840insATGA	EC12	Atypical Usher syndrome	Present study
ÅM1281	EC12	Usher syndrome type I	Bolz et al. (2001)
Q1294X	EC12	Usher syndrome type I	Bork et al. (2001)
D1341N	EC13	RNSD	Brouwer et al. (unpublished data)
Q1496H	EC14	Usher syndrome type I	Bolz et al. (2001)
R1502X	EC14	Usher syndrome type I, atypical Usher syndrome	Present study
A1586P	EC15	RNSD	Present study
E1595K	EC15	RNSD	Present study
R1746Q	EC16/EC17 ^c	Atypical Usher syndrome	Bolz et al. (2001); present study
D1846N	EC17	RNSD	Present study
T1904T	EC18	Usher syndrome type I	von Brederlow et al. (2002)
IVS45+1G→A	EC19	Usher syndrome type I	Bork et al. (2001)
IVS45-9G→A	EC19	Usher syndrome type I, atypical Usher syndrome	von Brederlow et al. (2002); present study
D2045N	EC19	RNSD	Bork et al. (2001)
6155delC	EC19	Atypical Usher syndrome	Present study
E2103X	EC20	Usher syndrome type I ^b	Present study
R2107X	EC20	Usher syndrome type I	Bork et al. (2001)
D2148N	EC20	RNSD	Brouwer et al. (unpublished data)
D2202N	EC21	RNSD	Bork et al. (2001)
6933delT	EC21	Usher syndrome type I	von Brederlow et al. (2002)
6968delC	EC22	Atypical Usher syndrome	Present study
IVS51+5G→A	EC23	Usher syndrome type I	Bolz et al. (2001); von Brederlow et al. (2002)
R2465W	EC23	RNSD	Present study
S2517G	EC23/EC24 ^c	Usher syndrome type I ^b	Present study
R2608H	EC24	RNSD	Present study
E2624E	EC25	Usher syndrome type I ^b	Present study
G2744S	EC26	Atypical Usher	Present study
R2833G	EC26	Atypical Usher syndrome	Present study
I2950N	EC27	RNSD	Bork et al. (2001)
R2956C	EC27	RNSD	Bork et al. (2001)
P3059T	EC27-TM ^d	RNSD	Bork et al. (2001)
IVS66+1G→A	CYTO	Atypical Usher syndrome	Bork et al. (2001)
R3175H	CYTO	Usher syndrome type I ^b	Present study
9626insC	CYTO	Usher syndrome type I	Present study

^a Further described in tables 4 and 5.

^b Family was referred with a diagnosis of Usher syndrome type I, but no specific clinical data were available.

^c Mutation lies within linker region between these two EC-repeat domains.

^d Mutation lies between EC27 and transmembrane domain.

frameshift leading to a subsequent premature stop codon were identified in seven probands with Usher syndrome. One mutation, 9626insC, is located in the cytoplasmic domain. Sequence analysis of family 216 identified a maternally inherited 4-base insertion of an ATGA between nucleotide position 3840 and nucleotide position 3841 in exon 31, resulting in a deduced TGA stop codon eight amino acids downstream. The insertion of these nucleotides also alters codon 1281, the same methionine codon that Bolz et al. (2001) previously had reported to be deleted in a patient who was compound heterozygous for Usher syndrome.

Splice-site mutations.—Seven splice-site defects were detected, two of which accounted for the most frequent mutations in *CDH23*. An IVS4+1G→A mutation of the splice-donor site of exon 4 was detected in eight Swedish families. The frequency of IVS4+1G→A in the Swedish probands suggests that it may also be a Swedish founder *CDH23* mutation. Haplotype conservation of the two Swedish mutations is currently under study.

The second most frequent *CDH23* mutation identified in the present study is an IVS45-9G→A mutation of the splice-acceptor region preceding exon 46. Exon-trapping experiments done by von Brederlow et al. (2002) have revealed aberrant splicing creating a novel acceptor splice site 9 bp upstream of the 5' end of the exon, consequently resulting in the insertion of 7 bp from the 3' end of intron 45. Another mutation disrupting the conserved donor splicing sequence is an IVS20+1G→A transition found in one allele of three independent families.

The four remaining predicted splicing defects are nucleotide substitutions occurring at the first or last nucleotide of an exon. One of these mutations, Q1496H, a mutation that has been reported elsewhere, is heterozygous in one of the Spanish probands. Published exon-trapping experiments have shown that this mutation causes the use of a cryptic intronic splice site used downstream of the G→C nucleotide substitution (Bolz et al. 2001).

Missense mutations.—A total of 19 different missense mutations were detected in our study; 12 were identified in families with RNSD, and the remaining 7 were identified in families with Usher syndrome type I. Each missense mutation identified in the probands with Usher syndrome was compound heterozygous with a presumed null allele, except for two cases: one family was homozygous for T1209A, and two families were homozygous for R1746Q; each presents an atypical Usher syndrome phenotype (Bolz et al. 2001; present study).

An American family with RNSD was compound heterozygous for two missense mutations in *CDH23*: D124G in exon 5 and R2465W in exon 52. Amino acid alignment of several cadherin 23 domains reveals that the D124G mutation disrupts a conserved DXNDN calcium-binding motif in the first EC domain. Likewise, the R2465W mu-

tation disrupts the LDRE calcium-binding motif in the 23rd EC domain (fig. 1). Molecular modeling (Brouwer et al., unpublished data) of the R2465W mutation shows, furthermore, an interrupted intra- and interdomain salt bridge, which should strongly reduce the interaction between EC23 and EC24 and which therefore may change the tertiary structure required for proper cadherin 23 function.

A homozygous D2148N was identified in one Dutch family with RNSD; an American family was heterozygous for the mutation. Restriction digestion with *Bsi* EI confirmed segregation of this *DFNB12* variant in both families with RNSD (data not shown). Additional EC alignments of these mutations revealed the amino acids to be conserved (fig. 1), and molecular modeling also indicates impairment in calcium binding (Brouwer et al., unpublished data). The remaining eight *DFNB12* mutations are listed in table 1.

Seven different missense mutations were identified among 11 typical and atypical families with Usher syndrome type I. Eight of these families were found to have missense mutations that were compound heterozygous with a null allele. Six of the seven missense mutations in probands with Usher syndrome type ID were identified in the EC domains; the seventh missense mutation was found in the cytoplasmic domain. All Usher syndrome missense mutations are listed in table 2. None of the *CDH23* missense mutations found in patients with Usher syndrome interfered with calcium-binding sites, in contrast to what was seen for the missense mutations found in patients with *DFNB12*; however, two of the substitutions did disrupt conserved amino acids (fig. 1).

Clinical Analyses

RNSD is defined as hearing impairment with no other identified organ-system involvement. Typical Usher syndrome type I is profound deafness, RP verified by an extinguished ERG, and absent bilateral vestibular function that first manifests as delayed (i.e., at age >18 mo) age at ambulation. Clinical investigations of both of our sample groups demonstrate a remarkable range of phenotypic variation. Clinical summaries presented in tables 4 and 5 contain only those families for which relevant audiologic, vestibular, and ophthalmologic examinations and documentation could be obtained; no anamnestic information is included, with the exception of the developmental reports of ages at independent ambulation.

DFNB12 phenotype.—Probands with *DFNB12* and their affected siblings show a less severe phenotype, compared with the probands with Usher syndrome. During their later years, they present a hearing loss ranging from moderate (40–60 dB) to profound (>90 dB) deafness. In two families, hearing loss had progressed from a mild

(<40 dB) loss during childhood. In table 4, six individuals with DFNB12 present with moderate to severe hearing loss, five individuals show severe to profound hearing loss, and one consanguineous family has profound hearing loss. The age at diagnosis of sensorineural hearing loss in affected members has been documented as being 3 mo–6 years, suggesting an onset that is not congenital in all cases. Intrafamilial variation in hearing is also evident, as demonstrated in family 1668, in which serial audiograms of two affected brothers show a progressive hearing loss, with onset at age 4 years in one and at age 6 years in the other; the hearing loss progressed from moderate to severe, bilaterally, in the older brother but progressed asymmetrically, from severe to profound, just in the left ear of the younger brother. All patients with DFNB12 had been able to walk at age \leq 12 mo, and subsequent examinations have shown normal vestibular function. However, ERG, visual-field analysis, and/or fundus examination results demonstrated a subjectively mild retinal attenuation and/or slightly subnormal, or low-amplitude, ERG response; however, there is no visual-field deficit, and only one of five families tested has night blindness. The retinal pathology can be subtle, as is the case in family 1194 (table 4): although ERG results for affected members were normal, one of two pediatric ophthalmologists noticed slightly narrowed retinal arterioles with underlying granular pigment epithelium; despite the fact that vision scores in dim light were below normal and that each individual complained of night-blindness difficulties, the subnormal, albeit mild, fundus-examination results were reported as falling within normal limits. Consequently, this family had been referred to the Boys Town National Research Hospital laboratory, with a diagnosis of RNSD.

Usher syndrome type I phenotype. — A remarkably diverse phenotype was observed in the patients with Usher syndrome type I. Although the mildest phenotype was consistently seen in families with DFNB12, families with Usher syndrome that carry an array of mutations demonstrated considerable variation in hearing, vision, and balance (table 5).

The majority of patients with Usher syndrome who were tested had congenital profound deafness. Six individuals with Usher syndrome type I deviated from this phenotype. Two families with Usher syndrome had severe hearing loss, two families had severe to profound hearing loss, one family had one affected individual with moderate to severe hearing loss and had a second affected individual with moderate to profound hearing loss. In these six individuals, serial audiologic data showed progression and asymmetry in two cases.

Age at ambulation was 10–36 mo in patients with Usher syndrome. Balance ability of 19 individuals with Usher syndrome type I was determined by ENG, calorics and/or examination in a rotary chair. All patients with typical Usher syndrome type I show a late age at am-

bulation, 18–36 mo, and no vestibular reflexes. One atypical patient, who had been able to walk at age 19 mo, presented with mild vestibular dysfunction. Balance-ability testing of five atypical cases, who had been able to walk at age 10–15 mo, demonstrated normal vestibular function.

Of 37 patients with Usher syndrome type I on whom ERG tests and/or fundus examinations were performed, 26 showed extinguished, or flat, ERG amplitudes, 19 had typical RP manifestations in the fundus, and 8 had subnormal ERG responses. Subnormal RP-like findings in the retina were found in four patients. One proband had a normal ERG response; the fundus examination of another proband with subnormal ERG amplitudes indicated a normal retina.

A collection of visual-field tests were examined, and rates of progression were quantified and categorized according to a classification system described by Grover et al. (1997). For those cases in which visual fields could not be determined, verbal descriptions of the testing results are given in table 5. Of the 34 individuals for whom visual-field data were available, 20 show a marked concentric loss of \leq 10° in both eyes, or oculi unitas (OU) (type 4). Eight patients presented a concentric central-field loss with a temporal island, or scotoma OU (type 3). Partial peripheral restrictions OU (type 5) were identified in two Swedish individuals with Usher syndrome type I, at ages 75 and 80 years. Another Swedish proband, at age 47 years, had a midperipheral complete ring scotoma OU (type 2) that had not progressed since age 19 years.

Clinical analyses of cases with the R1746Q missense mutation show a variable retinal phenotype similar to that described by Bolz et al. (2001). An American family, 3308, homozygous for the R1746Q mutation originally was referred to our laboratory through the RNSD research project. This 5-year-old boy presented with congenital profound deafness but had been able to walk at age 19 mo. Clinical investigation and examination in a rotary chair revealed a mildly hypoactive vestibular response. Although the results of an ERG at age 3.5 years were normal, follow-up examinations are being recommended, to determine whether a diagnosis of Usher syndrome type I is valid. An Irish family, 809, also homozygous for R1746Q, presented with mild late-onset RP. Ophthalmologic examinations of members of family 792, who were compound heterozygous for R1746Q and 1112delT, revealed a severe course of RP. Interestingly, late-onset RP was seen in the 73-year-old father, who was heterozygous for the R1746Q allele. Ophthalmologic examinations of the father revealed a flat ERG, a nonpigmentary and diffuse atrophy of the fundus, and visual fields of $<$ 5°. The father did not present with hearing loss. No other individuals in this family who were heterozygous for *CDH23* reported visual problems or any other carrier effect; how-

ever, the father has been tested only for mutations in exon 40 of *CDH23*, and the possibility exists that (a) another *CDH23* mutation exists or (b) mutations in another RP gene are responsible for his condition. Family 2790, heterozygous for R1746Q, presented atypical Usher features: the hearing loss is asymmetric and severe to profound, and results of ERG tests were subnormal at age 29 years, despite a documented 10° visual-field result. Fundus examination revealed typical RP, and, although vestibular testing was not completed, the affected individual in this family reportedly had been able to walk at age 13 mo.

The following clinical findings for families 980 and 2059 underscore the fact that the severity of the phenotypes can be highly variable and may overlap with Usher syndrome types II and III. Swedish family 980 was referred to our molecular-testing lab as having Usher syndrome type II. The 49-year-old singleton proband presented with a sloping moderate to profound hearing loss and normal developmental milestones and vestibular function. The serial visual data for this individual show a late onset and slow progression of RP. On the basis of the variable retinal phenotype associated with mutations of *CDH23*, these retinal findings prompted us to include this proband in our analysis, and, indeed, a heterozygous R2833G missense mutation was identified; however, the second mutation has not yet been identified. Family 2059 was originally referred to our laboratory as having RNSD. One of the two affected siblings had a severe asymmetrical hearing loss at age 14 years, and the other had a moderate to severe hearing loss at age 8 years; both showed progressive hearing loss. Subsequent testing for mutations in *GJB2* was negative. Genotyping analysis was consistent with linkage to the 10q region, and therefore our testing included this family, as putatively having *DFNB12*. While the mutation screening was in progress, visual exams revealed extinguished and subnormal ERG responses, as well as abnormal fundus-examination findings, in each affected individual. Because of reported normal developmental milestones and normal vestibular function, the diagnosis was changed to Usher syndrome type II, but testing for mutations in *CDH23* continued. Family 2059 simultaneously underwent preliminary analysis for mutations in *USH2A*, until two splice-site mutations, IVS20+1G→A and 3105A→C (T1035T), were identified in *CDH23*.

The remarkable phenotype of family 1054 has been reported elsewhere (Bitoun et al. 1991). Two affected children born of Sephardic Jewish first cousins have a heterozygous G2744S mutation and presented with retinal degeneration. A severe hearing loss and an extinguished ERG, typical of RP, were identified in the older proband; the younger brother has a subnormal ERG response, with normal fundus-examination findings and normal hearing. The lack of a homozygous *CDH23* mutation in this consanguineous family, the identification

of only one missense mutation, G2744S, and the additional clinical findings of developmental delay, dysmorphism, and severe ataxia suggest either a non-Usher syndrome diagnosis or the existence of another syndrome; therefore, until a second mutation is been identified, we are hesitant to claim that this family has Usher syndrome type ID.

Discussion

In 27 (56.3%) of 48 multiplex families with Usher syndrome and in 6 (30%) of 20 multiplex families with RNSD, we have identified *CDH23* mutations that link to chromosome 10q21.1-q22.1, which includes the *USH1D*, *DFNB12*, *USH1E*, and *DFNB23* loci (Chaib et al. 1996; Wayne et al. 1996, 1997; Hereditary Hearing Loss Homepage). Mutations were seen in six sporadic cases of Usher syndrome type I and in five sporadic cases of RNSD. Forty-one families with Usher syndrome type I were part of a previous study (Astuto et al. 2000) in which results of linkage and heterogeneity analyses suggested that the chromosome 10q-associated Usher syndrome type I subtype was the second most common Usher syndrome type I subtype. Our *CDH23* mutation analysis has corroborated those findings. On the basis of the proportion of *CDH23* mutations among families with RNSD, it should be noted that the sample is refined, since cases with mutations of *GJB2*, *SLC26A4*, or *OTOF* have been excluded. Extrapolating from an original population of 157 families with RNSD, we estimate that 5% of RNSD is caused by mutations in *CDH23*. Technical error or hidden mutations in gene regions that we did not explore—regions such as introns and regulatory regions—may account for the individuals who carry single *CDH23* heterozygous mutations. The possibility also exists that these individuals are *CDH23* carriers and that the causative mutation is at a different locus, such as *USH1E*.

The majority of *CDH23* mutations occur within the *CDH23*-gene region encoding the EC domain. Mutations were identified in all domains except the signal sequence. Only one mutation in the cytoplasmic domain had been previously identified (Bork et al. 2001); the remainder of the mutations occur in the EC repeats. We have identified two additional mutations in the cytoplasmic domain—R3175H and 9626insC—both associated with an Usher syndrome type I phenotype.

The distribution and location of the missense mutations identified in the present study illustrate that the mutations do not cluster within one domain. However, comparison of the locations between phenotypic groups show that 6 of 12 *DFNB12* missense mutations change the highly conserved peptide sequences LDRE, DXNDN, and DXD, which are responsible for calcium-binding and rigidification (fig. 1). Molecular

modeling of five of these amino acid substitutions strongly suggests that there is a defect in Ca^{2+} ion binding (Brouwer et al., unpublished data). No Usher syndrome type ID missense mutations change the conserved calcium-binding sequences, and only two of six EC-domain mutations substitute a conserved amino acid (fig. 1).

Conclusions drawn from the present study and from other groups (Bolz et al. 2001; Bork et al. 2001; Brouwer et al., unpublished data) show that missense mutations produce a less severe phenotype, both in families with Usher syndrome type I and in families with RNSD, whereas nonsense, splice-site, and frameshift mutations produce a more severe Usher syndrome type I phenotype (table 6). In our mutation data, a reduced frequency of null mutations of *CDH23* is seen as the phenotype becomes milder. Approximately 88%, 67%, and 0% of null mutations of *CDH23* are found in typical Usher syndrome type I, in atypical Usher syndrome type I, and in probands with RNSD, respectively. However, the genotype does not always predict the clinical phenotype, which varies both within and between families carrying the same *CDH23* mutations, implying the existence of other genetic and/or environmental factors that influence phenotype.

The lack of a distinct Usher syndrome type ID phenotype may complicate mutation analysis of all patients diagnosed with Usher syndrome types I–III. In the present study, we have identified a *CDH23* mutation in an individual diagnosed with Usher syndrome type II. We therefore plan to test a series of cases with an Usher syndrome type II phenotype, to determine the frequency of *CDH23* mutations within this diagnostic category. Identification of progressive hearing loss and variable balance dysfunction in patients excluded from harboring identified Usher syndrome type III mutations are also candidates for *CDH23* mutation screening. Efficient mutation testing will be difficult because of the complexity of the *CDH23*-gene structure. However, some mutations of *CDH23* occur with high frequency in certain populations. For example, R1502X and the IVS4+1G→A should be the first two *CDH23* mutations screened in patients with Usher syndrome who have a Swedish background.

The syndromic and nonsyndromic association found in *CDH23* alleles is not exclusive to this gene. Two other Usher syndrome type I subtypes are allelic with *DFNB* loci: *DFNB2* is associated with *USH1B* (Liu et al. 1997; Weil et al. 1997), and *DFNB18* is associated with *USH1C* (Ahmed et al., in press). Understanding the variations of pathophysiology underlying mutations of *CDH23*, as well as the other Usher syndrome genes, is paramount if effective treatments are to be developed.

Acknowledgments

We would like to thank all the patients and families, for their participation in the Usher syndrome and recessive non-syndromic-deafness research projects, and the physicians, including Drs. Martini and Iannoccone, for referring families to us. We would like to thank Dr. Rocky Young at Texas Tech University, for ERG testing of two patients with mutations in *DFNB12*, and Dr. Ed Cohn, for his advice and contribution. We also would like to recognize Mehdi Sadeghi, for helping to collect Swedish clinical information; Zubair Ahmed, for genotyping the Pakistani families; and Edward Wilcox, for his contributions. This work was supported in part by National Institutes of Health grant P01 DC01813, National Institute on Deafness and Other Communication Disorders grants R01 DC00677-07, and a Foundation Fighting Blindness grant (all to W.J.K.); National Institutes of Health grant P60 DC00982 (to L.M.A.); the University Grants Commission, Islamabad, Pakistan (support to S.K. and S.R.); National Institute on Deafness and Other Communication Disorders/National Institutes of Health intramural funds 1Z01 DC000035-05 and 1Z01 DC000039-05 (both to T.B.F.); a Research to Prevent Blindness Clinician-Scientist Award (to J.R.H.); and the Mgr Van Overbeek Foundation, De Drie Lichten, and De Gelderse Blinden Vereniging (support to C.W.R.J.C. and H.K.).

Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Berkeley Drosophila Genome Project Splice Site Prediction by Neural Network, http://www.fruitfly.org/seq_tools/splice.html
 GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for human *CDH23* [accession number AF312024])
 Hereditary Hearing Loss Homepage, <http://dnalab-www.uia.ac.be/dnalab/hhh/index.html>
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for *USH1D* [MIM 601067], *DFNB12* [MIM 601386], and *CDH23* [MIM 605516])
 Sequence Manipulation Suite, The, <http://www.ualberta.ca/~stothard/javascript/>

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