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RESEARCH ARTICLE

Mutational Spectrum of *MYO15A*: The Large N-Terminal Extension of Myosin XVA Is Required for Hearing

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Human *MYO15A* is located on chromosome 17p11.2, has 66 exons and encodes unconventional myosin XVA. Recessive mutations of *MYO15A* are associated with profound, nonsyndromic hearing loss DFNB3 in humans, and deafness and circling behavior in shaker 2 mice. In the inner ear, this motor protein is necessary for the development of hair cell stereocilia, which are actin-filled projections on the apical surface and the site of mechanotransduction of sound. The longest isoform of myosin XVA has 3,530 amino acid residues. Two isoform classes of *MYO15A* are distinguished by the presence or absence of 1,203 residues preceding the motor domain encoded by alternatively-spliced exon 2. It is not known whether this large N-terminal extension of myosin XVA is functionally necessary for hearing. We ascertained approximately 600 consanguineous families segregating hereditary hearing loss as a recessive trait and found evidence of linkage of markers at the *DFNB3* locus to hearing loss in 38 of these families ascertained in Pakistan (n = 30), India (n = 6), and Turkey (n = 2). In this study, we describe 16 novel recessive mutations of *MYO15A* associated with severe to profound hearing loss segregating in 20 of these *DFNB3*-linked families. Importantly, two homozygous mutant alleles—c.3313G>T (p.E1105X) and c.3334delG (p.G1112fsX1124) of *MYO15A*—located in exon 2 are associated with severe to profound hearing loss segregating in two families. These data demonstrate that isoform 1, containing the large N-terminal extension, is also necessary for normal hearing. Hum Mutat 28(10), 1014–1019, 2007. Published 2007 Wiley-Liss, Inc.†

KEY WORDS: *DFNB3*; hereditary deafness; genotype–phenotype; myosin; *MYO15A*

INTRODUCTION

Myosins are molecular motor proteins that hydrolyze ATP to generate a small conformational change in the globular motor domain that is translated into movement along actin filaments [Mooseker and Cheney, 1995; Mermall et al., 1998; Sellers, 1999; Schliwa and Woehlke, 2003]. Based upon phylogenetic analyses of motor domains, 37 distinct classes of heavy chain myosins have been cataloged in plants, fungi, amoebas, invertebrates, and vertebrates [Sellers, 2000; Berg et al., 2001; Richards and Cavalier-Smith, 2005; Foth et al., 2006]. Within the human genome, there are at least 39 myosin genes assigned to 12 classes [Berg et al., 2001].

Myosins are implicated in cellular functions including muscle contraction, cell movement, cytokinesis, exocytosis, endocytosis, transcription, vesicle and cargo trafficking, organelle localization, signal transduction, and anchoring and differential elongation of inner ear hair cell stereocilia [Baker and Titus, 1998; Vale, 2003; Belyantseva et al., 2005; Krendel and Mooseker, 2005;

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Grummt, 2006]. The conserved motor domain of myosin contains a nucleotide binding pocket, an actin binding site, and a converter region, which links the motor domain to the neck [Mooseker and Cheney, 1995]. The neck region can bind calmodulin or other light chains and is composed of a variable number of IQ motifs [Mooseker and Cheney, 1995]. Among unconventional myosins, the tail domain is highly divergent and may contain multiple protein or lipid binding motifs involved in subcellular localization of the myosin [Mermall et al., 1998; Krendel and Mooseker, 2005].

MYO15A (MIM# 602666) is composed of 66 exons distributed across 71 kb of DNA on chromosome 17p11.2. The longest *MYO15A* mRNA transcript encodes a predicted 3,530 amino acid protein with a deduced molecular weight of 395 kDa (NM_016239.3, NP_057323.3) [Liang et al., 1999; Belyantseva et al., 2003]. Two alternatively-spliced transcripts are distinguished by the presence (isoform class 1) or absence of exon 2 (isoform class 2) [Liang et al., 1999; Belyantseva et al., 2003]. Exon 2 is unusually large, encoding the 1,203 amino acid residues of the N-terminal extension. The biological function of the N-terminal extension is unknown.

Many inherited disorders in humans are due to mutant alleles of motor proteins including hereditary deafness, which is genetically heterogeneous [Friedman and Griffith, 2003]. At least seven different myosins are necessary for hearing (Supplementary Table S1; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat). Mutations of myosin XVA are associated with congenital, neurosensory deafness in both humans (*DFNB3*) and shaker 2 (*sh2*) mice [Friedman et al., 1995; Liang et al., 1998; Probst et al., 1998; Wang et al., 1998]. Mouse myosin XVa is highly restricted in its pattern of expression as it is detected only in neuroendocrine cells [Lloyd et al., 2001; La Rosa et al., 2002] and in the inner ear [Belyantseva et al., 2003]. In situ analyses

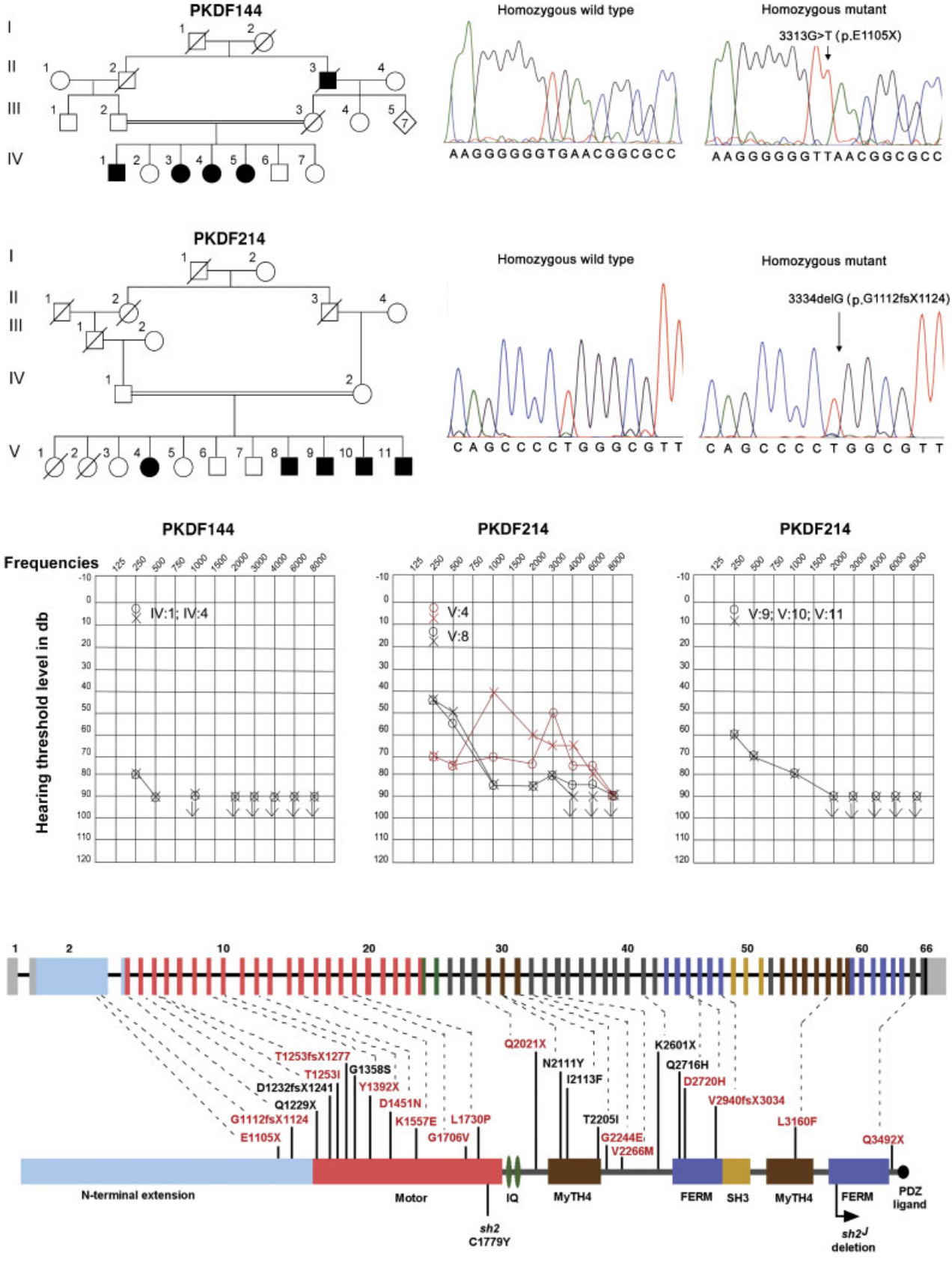
TABLE 1. **Novel Mutations of *MYO15A* Detected in This Study**

Site	Family	National origin	Max. LOD score[a]	Mutation[b]	Predicted protein effect	Allele frequency[c]
Exon 2	PKDF144	Pakistan	2.9	c.3313G>T	p.E1105X[d]	0/226
Exon 2	PKDF214	Pakistan	3.8	c.3334delG	p.G1112fsX1124[d]	0/226
Exon 5	HAP18	India	2.1	c.3758C>T	p.T1253I	0/296
Intron 5	PKDF459	Pakistan	5.1	c.3866+1G>A	p.T1253fsX1277[e]	0/264
Exon 10	PKDF336	Pakistan	2.0	c.4176C>A	p.Y1392X	0/276
Exon 12	DKB12	India	3.4	c.4351G>A	p.D1451N	0/278
Exon 15	PKDF323	Pakistan	3.0	c.4669A>G	p.K1557E	0/286
Exon 18	PKDF436	Pakistan	3.0	c.5117_5118GC>TT	p.G1706V	0/128
Exon 19	PKDF112	Pakistan	2.0	c.5189T>C	p.L1730P	1/242
Exon 28	PKDF201	Pakistan	3.4	c.6052G>A	p.G2018R	25/270[f]
Exon 28				c.6061C>T	p.Q2021X	0/270
Exon 31	PKDF322[g]	Pakistan	2.3	c.6614C>T	p.T2205I	2/294
Exon 32	PKSR13	Pakistan	2.7	c.6731G>A	p.G2244E	0/306
Exon 33	PKDF046	Pakistan	3.4	c.6796G>A	p.V2266M	1/280
	TRDF01	Turkey	3.7	c.6796G>A	p.V2266M	2/164
Exon 45	PKDF207	Pakistan	2.0	c.8158G>C	p.D2720H	0/288
	PKSR23	Pakistan	4.8	c.8158G>C	p.D2720H	
	PKB18	Pakistan	2.7	c.8158G>C	p.D2720H	
	PKB11	Pakistan	3.1	c.8158G>C	p.D2720H	
Exon 51	PKDF465	Pakistan	4.8	c.8821_8822insTG	p.V2940fsX3034	0/171
Exon 57	PKSR8	Pakistan	2.9	c.9478C>T	p.L3160F	1/266
Exon 65	PKDF322[g]	Pakistan	2.3	c.10474C>T	p.Q3492X	0/270

[a]LOD scores were calculated assuming equal marker allele frequencies.
[b]Nucleotide positions are numbered relative to the first nucleotide of the translational open reading frame found in RefSeq NM_016239.3; Nucleotide +1 corresponds to the "A" of the translation start codon at position 339.
[c]Number of chromosomes; genomic DNA from ethnically matched controls were used to estimate the carrier frequency of the mutant alleles of *MYO15A* observed in families from Pakistan, India, and Turkey.
[d]Nonsense and frameshift mutations in exon 2 are expected to be null for isoform class 1 of myosin XVA that includes the N-terminal domain. These two mutant alleles are assumed to not alter the expression of class 2 isoforms of *MYO15* that do not include exon 2 [Belyantseva et al., 2003].
[e]Location of the nonsense codon in exon 6 assumes that only exon 5 is skipped due to the donor splice site mutation 3866+1G>A.
[f]Includes one homozygote among normal hearing controls.
[g]Affected individuals in Family PKDF322 are homozygous for p.T2205I and p.Q3492X (see exons 31 and 65), raising the possibility that p.T2205I is a benign polymorphism, although previously reported by us as a possible pathogenic allele [Liburd et al., 2001].

FIGURE 1. **Two families segregating hearing loss DFNB3, audiograms of affected members, novel mutant alleles of *MYO15A* and protein domain organization of myosin XVA. A:** Pedigrees of Families PKDF144 and PKDF214. Affected individuals are homozygous for mutant alleles of exon 2 of *MYO15A* and are shown as black-filled circles (females) and filled squares (males). **B:** Two mutant alleles, c.3313G>T (p.E1105X) and c.3334delG (p.G1112fsX1124), are homozygous in affected members of Families PKDF144 and PKDF214, respectively. **C**: Audiograms from a total of seven affected members of Families PKDF144 and PKDF214 showing some retention of hearing at low frequencies (X = left ear; O = right ear). Patients V:9, V:10, and V:11 in the right most audiogram had the same hearing profile. **D:** Genomic structure of *MYO15A*, mutant alleles and the protein domains of myosin XVA (cDNA, RefSeq number: NM_016239.3; protein RefSeq number: NP_057323.3). See Supplementary Table S3 for residues defining the boundaries encoding each domain of myosin XVA. The motor domain and neck region of myosin XVA are followed by a unique tail domain composed of two MyTh4 domains (MyosinTail homology 4, PF00784), two FERM domains (F for 4.1 protein, ezrin, radixin and moesin; PF00373), a SH3 domain (Src homology 3, PF00018), and predicted class I PDZ peptide ligand at the carboxy-terminus [Belyantseva et al., 2003, 2005]. A PDZ ligand is a short motif usually at the carboxy-terminus of a protein that binds to a PDZ domain. Sixteen novel mutations of *MYO15A* are shown in red and eight previously reported mutations and *sh2* and $sh2^J$ alleles are in black font [Probst et al., 1998; Wang et al., 1998; Anderson et al., 2000; Liburd et al., 2001].

using mouse inner ear tissue demonstrate that messenger RNA for both class 1 and 2 isoforms of myosin XVa are expressed only in neurosensory hair cells [Liang et al., 1999; Anderson et al., 2000]. In wild-type inner ear hair cells, myosin XVa immunoreactivity is present at the tips of stereocilia but absent from stereocilia of homozygous *sh2* mice, which have abnormally short stereocilia



[Anderson et al., 2000; Belyantseva et al., 2003, 2005; Rzadzinska et al., 2004; Delprat et al., 2005]. One function of myosin XVa in hair cells is to deliver whirlin, a multi-PDZ domain–containing scaffold protein, to stereocilia tips [Belyantseva et al., 2005]. Both myosin XVa and whirlin are necessary for elongation and staircase formation of the stereocilia bundle [Belyantseva et al., 2005].

To date, only eight recessive mutations of *MYO15A* associated with profound, congenital deafness (*DFNB3*; MIM# 600316) have been reported [Wang et al., 1998; Liburd et al., 2001]. Here, we describe 16 novel mutations of *MYO15A* cosegregating with DFNB3 hearing loss in 20 families from Turkey, India, and Pakistan. Significantly, a nonsense mutation and a frameshift mutation, E1105X and G1112fs1124X, are located in alternatively-spliced exon 2 that encodes the large N-terminal extension. These data indicate that the class 1 isoform of myosin XVA that contains the N-terminal extension is necessary for normal auditory function.

MATERIALS AND METHODS

Subjects

The study was approved by Institutional Review Boards (IRBs) in Turkey (Akdeniz University, Antalya), Pakistan (Center for Excellence in Molecular Biology, Punjab University, Lahore), India (All India Institute of Medical Sciences, New Delhi), and also from the National Institute on Deafness and Other Communication Disorders (NIDCD)/National Institute of Neurological Disorders and Stroke (NINDS) IRB at the National Institutes of Health, Bethesda, MD (OH93-N-016). Family members were ascertained after obtaining written informed consent. All of the families from India, Pakistan, and Turkey reported herein are segregating severe to profound hearing loss, and all these families have consanguineous marriages.

Genotyping and Nucleotide Sequence Analysis

Approximately 600 families segregating hereditary deafness were screened for markers linked to *DFNB3* on chromosome 17p11.2 as previously described [Wang et al., 1998; Liburd et al., 2001]. After identifying families segregating deafness linked to markers for *DFNB3*, two affected individuals from each family were screened for mutations by direct sequencing of each of the 66 exons of *MYO15A* using big dye terminators (v3.1; Applied Biosystems, Foster City, CA) and a 3730XL DNA Analyzer (Applied Biosystems). PCR reactions had a total volume of 20 μl with 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 U thermostable polymerase, and 0.25 μM forward and reverse primers (Supplementary Table S2). After an initial denaturation at 95°C for 1 minute and 30 seconds, cycling parameters were 95°C for 45 seconds, with annealing for 45 seconds at 60°C. Extension times were 2 minutes at 72°C for 35 cycles followed by a final extension of 72°C for 5 minutes. Primers used for sequencing *MYO15A* are shown in Supplementary Table S2. Nucleotide positions are numbered relative to the first nucleotide of the translational open reading frame found in RefSeq NM_016239.3. Nucleotide+1 corresponds to the "A" of the translation start codon at position 339.

RESULTS AND DISCUSSION

The 66 exons of *MYO15A* from affected individuals from 38 *DFNB3*-linked families were sequenced. We identified likely pathogenic mutant alleles in 20 of these 38 families. Hearing loss in 11 of these 20 families was statistically significantly linked (logarithm of the odds [LOD] score ≥ 3.0) to markers on chromosome 17p11.2 and hearing loss in nine families was consistent with linkage to this locus (LOD score between 1.5 and 2.9). In affected individuals in one Turkish family, two Indian families, and 17 Pakistani families, there were 16 novel mutations of *MYO15A* (Table 1). We found a nonsense mutation, p.E1105X, segregating in Family PKDF144, and a frameshift mutation that results in a predicted premature stop codon (G1112fsX1124) in Family PKDF214 (Fig. 1A, B). These mutant alleles are homozygous in affected individuals and heterozygous in normal-hearing obligate carriers. The degree of hearing loss ranged from severe to profound. The audiometric data were obtained in the homes of family members and because of ambient noise we may have underestimated hearing capacity (Fig. 1C).

The two mutant alleles in exon 2 of *MYO15A* provide the first evidence that the class 1 isoform of myosin XVA is required for normal hearing in humans. In the mouse inner ear, messenger RNA for the class 1 isoform of myosin XVa is expressed in hair cells of the inner ear [Liang et al., 1999]. However, the class 1 isoform of myosin XVa is not necessary either for elongation of stereocilia or for establishing the staircase architecture of the hair bundle [Belyantseva et al., 2005]. When we transfected a class 2 myosin XVa isoform (no N-terminal extension) into shaker 2 hair cells that have abnormally short stereocilia, elongation of stereocilia was reinitiated and a wild type-like staircase architecture of the hair bundle was observed [Belyantseva et al., 2005]. It is possible that in the absence of the class 1 isoform there are functional abnormalities or subtle structural aberrations in the architecture of a mature hair bundle. Alternatively, the class 1 isoform of myosin XVa may perform a function in hair cells unrelated to stereocilia development or function. Despite the disablement of myosin XVa due to a missense mutation in the motor, which presumably would affect both class 1 and 2 isoforms, the abnormally short mutant stereocilia of hair cells from homozygous shaker 2 mice have a wild-type mechanotransduction current [Stepanyan et al., 2006]. These data indicate that neither isoform of myosin XVa delivers an essential component of the mechanotransduction complex to the tips of stereocilia.

When queried against protein and nucleic acid databases such as Pfam (www.sanger.ac.uk/Software/Pfam) and Prosite (http://us.expasy.org/prosite), and the PredictProtein (http://cubic.bioc.columbia.edu/predictprotein) program [Rost et al., 2003], the large N-terminal extension of myosin XVA is unique and has no obvious domains or motifs. The majority of myosins with N-terminal extensions, although short by comparison to myosin XVa, contain a predicted protein motif including a Serine/Threonine kinase domain preceding myosins IIIa and IIIB proteins [Dose and Burnside, 2000, 2002], a Ras-GTP binding domain in myosins IXa and IXb proteins [Gorman et al., 1999; Grewal et al., 1999], an ankyrin repeat domain in myosin XVI [Patel et al., 2001], and a PDZ domain in myosin XVIIIa [Furusawa et al., 2000]. The N-terminal extension of myosin XVa is rich in proline and tyrosine and we have speculated [Friedman and Griffith, 2003] that it may have elastomeric properties [Tatham and Shewry, 2000]. Insight into the function of the class 1 isoform of myosin XVa awaits biophysical characterization of the N-terminal extension and a mouse model deficient for isoform 1, while retaining normal expression of the class 2 isoform.

In addition to the two mutant alleles in exon 2, we found three novel nonsense mutations (p.Y1392X, p.Q2021X, and p.Q3492X), nine novel missense mutations (p.T1253I, p.D1451N, p.K1557E, p.G1706V, p.L1730P, p.G2244E, pV2266M, p.D2720H, and p.L3160F), a frameshift mutation (p.V2940fsX3034), and a splice donor site mutation in intron 4

(c.3866+1G>A), which is predicted to cause a frameshift in exon 5 (p.T1253fsX1277) (Table 1). We do not know exactly how this splice donor site mutation alters splicing of *MYO15A* mRNA in the human inner ear.

The mutant alleles of *MYO15A* causing deafness are distributed across the length of the gene including regions encoding the motor and the various domains of the tail (Fig. 1D). For most of these mutant alleles of *MYO15A* (Table 1), we found no carrier chromosomes from control hearing individuals that are ethnically matched from populations in Pakistan, India, and Turkey (Table 1). The p.V2266M allele is segregating in a Pakistani family (Family PKDF046) and a Turkish family (Family TRDF01) both with LOD scores over 3.0 indicating significant linkage of deafness segregating in these two families to markers for *DFNB3*. Other than p.V2266M, we found no additional mutations of the protein coding sequence of *MYO15A* in these two families. We did find two carriers of p.V2266M among 82 control hearing individuals from Turkey and one carrier in 140 control hearing individuals from Pakistan (Table 1). A functional assay of the affect of this substitution on myosin XVA function is required to distinguish between the possibility that V2266M is a benign polymorphism or a pathogenic allele with a significant overall carrier frequency of 1.35%. If p.V2266M is a pathogenic allele, a carrier frequency of 2 out of 82 hearing individuals (2.4%; 95% confidence interval is 0.01–7.0%) would predict that p.V2266M in the Turkish population is a significant contributor to hereditary deafness. Historically, Turkey has attracted migrations from many different populations [Di Benedetto et al., 2001] and this admixture may be the reason for finding the same mutations in a Pakistani family and in a Turkish family segregating deafness. However, in these two families segregating V2266M, the haplotypes differ at five intronic SNPs in *MYO15A*, and so p.V2266M is not likely to have a recent common origin (data not shown). By comparison, four Pakistani families (Families PKDF207, PKSR23, PKB18, and PKB11; Table 1) are segregating p.D2720H and have the same haplotype for three STR markers (D17S2196, D17S2206, and D17S2207; data not shown), one of which is just upstream of this gene and two of which are located in introns of *MYO15A*. These data suggest a single origin of p.D2720H and a common ancestor among the four families.

Mutations in the protein coding exons or in the splice junctions of *MYO15A* were not detected in 18 other *DFNB3*-linked families. There are at least three possible reasons for our failure to detect mutations of *MYO15A* in these additional *DFNB3* families segregating hearing loss. First, mutations may alter sequence of a *cis*-acting regulatory or splicing element of *MYO15A* that is necessary for expression of this gene in the inner ear. Presently we do not know the location of the regulatory elements of *MYO15A*, although there is conserved sequence in some of the introns that may be important for control of transcription of this gene [Liang et al., 1999]. Second, there may be an additional gene in the *DFNB3* interval in which mutant alleles cause hearing loss. There are examples of closely linked deafness genes and mutant alleles of likely *cis*-acting regulatory elements [Wilch et al., 2006]. Third, the hearing loss segregating in these 18 families may be spuriously linked to markers at 17p11.2.

In summary, we have identified 16 novel mutations of unconventional *MYO15A* that are associated with neurosensory DFNB3 hearing loss. Two of these mutant alleles result in translation stop codons in exon 2, which alone encodes the large N-terminal extension. Assuming that these mutations do not affect the expression of the class 2 MYO15A isoform, our data indicate that the class 1 MYO15A isoform is necessary for normal hearing function. Functional studies of the large N-terminal extension of myosin XVa in the inner ear will benefit from expression studies, immunolocalization using antisera specific to this domain, and from a mouse model in which exon 2 is disabled. This study also demonstrated that mutant alleles of *MYO15A* are responsible for hearing loss in many different ethnic groups.

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