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ORIGINAL INVESTIGATION

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Novel mutations of *MYO15A* associated with profound deafness in consanguineous families and moderately severe hearing loss in a patient with Smith-Magenis syndrome

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Abstract Mutations in myosin XVA are responsible for the *shaker 2* (*sh2*) phenotype in mice and nonsyndromic autosomal recessive profound hearing loss *DFNB3* on chromosome 17p11.2. We have ascertained seven families with profound congenital hearing loss from Pakistan and India with evidence of linkage to *DFNB3* at 17p11.2. We report three novel homozygous mutations in *MYO15A* segregating in three of these families. In addition, one hemizygous missense mutation of *MYO15A* was found in one of eight Smith-Magenis syndrome (del(17)p11.2) patients from North America who had moderately severe sensorineural hearing loss.

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Introduction

Myosins are molecular motors that bind actin and use ATP to generate force for movement along actin (Sellers 1999, 2000; Baker and Titus 1997). Myosins typically have a conserved motor domain and a variable number of regulatory light-chain binding domains (IQ motifs), which separate the motor from the tail region (Fig. 1). The large family of myosin genes is subdivided into more than 18 classes on the basis of evolutionary relationships among amino acid sequences of the motor domain (Sellers 1999).

Four motor protein genes encoding myosin VI, myosin VIIA, myosin XVA, and nonmuscle myosin IIA (*MYH9*) are essential for normal auditory function. Mutant alleles of these four myosin genes are associated with hearing loss (Avraham et al. 1995; Friedman et al. 1999; Lalwani et al. 2000). Mutations in myosin XVA are responsible for the *shaker 2* (*sh2*) phenotype in mice and nonsyndromic autosomal recessive profound hearing loss *DFNB3* in humans (Friedman et al. 1995). A genome-wide homozygosity mapping strategy has localized *DFNB3* to 17p, which has subsequently been refined to a 3-cM interval within the 17p11.2 Smith-Magenis syndrome interval (SMS, MIM 182290; Friedman et al. 1995; Liang et al. 1998). Myosin XVA was identified through bacterial artificial chromosome rescue of the shaker 2 deafness phenotype, and its human ortholog was shown to underlie *DFNB3* deafness (Liang et al. 1998; Wang et al. 1998).

The full-length myosin XVA cDNA is approximately 12 kb and encodes a protein with a deduced molecular mass of 365 kDa (Liang et al. 1999). Myosin XVA is structurally unique among all known myosins because of an alternatively spliced isoform (encoded by exon 2) with a 1223-amino-acid N-terminal extension preceding the motor domain. In addition, myosin XVA has a long tail composed of 1587 amino acids. Immunofluorescence light microscopy and in situ hybridization have localized myosin XVA protein and mRNA, respectively, in the anterior pituitary gland (Liang et al. 1999; Lloyd et al. 2001) and in the inner and outer hair cells of the cochlea (Anderson et al. 2000; Liang et al. 1999).

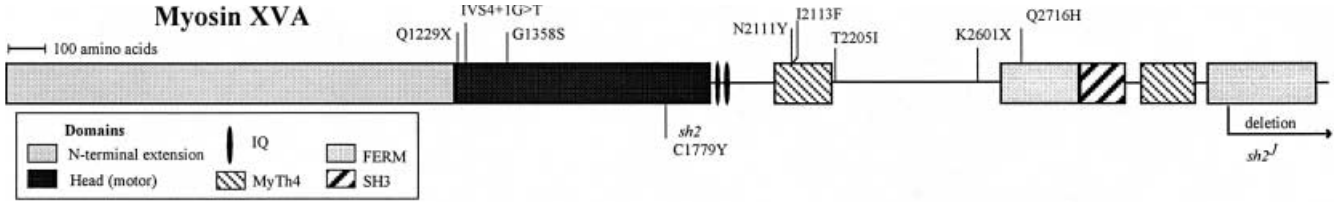

Fig. 1 Diagram of the protein domains of human myosin XVA. Myosin XVA consists of an alternatively spliced 1223-amino-acid N-terminus encoded by exon 2, a motor domain, two IQ-motifs, and a tail with two MyTh4 domains, two FERM domains and an SH3 domain. We have included the four new myosin XVA mutations reported here and four published mutations of human myosin XVA (Wang et al. 1998; Liang 1999) and two shaker 2 mutant alleles (*sh2* and *sh2^J*; Probst et al. 1998; Anderson et al. 2000)

Three mutations of *MYO15A* segregating in a Balinese village and in two consanguineous families have been described previously (Fig. 1; Wang et al. 1998). Here, we report one nonsense, one splice site, and one missense mutation of *MYO15A* segregating in three consanguineous *DFNB3* families with hearing loss. We have also examined *MYO15A* in a selected group of eight patients with SMS with mild to moderate sensorinueral hearing loss. SMS is a multiple congenital anomalies/mental retardation syndrome (Smith et al. 1986; Stratton et al. 1986) associated with a 4-Mb interstitial deletion of chromosome 17p11.2, an interval that includes *MYO15A*. Thus, *MYO15A* is predicted to be hemizygous in SMS patients (Chen et al. 1997; Juyal et al. 1996; Liang et al. 1998). The majority of SMS patients has minor craniofacial and ocular anomalies, a unique neurobehavior phenotype, and sleep disturbances (Greenberg et al. 1996; Potocki et al. 2000). Approximately 68% of SMS patients have at least some degree of hearing impairment, which can be conductive, sensorineural, or mixed in nature (Greenberg et al. 1996; Chen et al. 1996). Hemizygosity for one or more genes in the SMS deletion interval may be the cause of this mild to moderate hearing loss. We hypothesize that more severe hearing loss among SMS patients may be attributable to mutant alleles of *MYO15A* on the normal chromosome 17 uncovered by the SMS deletion. A hemizygous missense mutation of *MYO15A* has been identified in one of eight individuals with SMS affected with sensorineural hearing impairment.

Materials and methods

Subjects

DFNB3 families

This study was approved by the Institutional Review Boards (IRB) at the National Institutes of Health and from the Center of Excellence in Molecular Biology, Lahore, Pakistan. All subjects gave informed consent in their native language. Probands were initially identified at schools for the deaf in Lahore and Karachi, Pakistan, or in Maharashtra, India. The consanguineous families segregating for hearing loss were first screened for linkage to markers for the published nonsyndromic recessive deafness loci (DFNB loci) including D17S2206 and D17S2207 for *DFNB3* on chromosome 17p11.2.

Smith-Magenis syndrome

Eight SMS patients with sensorineural or mixed hearing impairment (ascertained by L.P., J.R.L and A.C.M.S.) were screened for mutations in *MYO15A*. All SMS subjects were enrolled in IRB-approved studies at the Baylor College of Medicine (6 SMS subjects) or at the National Institutes of Health (2 patients). Pure tone audiometry was obtained for congenitally, profoundly deaf individuals in each of the consanguineous families, for the SMS patients, and selected hearing parents and siblings.

Genotyping

Genomic DNA was isolated from peripheral venous blood. Linkage calculations were conducted with the FASTLINK version of LINKAGE (Lathrop and Lalouel 1984; Schaffer 1996). All polymerase chain reactions (PCRs) had a final concentration of 1.5 mM $MgCl_2$, 200 μmM each dNTP, 0.5 U thermo-stable polymerase, and 0.25 μmM forward and reverse primers (Table 1) in a total volume of 10 μl. After initial denaturation at 95°C for 2 min, cycling parameters were 95°C for 45 s to 1 min, 56°C for 45 s to 1 min, 72°C for 45 s to 1 min for 35 or 36 cycles, followed by 72°C for 5 min.

More than 100 consanguineous families segregating recessively inherited hearing loss were genotyped for short tandem repeat (STR) markers linked to reported DFNB loci (http://dnalab-

Table 1 Primer pairs used for PCR amplification of four exons of *MYO15A* for DNA sequencing

MYO15A exons	Primer names	Forward primers (5′ to 3′)	Reverse primers (5′ to 3′)	Amplimer sizes (bp)
3[a]	AW 160/161	TCT GTA ATG AGG AGA CCT GC	ACT GAG GCT CTG AAA ATG TC	350
3[a]	60U20/310L19	TAG CAG GCC CCA GGT ATG AC	GGG AGG TGA GAG GCA ACT G	270
4	AW 162/163	CTT GAA TTC AGG CCT ATC TG	ACA AGA GAA ATC TGT GCG TC	258
31	AW216/217	AGC TAG CTT CAG ATC CTT CTT	CTG ACT CCC ACT GCC CTC	273
44	151U19/346L9	GGC GGA ACT GCA TTT AGG G	GCT CAT CTT TGC GTC CAC A	213

[a]Primers AW160/161 were used for PCR amplifying and sequencing exon 3. Primer pair 60U20/310L19 was used for synthesizing amplimers of exon 3 for restriction endonuclease digestion to detect the 4023C→T allele. Primer pairs for amplifying each of the 66 exons of *MYO15A* exons are available upon request (friedman@nidcd.nih.gov)

www.uia.ac.be/dnalab/hhh/). To identify DFNB3 families, we genotyped families for D17S2206 and D17S2207, which are located in the intronic sequence of *MYO15A* (Liang et al. 1999). In addition, these families were genotyped with at least three of the following markers: D17S953, D17S2196, D17S1794, or D17S805, all of which are closely linked to *DFNB3* (Liang et al. 1998). Amplimers for markers with a fluorescently tagged primer were separated on an ABI 377, and alleles were analyzed by using Genescan (3.1.2) and Genotyper (2.0).

To determine allele frequencies, 96 DNA samples were also obtained from normal hearing individuals from Lahore, 94 DNA samples from individuals from Karachi, Pakistan, 120 DNA samples from individuals from Maharashtra, and 80 Pan-ethnic DNA samples (Human Variation Panel, Coriell Institute for Medical Research, Camden, N.J.).

Nucleotide sequence analysis

A total PCR volume of 25–50 μl was used to generate amplimers for sequencing or restriction endonuclease digestions. After an initial denaturation at 95°C for 2 min, cycling parameters were 95°C for 30 s to 1 min with annealing for 30 s to 1 min at temperatures of 67°C for exon 3, 54°C for exon 4, 64°C for exon 44, and 55°C for exon 31. An annealing temperature of 45°C was used for myosin XVA exon 31 from the Rhesus monkey, Chinese hamster, cow, hyena, and mouse. Extension times were 30 s to 1 min at 72°C for 35 cycles followed by a final extension of 72°C for 5–6 min. Prior to sequencing, PCR amplimers from *MYO15A* exons 3, 4, and 44 were incubated with 0.30 μl each of 1 U/μl shrimp alkaline phosphatase and 10 U/μl exonuclease I at 37°C for 1 h and sequenced by using Applied Biosystems Big Dye Terminator. Amplimers were purified (Qiaquick, Qiagen, Valencia, Calif.) and sequenced (Thermo Sequenase Radiolabeled Terminators; Amersham, Piscataway, N.J.).

Two-color fluorescence in situ hybridization

Lymphoblastoid cells from SMS patient BAB1123 were cultured in RPMI supplemented with 10% fetal calf serum, according to standard cytogenetic laboratory procedures. Although the analysis was performed on interphase nuclei, cultures were exposed to colcemid in order to obtain metaphase chromosomes in addition to interphase nuclei. The metaphase chromosomes were used to test hybridization efficiency and to confirm apparent deletions. This strategy allows for the identification of both duplications and deletions (Shaffer et al. 1997).

Two-color fluorescence in situ hybridization (FISH) was performed as previously described (Shaffer et al. 1997) with the following modifications. Two probe combinations were used; one was a cosmid contig for the *PMP22* locus at 17p12 (cosmids 103B11, 132G8, 77F4; Roa et al. 1996) and the other was a single cosmid (c155D2) for the *MYO15A* locus, which is derived from the commonly deleted 17p11.2 region in SMS. The *PMP22* contig served as a positive control for the efficiency of hybridization. The *PMP22* cosmid contig was labeled with digoxigenin-11-dUTP by nick-translation and detected with anti-digoxigenin conjugated to rhodamine (fluoresces red). Cosmid c155D2 was labeled with biotin-16-dUTP by nick-translation and detected with avidin conjugated to fluorescein isothiocyanate (fluoresces green). The signals were amplified as described (Roa et al. 1996). For each probe, 20–30 ng/μl DNA was used per hybridization reaction. Cells were counter-stained with 4,6-diamidino-2-phenylindole and viewed with a Zeiss Axiophot fluorescent microscope equipped with a triple-band-pass filter that allows one to visualize multiple colors simultaneously. Digital images were captured and stored by using a PSI Powergene 810 probe system and printed on a Tektronix Color/Monochrome Phasar II SDX printer. A total of 25 nuclei were scored on cells from SMS patient BAB1123.

Results and discussion

Three different mutant alleles of *MYO15A* were previously identified in families segregating recessive nonsyndromic profound congenital deafness (Wang et al. 1998). Subsequently, seven additional families with hereditary deafness were ascertained and found to be consistent with linkage to *DFNB3* markers on 17p11.2. Estimated maximum lod scores for these seven families ranged from 2.4 to 4.8. The 66 exons of *MYO15A* of two affected individuals from each of the seven putative *DFNB3* families were sequenced. No mutation was found in the 66 exons of *MYO15A* from a deaf individual in four of these seven putative *DFNB3* families. One of these families in which we did not find a mutant allele of *MYO15A* had five deaf individuals in two consanguineous marriage loops and was linked to this region with a simulated maximum lod score of 4.8.

In each of three *DFNB3* families (PKSN33, PKSR4, PKSK19), a novel mutation of *MYO15A* was found to be homozygous in affected individuals and co-segregated with the hearing loss phenotype (Figs. 1, 2). The three mutant alleles of *MYO15A* were not found in approximately 200 chromosomes from 100 random individuals from Pakistan and India.

Family PKSN33

In profoundly deaf members of family PKSN33, a homozygous 4023C→T transition mutation was found in codon 1229 of exon 3 (accession no. AF144094). The 4023C→T mutation creates a translation stop codon (Q1229X) and also removes a *Pvu*II site (Fig. 2). Exon 3 encodes the region that connects the N-terminal extension of MYO15A to the first seven amino acids of the motor domain (Fig. 1; Liang et al. 1999). Homozygosity for Q1229X has been confirmed in the five available affected children by direct sequencing and *Pvu*II endonuclease digestion of PCR amplicons of exon 3. Both parents and an unaffected sibling are heterozygotes (Fig. 2). This nonsense mutation (Q1229X) in exon 3 is predicted to result in a truncated MYO15A protein and, if exon 3 is included in all mRNA splice variants, MYO15A function would presumably be abolished in Q1229X homozygotes. Although there are many different splicing isoforms of *MYO15A*, an isoform that is missing exon 3 has not been observed (Liang et al. 1999).

Family PKSR4

For family PKSR4, a homozygous splice donor site mutation (IVS4+1G→T) was found at the genomic nucleotide position 33,104 (accession no. AF051976) in intron 4 of the four affected children (Fig. 2; IV-6 and IV-7 were unavailable). This mutation destroys an *Sna*BI restriction site and creates an *Afl*II site. (Fig. 2). The parents are het-

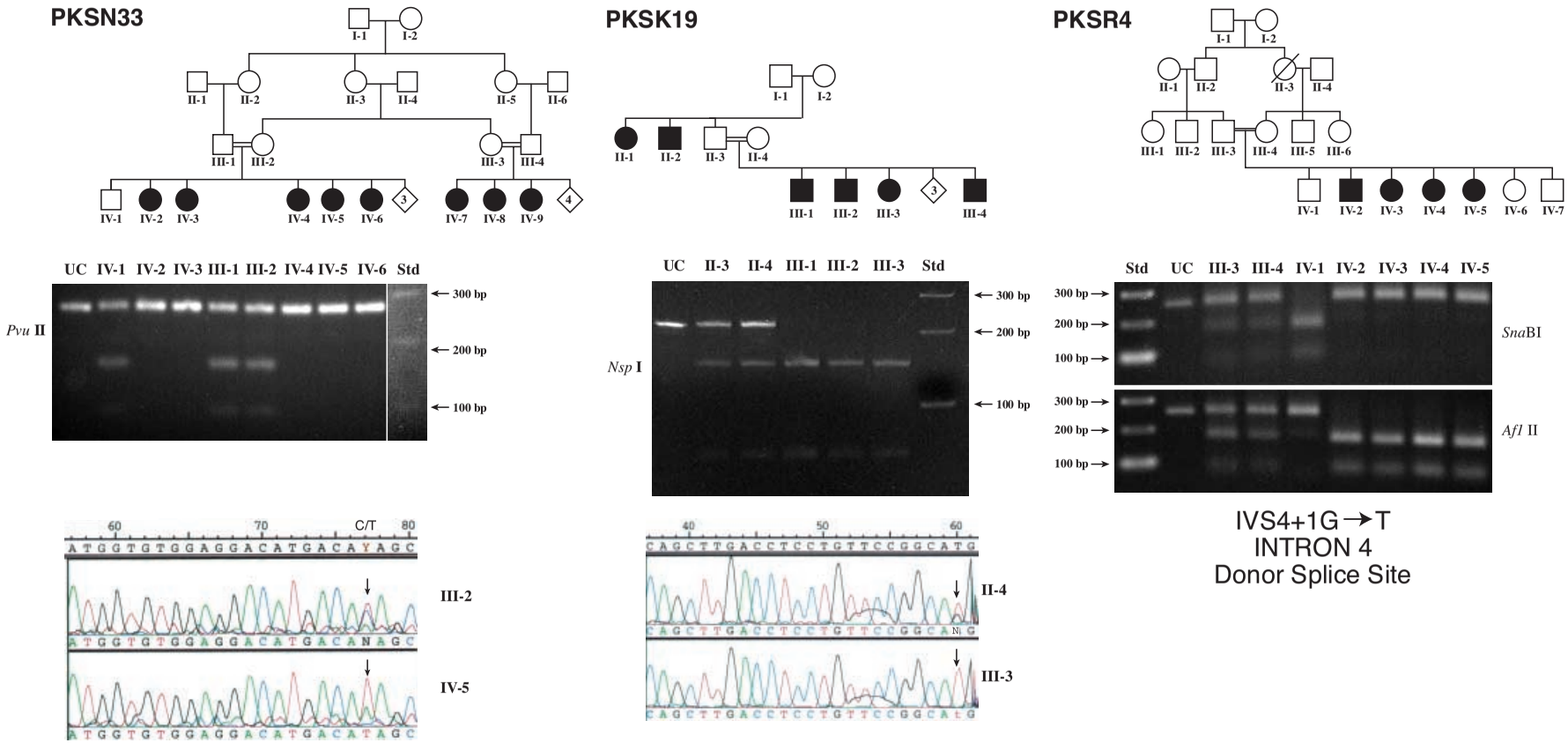

Fig. 2 Three different homozygous mutations segregating in consanguineous Pakistani families were identified. Each mutation was detected by sequencing with primers in the flanking intronic sequence of the exon. The 4023C→T transition mutation, which removes the *Pvu*II site, co-segregated with hearing loss in family PKSN33. Primer pair 60U20 and 310L9 amplifies a 270-bp fragment that includes exon 3. Exon 3 amplimers of affected individuals (*IV-2*, *IV-3*, *IV-4*, *IV-5*, *IV-6*) were uncut by *Pvu*II, whereas the *Pvu*II site in PCR fragments of both parents (*III-1*, *III-2*) and one unaffected child (*IV-1*), who are carriers of 4023C→T, is restricted into 167-bp and 103-bp fragments. Sequencing chromatograms of the unaffected mother (*III-2*) and an affected child (*IV-5*) show that the mother is heterozygous for the 4023C→T mutation, and the child (*IV-5*) is homozygous for 4023C→T. A 8486G→T mutation was identified co-segregating with deafness in family PKSK19. Primer pairs 151U19 and 346L19 for exon 44 amplify a 213-bp product. *Nsp*I restricts the mutant allele in the affected children (*III-1*, *III-2*, *III-3*, *III-4*), who are homozygous for 8486G→T, yielding fragments of 149 bp and 64 bp. The parents (*II-3*, *II-4*) are heterozygous for 8486G→T. Chromatograms of the unaffected mother (*II-4*) and affected child (*III-3*) show that the mother is heterozygous for the 8486G→T, whereas III-3 is homozygous for 8486G→T. A splice site mutation IVS4+1G→T was identified as segregating in family PKSR4. Primers AW162/163 amplify a 258-bp fragment containing exon 4. *Sna*BI cuts the wild-type allele in the parents (*III-3*, *III-4*), who are heterozygous carriers, and in one unaffected child (*IV-1*), yielding fragments of 258 bp, 171 bp, and 87 bp. PCR amplimers from affected children *IV-2*, *IV-3*, *IV-4*, and *IV-5* do not contain an *Sna*BI site. Conversely, *Afl*II cuts only the mutant allele. The parents are carriers, whereas the unaffected child (*IV-1*) is homozygous for the wild-type allele. *UC* Uncut PCR amplimer from a carrier parent, *Std* standard

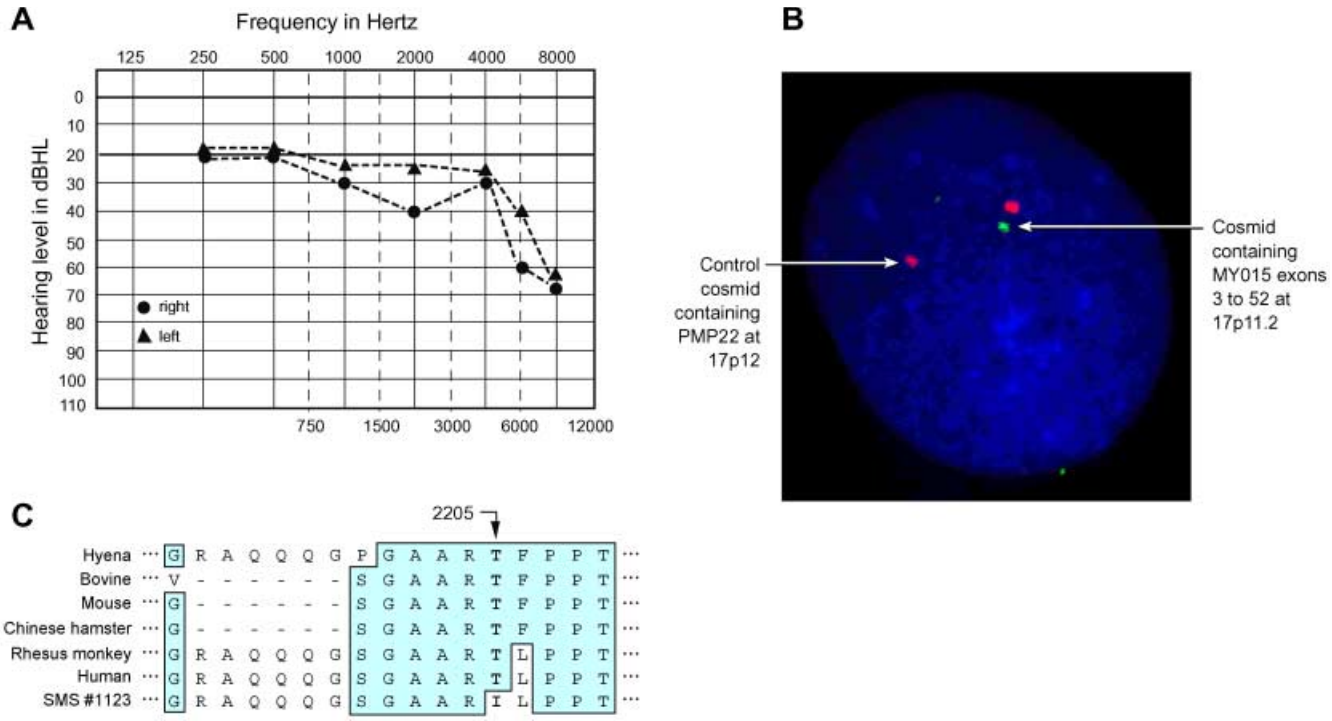

Fig. 3A–C Audiogram, FISH analysis of Smith-Magenis syndrome (SMS) subject 1123, and conservation of threonine 2205. **A** Pure tone audiometry of SMS patient BAB1123 at age 33 years shows a sloping, high frequency, sensorineural hearing loss that is moderate to moderately severe in both ears. Middle ear function was normal (data not shown). **B** FISH analysis of interphase lymphoblast cells from SMS patient BAB1123 shows a nuclei with two *red* signals representing *PMP22*, consistent with the retention of *PMP22* on both chromosomes 17. When cosmid c155D2 containing exons 3–52 of *MYO15A* (Liang et al. 1999) was used as a probe, only one copy (*green* signal) of *MYO15A* was observed. **C** Alignment of amino acid sequences from a portion of exon 31 of *MYO15A* from hyena, cow, mouse, Chinese hamster, Rhesus monkey, and human. The threonine at amino acid residue 2205 (cDNA nucleotide position 6952, accession no. AF144094) is conserved in all six species. A missense mutation (T2205I) in SMS patient BAB1123 is at a conserved threonine residue in a region of the MYO15A tail just beyond the end of the first Myth4 domain (Fig. 1), which is thought to end at residue 2174 (see Table 2 in Liang et al. 1999)

erozygous for IVS4+1G→T, and one unaffected child is homozygous for the wild-type allele (Fig. 2). The consequence of this mutation was not directly examined by sequencing *MYO15A* cDNA from the affected subject, since a source of mRNA was not available. *MYO15A* is rarely and only weakly expressed in lymphoblastoid cell lines (unpublished observation). The in vivo splicing consequences of IVS4+1G→T are unknown, although the phenotype of IVS4+1G→T homozygotes indicates that it is sufficient to disrupt *MYO15A* function and thus cause profound hearing loss.

Family PKSK19

In family PKSK19 from Karachi, Pakistan, the sequence of exon 44 revealed an 8486G→T transversion mutation in codon 2716 (accession no. AF144094) that creates an *Nsp*I restriction site and results in the substitution of histidine for glutamine (Q2716H; Fig. 1; see Table 2 in Liang et al. 1999). This mutation was homozygous in three affected children and heterozygous in both parents (Fig. 2). We did not find 8486G→T in 188 chromosomes from hearing individuals from Karachi, Pakistan.

Exon 44 encodes part of the first FERM domain in the *MYO15A* tail. FERM domains of *MYO15A* are similar to motifs found in the tails of myosins of other classes and in the kinesin-like proteins of plants (Baker and Titus 1997), suggesting that the tails of all these molecular motors form interactions with a similar class of intracellular targets. The FERM domain was originally identified in proteins thought to link the cytoskeleton to specific integral membrane proteins (Chishti et al. 1998). The function of the two FERM domains in the tail of MYO15A is unknown.

We were unable to find mutations of *MYO15A* in its 66 exons or in the donor and acceptor splice sites in four consanguineous families segregating profound congenital deafness linked to the *DFNB3* locus. Sequence analysis has been the nonpareil for mutation detection. Denaturing high-pressure liquid chromatography (DHPLC) is another sensitive method for detecting mutations but was not used in this study. Nevertheless, both direct sequence analysis and DHPLC may be unable to detect all disease-associated mutations of a gene (Takashima et al. 2001; Yatsenko et al. 2001). In these four *DFNB3*-linked families, it is also possible that the hereditary deafness is attributable to a DNA rearrangement involving *MYO15A* or mutations in a non-coding region of *MYO15A*. Alternatively, the mutations associated with hearing loss in these four families may reside in a closely linked gene, such as *KCNJ12* at 17p11.1 (Liang et al. 1998). *KCNJ12* encodes an inward

rectifying potassium channel (Hugnot et al. 1997). Genotype analysis of linked STR markers in one of these four families showed recombination between *KCNJ12* and the deafness phenotype. A mutation screen of *KCNJ12* in the other three „*DFNB3*“ families is warranted, as locus heterogeneity has previously been reported for other deafness loci, such as *DFNA2* and *DFNA3* (Denoyelle et al. 1998; Grifa et al. 1999; Van Hauwe et al. 1999).

SMS and hearing loss

Variation in the degree of hearing loss is present in approximately 68% of SMS patients. Conductive hearing impairment is more common than sensorineural hearing loss (SNHL) in SMS, whereas mixed hearing loss is also a feature of this disorder (Greenberg et al. 1996). As patients with the common SMS deletion and the smaller but less common SMS deletions are hemizygous for *MYO15A*, we have hypothesized that a mutant allele of *MYO15A* on the intact chromosome 17 could be a cause for SNHL in some SMS patients. Eight SMS patients were evaluated for mutations of *MYO15A* by sequencing 66 exons. Seven of the SMS patients had mild SNHL, whereas one patient (BAB1123) had moderately severe high-frequency hearing loss (Fig. 3). This patient, who had the SMS common deletion (Chen et al. 1997) and thus only one copy of *MYO15A* (Fig. 3), was the only one of the eight SMS patients with SNHL whom we found to have a mutation of *MYO15A*. The T2205I mutation (6952C→T; accession no. AF144094) identified in BAB1123 was maternal in origin in that the normal hearing mother was heterozygous for T2205I (data not shown), and therefore, the SMS deletion in this patient appears to have occurred in a gamete from the subject's father.

We recognize that there is only an association of T2205I with the moderately severe hearing loss in SMS patient BAB1123. To explore the possibility that this was a polymorphism, exon 31 was sequenced from 720 chromosomes derived from individuals from a variety of ethnic backgrounds. Threonine was present at this position in all 720 chromosomes. Although there are substitutions at many adjacent amino acids, threonine 2205 of myosin XVA is conserved in the mouse, Chinese hamster, Rhesus monkey, hyena, and cow, suggesting that threonine 2205 is important for *MYO15A* function (Fig. 3C). Exon 31 encodes the first of two MyTH4 domains of the myosin XVA tail (Fig. 1), and threonine 2205 lies just downstream of the first MyTH4 domain. Although the function of the MyTH4 domain has not been determined in *MYO15A*, it is found in the tail regions of many unconventional myosins and kinesin-like motor protein tail regions (Mermall et al. 1998). The functional consequence, if any, of the T2205I allele may be evaluated when the protein partners of the MyTH4 domains of myosin XVA are identified and/or an appropriate functional assay for myosin XVA is developed.

In summary, we have identified three additional novel *MYO15A* mutations that cause congenital profound neurosensory hearing loss and a possible hypomorphic allele of *MYO15A* that is associated with moderately severe hearing loss in one of eight SMS patients. In a group of families segregating profound congenital hearing loss from Pakistan, approximately 10% (11 of 112 DFNB families) are linked to *DFNB3* with suggestive (lod 2.4–2.9) or statistically significant support for linkage (lod=3.0). In a half of these *DFNB3* families, we found a mutation in one of the 66 exons of *MYO15A*. Mutations of myosin XVA are therefore responsible for at least 5% of recessively inherited, profound hearing loss in Pakistan. The extent to which mutations of *MYO15A* contribute to hereditary hearing loss in other populations remains to be determined.

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