

Report

Nonmuscle Myosin Heavy-Chain Gene *MYH14* Is Expressed in Cochlea and Mutated in Patients Affected by Autosomal Dominant Hearing Impairment (DFNA4)

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Myosins have been implicated in various motile processes, including organelle translocation, ion-channel gating, and cytoskeleton reorganization. Different members of the myosin superfamily are responsible for syndromic and nonsyndromic hearing impairment in both humans and mice. *MYH14* encodes one of the heavy chains of the class II nonmuscle myosins, and it is localized within the autosomal dominant hearing impairment (DFNA4) critical region. After demonstrating that *MYH14* is highly expressed in mouse cochlea, we performed a mutational screening in a large series of 300 hearing-impaired patients from Italy, Spain, and Belgium and in a German kindred linked to DFNA4. This study allowed us to identify a nonsense and two missense mutations in large pedigrees, linked to DFNA4, as well as a de novo allele in a sporadic case. Absence of these mutations in healthy individuals was tested in 200 control individuals. These findings clearly demonstrate the role of *MYH14* in causing autosomal dominant hearing loss and further confirm the crucial role of the myosin superfamily in auditive functions.

Hereditary hearing loss is caused, in >50% of cases, by mutations in single genes, including myosins, connexins, transcription factors, potassium channels, and other cellular components that play an important function in ear cells (see Hereditary Hearing Loss Homepage). Different members of the myosin superfamily are responsible for syndromic and nonsyndromic hearing impairment transmitted as an autosomal dominant or recessive trait. Phylogenetic analyses have identified 18 distinct classes of myosins: 17 classes represent unconventional myosins, and class II includes conventional muscle and nonmuscle

myosins (Berg et al. 2001). Among unconventional myosins, mutations in the *MYO7A* gene are responsible for type 1B Usher syndrome (MIM 276903), a syndromic deafness combined with retinitis pigmentosa and vestibular abnormalities. *MYO7A* is also implicated in two nonsyndromic forms of dominant and recessive hearing loss, DFNA11 and DFNB2, as well as in a strain of deaf mice called “shaker-1” (Liu et al. 1997; Weil et al. 1997). The *MYO6* gene is mutated in human DFNA22 and in the *Snell’s waltzer* mouse (Avraham et al. 1995; Melchionda et al. 2001). The *MYO15A* gene is mutated in human DFNB3 and in mouse shaker-2 (Probst et al. 1998; Wang et al. 1998). Most recently, it has been shown that normal hearing in humans requires myosin IIIA (*MYO3A*) and myosin IA (*MYO1A*), which are implicated in two nonsyndromic forms of hearing impairment, DFNB30 and DFNA48, respectively (Walsh et al. 2002; Donaudy et al. 2003).

The conventional myosins of class II consist of 15

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genes, including 6 skeletal-muscle myosin heavy chains, 2 cardiac myosin heavy chains, a smooth-muscle myosin heavy chain, and 3 nonmuscle myosin heavy chains II (NMHC IIA, NMHC IIB, and NMHC IIC) (Berg et al. 2001). The three nonmuscle myosins are encoded by the *MYH9*, *MYH10*, and *MYH14* genes located on chromosomes 22q11.2, 17p13.3, and 19q13.3, respectively (Simons et al. 1991; Leal et al. 2003). Among nonmuscle myosins, *MYH9* was the only gene known to be responsible for a human pathology, including four autosomal dominant diseases reported elsewhere as May Hegglin anomaly and as Sebastian, Fechtner, and Epstein syndromes (Seri et al. 2003). Patients with these diseases have congenital macrothrombocytopenia and aggregates of NMHC IIA in neutrophils. During life, they can develop deafness, nephritis, and cataract. The same gene is also mutated in a family affected by nonsyndromic hearing impairment, DFNA17 (MIM 603622) (Lalwani et al. 2000).

It is interesting that *MYH14* is located within a candidate region in which a nonsyndromic autosomal dominant form of hearing impairment, DFNA4, has been mapped independently in two unrelated families (Chen et al. 1995; Mirghomizadeh et al. 2002). The two candidate regions partly overlap, defining a critical region of ~4 Mb between markers D19S412 and D19S246. Therefore, the *MYH14* gene was considered a strong candidate for hearing loss. First, we ascertained its expression in cochlea, using a specific anti-NMHC IIC antibody. We then performed mutational screening of the gene in a large series of 300 sporadic or familial hearing-impaired patients from Italy, Belgium, and Spain. The identification of one nonsense and three missense mutations clearly indicates a fundamental contribution of *MYH14* to autosomal dominant hearing loss.

To determine whether the *MYH14* gene is expressed in cochlea, we first assayed for its expression in mice. Total RNA from mouse cochlea, from mouse melanoma cell line B16 (Chirivi et al. 1994), and embryonic stem (ES) TVB2 cells, was reverse transcribed to perform RT-PCR. The correct fragment length of 768 bp was amplified from cochlear cDNA (fig. 1).

The NMHC IIC protein was detected in mouse cochlear neonatal sections (postnatal day [P0]) by use of polyclonal antibodies raised against mouse C-terminal NMHC IIC (Golomb et al. 2004). High expression was detected in the organ of Corti and the stria vascularis, as well as in cells of the cochlear duct, such as Hensen and Claudius cells, external sulcus cells, and epithelium of the spiral prominence (fig. 2). It is interesting that low or no expression of NMHC IIC was detected in the Reissner membrane and spiral ligament, in contrast with the *MYH9* expression in the latter structures in the rat (Lalwani et al. 2000). It is also interesting that no expression was detectable in vestibular epithelia (data not

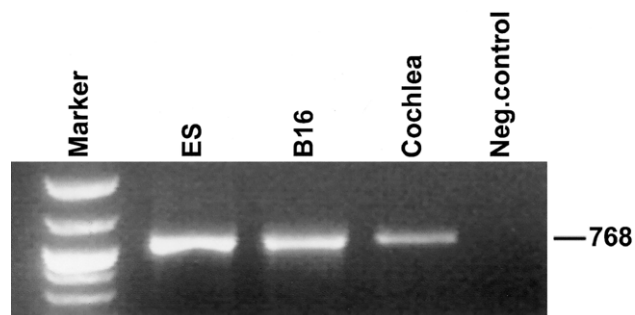


Figure 1 RT-PCR of the *Myb14* gene in mouse cochlea. Specific oligonucleotides C1F (5'-AGCATTGGAAGAGGAGTCCC-3') and C2R (5'-GCTCCAGTCGTTCTACAGC-3'), spanning coding exons 26–30, were used to detect the expression of *Myb14* in mouse cochlea RNA, as well as in two mouse cell lines, B16 (melanoma) and ES cells. Cochlea RNA was kindly provided by K. Avraham (Tel Aviv). In brief, it has been prepared from inner ears dissected out of the temporal bones of mice at embryonic day (E) 14, E16, E18, and P0. Negative control was performed without cDNA aliquot in the reaction. Marker: 1-kb DNA ladder (BioLabs).

shown), in agreement with phenotypic findings in patients (see below).

An overall number of 300 subjects were analyzed for mutations in the *MYH14* gene. Inclusion criteria were: (a) absence of the most common mutations within the *GJB2* gene, (b) sensorineural hearing loss, and (c) normal tympanometric evaluation. All subjects underwent a physical examination, including otoscopy; pure-tone audiometry with air and bone conduction performed at 125, 250, 500, 1,000, 2,000, 4,000, and 8,000 Hz with a Beltone 2000 audiometer; and tympanometry. The series includes subjects with a variable degree of hearing loss, ranging from mild to profound, and with a variable age at onset, ranging from congenital to late onset. Familial records were available, in most cases. The majority of patients came from central and southern Italy (200), whereas 48 and 52 were from Spain and Belgium, respectively. A large German family (Tü-N) that made possible the mapping of the DFNA4 locus to a 2.7-Mb map segment in 19q13.33 was also included in the screening (M.P., personal communication). After receipt of informed consent, peripheral blood was obtained from all subjects, and DNA was isolated from blood leukocytes according to standard methods.

An overall number of 40 primer pairs were designed to amplify the 40 coding exons of the *MYH14* gene, including the splice sites (PCR primer sequences and conditions available on request). All amplicons were screened by DHPLC on a WAVE Nucleic Acid Fragment Analysis System HSM (Transgenomic), according to supplier protocols. DHPLC data analysis was based on a subjective comparison of sample and reference chromatograms. PCR products showing an abnormal chro-

matographic profile were directly sequenced on an automated sequencer (ABI 3100 [Perkin Elmer]).

The mutational screening of the *MYH14* gene allowed us to identify one nonsense and three missense mutations (table 1). The first mutation is a C→A nucleotide change at position 20, which replaces a serine residue at position 7 with a stop codon (S7X) (fig. 3A). This mutation was detected in the German 4-generation kindred Tü-N that mapped to the *DFNA4* locus and defined a 2.7-Mb candidate region containing the *MYH14* gene (M.P., unpublished data). In this pedigree, the affected individuals show a progressive sensorineural hearing impairment that starts in the 1st or 2nd decade of life and leads to severe-to-profound hearing loss in the 4th decade of life.

A G→T nucleotide change was detected at position 1126 of the cDNA, leading to a glycine→cysteine amino acid substitution at position 376 (G376C) in the motor

Table 1

Mutations Identified in the *MYH14* Gene

Mutation ^a	Exon	Effect	
		on Protein	Protein Domain
C20A	1	S7X	Motor domain
G1126T	9	G376C	Motor domain
C2176A	16	R726S	Tail
C2926T	22	L976F	Tail

^a GenBank accession number NM_024729. (The A of the first methionine is referred to as nucleotide +1.)

domain (data not shown). It was detected in a 9-year-old Italian patient affected by a moderate bilateral sensorineural hearing loss without vestibular involvement. There was no family history of hearing loss, and neither

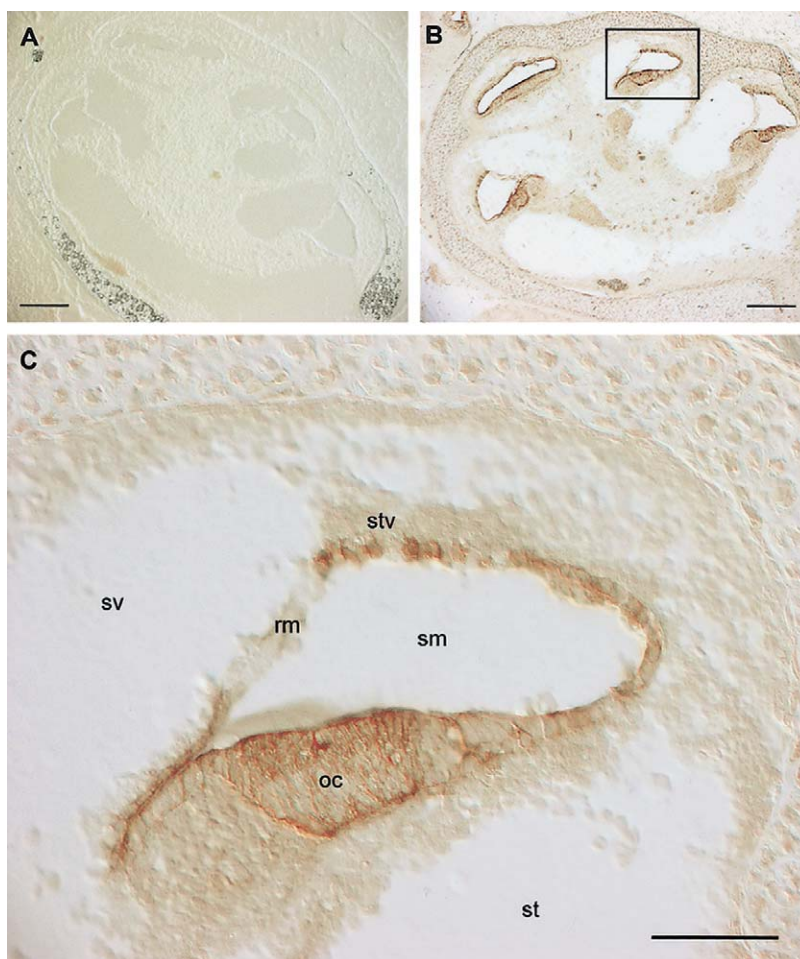


Figure 2 Immunohistochemistry of NMHC IIC in mouse neonatal cochlea. Gelatin tissues at P0 were sectioned (10 μ m) and fixed with 4% paraformaldehyde. A, Negative control obtained using only biotin-conjugated immunoglobulin G secondary antibodies. B, Section immunolabeled with anti-NMHC IIC antibodies (Buxton et al. 2003; Golomb et al. 2004), revealing a high expression in the cells surrounding the scala media. The insert is enlarged in panel C. sv = scala vestibuli; sm = scala media; st = scala tympany; rm = Reissner's membrane; oc = organ of Corti; stv = stria vascularis. No expression in the vestibular epithelia has been detected (data not shown). Scale bars = 20 μ m.

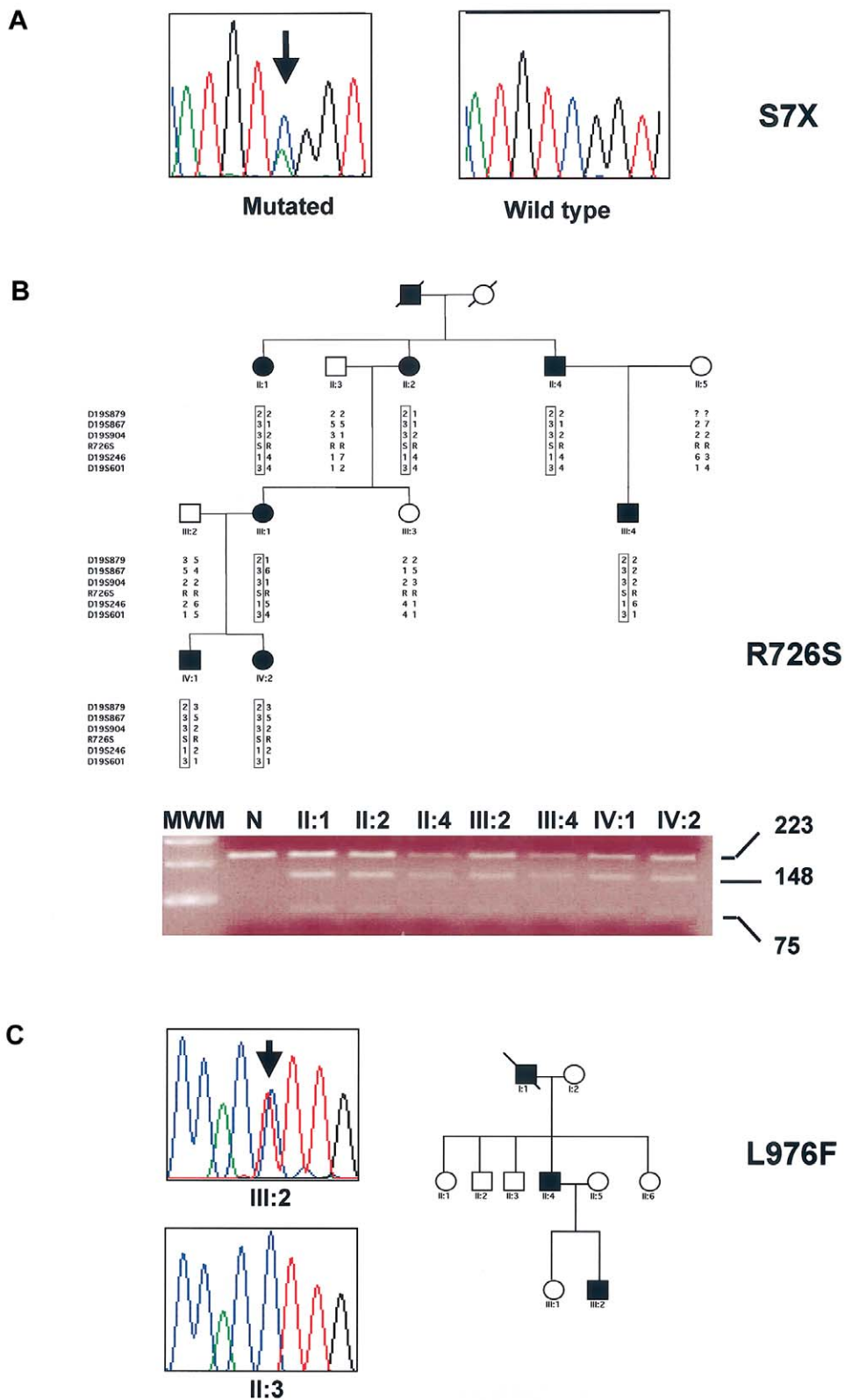


Figure 3 *MYH14* nonsense and missense mutations. **A**, Sequence data of the nonsense S7X mutation. **B**, Pedigree of the Belgian family with the R726S mutation and results showing its presence after *Alu*NI restriction-enzyme digestion. Haplotypes were constructed with markers around the *MYH14* gene. Shared haplotypes are indicated with rectangles. MWM = molecular weight marker; N = normal. **C**, Pedigree of the Italian family with the L976F mutation and results showing sequence data in the proband (III:2) and in a healthy member (II:3).

parent had the G376C mutation. Repeated analysis in the proband confirmed the presence of this mutated allele, which affects a residue highly conserved across species (fig. 4), was absent in control chromosomes and is, therefore, described as a de novo alteration. Paternity has been confirmed using a panel of highly informative microsatellite markers.

Another C→A change affecting nucleotide 2176 was detected in a large Belgian kindred. This change leads to substitution of a highly conserved arginine residue with serine at position 726 (R726S) (fig. 4). The presence of the mutation can be easily detected by PCR and subsequent cleavage with the restriction endonuclease *AlwNI*. We constructed haplotypes for a 3-cM region surrounding the *MYH14* gene, including the mutation and a total of five dinucleotide markers (fig. 3B).

We found that all affected family members shared the same haplotype from markers D19S879 (centromeric) to D19S601 (telomeric), indicating that this family showed linkage with the DFNA4 region and that the mutation cosegregated with hearing impairment. Patients show a mild-to-moderate progressive hearing loss without vestibular involvement.

Finally, the sequence from the affected member (III-2) of a small Italian family (fig. 3C) displayed the heterozygous nucleotide transition C→T at position 2926 in exon 22. This leads to the amino acid substitution L976F affecting the NMHC IIC tail. Although leucine and phenylalanine are both nonpolar residues, this particular position appears to be evolutionarily conserved in the species available in the databases (fig. 4). The patient inherited the mutation from his hearing-impaired father (II-4). Sequence analysis confirmed the absence of this mutation in the healthy members of the pedigree. Affected individuals present with a mild-to-moderate bilateral sensorineural hearing impairment without vestibular symptoms.

The presence of these mutated alleles was tested on 200 normal chromosomes of individuals from the same

Table 2

Nucleotide Substitutions Identified in the Coding Region of the *MYH14* Gene

Nucleotide Variant	Exon	Effect on Protein
T474C	2	Silent
A796G	6	I266V
C1128T	9	Silent
C1731T	13	Silent
C1863T	14	Silent
C2082T	15	Silent
G1506A	21	Silent
A2895G	21	Silent
G4722A	32	Silent
A4676G	32	N1559S
A5307G	36	Silent
G5406T	37	Silent
T5745C	39	Silent
A5889T	40	Silent

NOTE.—The following intronic variants have also been detected: IVS2-44G→A, IVS5+19G→A, IVS7+8G→C, IVS8+13C→T, IVS12-7G→C, IVS21+11T→C, and IVS25+9C→T.

geographical area as that of patients. None of the alleles was detected in any normal chromosome. We also detected two polymorphic variants, I266V and N1559S, 12 synonymous substitutions, and 7 intronic nucleotide substitutions (table 2). Since these latter changes do not create putative cryptic splice sites and/or did not segregate with deafness (data not shown), they have been excluded as causative mutations for deafness in our cohort of patients.

The *MYH14* gene was an excellent candidate for hearing impairment because of its chromosomal location within the DFNA4 critical region and its high expression in the cochlea. One nonsense and three missense heterozygous mutations have been identified in 4 patients be-

	G376	R726	L976
NMHC IIC Hs	<u>SAVLQ<u>F</u>GNIALKR</u> 382	<u>GVLEGI<u>R</u>ICRQGF</u> 732	<u>QELEAH<u>L</u>EAEEGA</u> 982
Mm	<u>SAVLQ<u>F</u>GNIVLKK</u> 373	<u>GVLEGI<u>R</u>ICRQGF</u> 723	<u>QELEAH<u>L</u>EAEEGA</u> 973
NMHC IIA Hs	<u>SGVLQ<u>L</u>GNIVFVK</u> 356	<u>GVLEGI<u>R</u>ICRQGF</u> 708	<u>QELEE<u>Q</u>LEEEESA</u> 958
Rn	<u>SGVLQ<u>L</u>GNIVFVK</u> 356	<u>GVLEGI<u>R</u>ICRQGF</u> 708	<u>QELEE<u>Q</u>LEEEESA</u> 958
NMHC IIB Hs	<u>SSVLQ<u>F</u>GNISFVK</u> 363	<u>GVLEGI<u>R</u>ICRQGF</u> 718	<u>QDLEE<u>Q</u>LDEEEGA</u> 968
Rn	<u>SSVLQ<u>F</u>GNISFVK</u> 363	<u>GVLEGI<u>R</u>ICRQGF</u> 715	<u>QDLEE<u>Q</u>LDEEEGA</u> 965
Mm	<u>SSVLQ<u>F</u>GNISFVK</u> 363	<u>GVLEGI<u>R</u>ICRQGF</u> 715	<u>QDLEE<u>Q</u>LDEEEGA</u> 965
SMM _Y HC Hs	<u>SSVLQ<u>L</u>GNIVFVK</u> 363	<u>GVLEGI<u>R</u>ICRQGF</u> 715	<u>LDLEE<u>Q</u>LEEEESA</u> 965
Rn	<u>SSVLQ<u>L</u>GNIVFVK</u> 358	<u>GVLEGI<u>R</u>ICRQGF</u> 709	<u>LDLEE<u>Q</u>LEEEESA</u> 965
Mm	<u>SSVLQ<u>L</u>GNIVFVK</u> 363	<u>GVLEGI<u>R</u>ICRQGF</u> 715	<u>LDLEE<u>Q</u>LEEEESA</u> 965

Figure 4 ClustalW (European Bioinformatics Institute) alignment of the amino acid sequences of smooth muscle and nonmuscle myosins of class II; sequence alignments for *MYH14* missense mutations. The residues mutated in NMHC IIC and their conservation across species are underlined. Hs = *Homo sapiens*; Rn = *Rattus norvegicus*; Mm = *Mus musculus*.

longing to a cohort of 300 affected individuals from different European countries. Apart from patients carrying the nonsense allele who presented a profound hearing loss from the 4th decade of life, patients with the missense mutations were affected by sensorineural bilateral hearing loss of variable degree, usually ranging from moderate to severe. This finding might indicate a genotype-phenotype correlation, in which a more-severe clinical course is most likely to be associated with reduced levels of NMHC IIC.

It is interesting that one of the three missense mutation is a *de novo* allele altering an evolutionarily conserved residue. This mutated allele was never detected in healthy control individuals, and paternity was confirmed at molecular levels. The patient is the only affected member of his family, as negative records (i.e., audiological and clinical data) have been obtained for all members. These findings strongly support the pathogenic role of G376C. Two other missense mutations were detected in families affected by autosomal dominant hearing loss. Similar to most of the *MYH9* mutations (Seri et al. 2003), the majority of mutations identified in the *MYH14* gene comprises amino acid substitutions, suggesting that they might exert a dominant negative effect. For the three missense mutations found, there is a remarkable conservation of the residues' positions across species, which probably highlights their functional importance in the peptide (fig. 4). However, a functional analysis of all missense mutations will definitively prove their molecular mechanisms on pathogenesis.

Myosins of class II are hexameric enzymes composed of two heavy chains and two pairs of light chains. Dimerization of the heavy chains yields a polar structure. The N-termini form two globular heads with actin- and ATP-binding domains required for motor activity, whereas the α -helical C-termini form a single rodlike tail, which allows the molecules to polymerize into bipolar filaments in both muscle and nonmuscle cells (Sellers 2000). Besides the well-characterized role of myosin in contraction and force production in muscles, little is known about the specific functional role of nonmuscle myosins, although they are likely to participate in motility, cytokinesis, phagocytosis, maintenance of cell shape, and organelle/particle trafficking.

The NMHC IIC myosin has only recently been characterized, and information on its biological role is very limited. The expression pattern defined in human and mouse tissues showed that the gene is ubiquitously expressed at higher levels in adults than during development (Buxton et al. 2003; Leal et al. 2003; Golomb et al. 2004). In the mouse cochlea, with the exception of the Reissner membrane, the protein is localized in all cells of the scala media wall, with a relatively higher level in the organ of Corti and the stria vascularis. It is remarkable that the NMHC IIC distribution is different from

the expression of the *MYH9* (Lalwani et al. 2000), which immunolocalizes specifically in outer cells of the organ of Corti, spiral ligament, and Reissner membrane. Although the role of both nonmuscle myosins in the cochlear cells remains unclear, taken together, the expression data indicate that different physiopathogenetic mechanisms are probably implicated in hearing impairment owing to mutations in the *MYH9* and *MYH14* genes.

Finally, the identification of four different mutations suggests that the *MYH14* gene distinctly contributes to autosomal dominant cases of hearing impairment. As a matter of fact, only a few genes among those so far described present with mutations in more than one single family (see Hereditary Hearing Loss Homepage). Additional population-based and functional studies are required to determine the precise contribution of *MYH14* to deafness.

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Electronic-Database Information

The accession number and URLs for data presented herein are as follows:

European Bioinformatics Institute, <http://www.ebi.ac.uk/clusterw/> (for ClustalW)
 GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for human *MYH14* [accession number NM_024729])
 Hereditary Hearing Loss Homepage, <http://www.uia.ac.be/dnalab/hhh/>
 Online Mendelian Inheritance in Man (OMIM) <http://www.ncbi.nlm.nih.gov/Omim/> (for type 1B Usher syndrome and DFNA17)

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