Espin gene (*ESPN*) mutations associated with autosomal dominant hearing loss cause defects in microvillar elongation or organization

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Key words: Espin, ESPN, microvilli, actin, hearing loss, autosomal dominant deafness
Abstract

Introduction
Espins are actin-bundling proteins present in hair cell stereocilia. A recessive mutation in the espin gene (Espn) has been detected in the jerker mouse and causes deafness, vestibular dysfunction and hair cell degeneration. More recently mutations in the human espin gene (ESPN) have been described in two families affected by autosomal recessive hearing loss and vestibular areflexia.

Methods
An overall number of 450 hearing impaired subjects were analyzed for mutations in the espin gene by DHPLC on a WAVE® Nucleic Acid Fragment Analysis System HSM (Transgenomic). To determine whether the mutated ESPN alleles affected the biological activity of the corresponding espin proteins in vivo, we investigated their ability to target and elongate the parallel actin bundles of brush border microvilli in transfected LLC-PK1-CL4 epithelial cells.

Results
Here, we report the identification of 4 additional ESPN mutations (S719R, D744N, R774Q, delK848) in patients affected by autosomal dominant hearing loss without vestibular involvement. For three mutated alleles clear abnormalities in microvillar length or distribution were obtained after investigating their ability to target and elongate the parallel actin bundles of brush border microvilli in transfected LLC-PK1-CL4 epithelial cells.

Discussion
These results further strengthen the causative role of the espin gene in nonsyndromic hearing loss and add new insights into espin structure and function.
Espins represent a family of multifunctional actin cytoskeletal regulatory proteins with the potential to differentially influence the organization, dimensions, dynamics, and signaling capabilities of the actin filament-rich, microvillus-type specializations that mediate sensory transduction in various mechanosensory and chemosensory cells (1). They are associated with the parallel actin bundles of hair cell stereocilia and are the target of mutations that cause deafness and vestibular dysfunction in mice (jerker) and humans (DFNB36) (2,3). One striking illustration of espin activity in vivo is the ability to cause a dramatic, concentration-dependent lengthening of brush border microvilli and their parallel actin bundles in transfected LLC-PK1-CL4 epithelial cells (4). The espin COOH-terminal peptide, which contains the 116-amino acid actin-bundling module (5), is necessary and sufficient for this microvillar lengthening activity (4). This ability to increase microvillar length in transfected epithelial cells has led to the hypothesis that espins play an essential role in determining the length of hair cell stereocilia (4). In support of this hypothesis, espin protein level is positively correlated with stereocilium length (4), and abnormally short hair cell stereocilia are observed in homozygous jerker mice, which lack espin protein (2). A human deafness recessive locus DFNB36 has been recently mapped to chromosome 1p36.3 in two consanguineous families affected by deafness and vestibular areflexia (3). The phenotype segregates with mutations in ESPN, which encodes espin. These findings establish espin as an essential protein for hearing and vestibular function in humans. Here we describe the identification of new functionally abnormal ESPN alleles associated with dominant forms of hearing loss without vestibular involvement.

**Methods**
An overall number of 450 subjects were analyzed for mutations in the espin gene. Inclusion criteria were: a) absence of the most common mutations within the GJB2 gene, b) sensorineural hearing loss, and c) normal tympanometric evaluation (6). In all cases vestibular data were obtained by clinical examination and routine vestibular tests (one or more of the following tests: caloric, rotatory, optokinetic, swinging torsion, statokinesimetric and vestibulo-vegetative). The series includes cases with a variable degree of hearing loss, ranging from mild to profound and with a variable age of onset, from congenital to late onset. Familiar records were available in most cases. The majority of patients came from Central and Southern Italy (200), while 140 were from Spain, 54 from Belgium, and 50 from Israel. After informed consent, peripheral blood was obtained from all subjects, and DNA was isolated from blood leukocytes according to standard methods.

Thirteen different primer pairs were designed in order to amplify the 13 coding exons of the ESPN gene, including the splice sites (PCR primer sequences and conditions available upon 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 chromatographic profile were directly sequenced on an automated sequencer (ABI 3100; Perkin Elmer).
Human espin 3A cDNA was obtained by reverse transcription PCR from human testis RNA (BD Biosciences, Clontech). Mutations were introduced by PCR. All cDNAs were checked by DNA sequence analysis before introduction into the SmaI site of the pEGFP-C2 vector for transfection studies in differentiated LLC-PK1-CL4 epithelial cells (4). Briefly, cells were cultured at 37°C in minimum essential medium alpha medium (with L-glutamine, without nucleosides) supplemented with 10% fetal bovine serum (Invitrogen) and 100 U/ml penicillin and streptomycin. Cells cultured on glass coverslips for 8-10 d (~75% confluency) were transfected for 4 hours with a fixed amount of plasmid DNA using Lipofectamine (Invitrogen) and examined 20-24 hours later. Cells were fixed with 2% paraformaldehyde, treated briefly with 0.1% Triton X-100, labeled with Texas Red–phalloidin (Molecular Probes) to detect F-actin, and mounted in 5% n-propylgallate, 90% glycerol. Transfected cells were identified by GFP fluorescence. 0.5-µm confocal z-sections were collected at room temperature using a confocal microscope (model LSM 510 META; Carl Zeiss Microimaging, Inc.) and a 100X, 1.4 NA oil immersion objective. LSM510 imaging software was used to generate stacks, from which measurements of microvillus length were made. Images were saved in TIF format, transferred to Photoshop (Adobe Systems), assembled into composites and converted to CMYK color format with minor adjustments of brightness and contrast. Antibody to purified recombinant rat espin 2B, previously called rat Purkinje cell espin 1 (GenBank accession number AF540946) was prepared in rabbits and affinity purified on columns of rat espin 2B-Sepharose 4B. This antibody has been used extensively to detect espin isoforms on western blots and by immunocytochemistry (1).

Western blotting was performed using the ECL method (Amersham Biosciences) on extracts prepared from replicate dishes by boiling (or heating at 100 C) for 3 min in SDS gel sample buffer (4).

Results
Mutational screening of the ESPN gene by denaturing high performance liquid chromatography (DHPLC) and sequencing of 450 subjects from different countries allowed us to identify 1 deletion and 3 missense mutations that were associated with autosomal dominant hearing loss (Table 1). The first such mutation to be identified was an A to C nucleotide change at position 2155. This replaces a conserved serine residue at position 719 with an arginine (S719R) (Fig. 1). [The numbering schemes used are those of prototypical espin (GenBank accession number NM_031475), which is referred to as espin 1 in the revised nomenclature for espin isoforms (1)]. This mutation was detected in a small Italian kindred, in which two affected individuals, in two generations, show a dominant form of progressive sensorineural hearing impairment that starts in the second decade and leads to a mild to moderate hearing loss in the fourth decade (high frequencies mainly involved). No vestibular involvement was detected using standard
vestibular tests. Normal members of the family (two additional subjects) were completely negative for the presence of the mutation.

A G->A nucleotide change was detected at position 2230, leading to an amino acid substitution from aspartic acid to asparagine at position 744 (D744N). This aspartic acid residue is highly conserved across species (Fig. 1). The mutation was detected in an Italian patient affected by severe bilateral sensorineural hearing loss involving all frequencies without vestibular involvement. Although there was a family history of hearing loss, no additional genetic and instrumental tests could be performed in other family members.

A G->A nucleotide change affecting position 2321 was detected in a sporadic Italian case. This change leads to substitution of a highly conserved arginine residue with glutamine at position 774 (R774Q) (Fig. 1). The patient is affected by late onset (age 42) mild bilateral sensorineural hearing loss involving all frequencies but mainly the high ones.

Finally, the sequence from a Spanish sporadic case displayed a 3-nucleotide deletion (2541-2543delAAG), leading to the loss of a lysine residue at position 848 in the COOH-terminal peptide (delK848). This patient (now age 4) exhibits a severe bilateral sensorineural hearing impairment without vestibular involvement. Independent analysis of the proband sample confirmed the presence of this mutated allele, which affects a residue highly conserved across species (Fig. 1) and must be catalogued as a de novo alteration.

Two hundred chromosomes of individuals from the same geographical areas as the patients were examined for the presence of the above-described mutant alleles. None of the changes was detected in the control samples. We also detected six synonymous substitutions (Table 1) in the cohort of hearing subjects. 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.

To determine whether the espin gene mutations associated with autosomal dominant sensorineural hearing loss had a discernible effect on espin function in vivo, we compared the effects of the wild-type (wt) and mutated espin proteins on the microvilli of transiently transfected LLC-PK1-CL4 epithelial cells (4). When maintained as confluent islands for 8-10 d in culture, LLC-PK1-CL4 epithelial cells invariably express uniform brush border microvilli 1–1.5 µm in length (4,7,8). When expressed exogenously in these cells by transient transfection, wt espins are efficiently targeted to the microvilli and bring about a dramatic elongation of the microvilli and the parallel-actin-bundle scaffold at their core (4). This activity, which is believed to stem directly from espin cross-links in the core actin bundle, could explain the positive correlation noted between espin protein level and stereocilium length and the shortening of hair cell stereocilia observed in the espin-deficient homozygous jerk mouse (2,4).

After 8-10 d in culture, LLC-PK1-CL4 cells were transfected with plasmids encoding green fluorescent protein (GFP)-tagged wt or mutated human espin 3A under the control of the cytomegalovirus promoter. Espin 3A is the major espin isoform found in cochlear hair cells (1). Twenty to twenty-
four hours after transfection, the cells were fixed, labeled with Texas Red-phalloidin to localize F-actin and imaged by confocal microscopy. The wt GFP-human espin 3A was found to be highly concentrated in the microvilli and caused an ~5-fold elongation of the microvilli from $1.33 \pm 0.04 \mu m$, the length in untransfected control cells, to $6.28 \pm 0.09 \mu m$ (mean $\pm$ SE, $n = 167-189$ microvilli, 14-17 cells) (Fig. 2A-C). The long microvilli of the transfected cells appear yellow because the GFP-espin is colocalized with the microvillar F-actin bundles, which are brightly labeled with the Texas Red-phalloidin. The construct bearing the R774Q mutation gave results (length, $6.2 \pm 0.1 \mu m$, mean $\pm$ SE, $n = 153$ microvilli, 14 cells) that were not significantly different from those obtained with the wt (Fig. 2J-L). In contrast, constructs bearing the S719R mutation or the D744N mutation made long microvilli, as long as or longer than wt, but they frequently appeared in clumps that occupied only a relatively small percentage of the apical cell surface (Fig. 2D-I). A simple quantification of the behavior of these latter two mutations revealed an 8- to 10-fold increase in the number of cells showing such a disorganized microvillar phenotype relative to wt: while only 2-3% of cells transfected with wt human espin 3A showed disorganized long microvilli, 25-30% of cells transfected with the D744N or S719R construct showed the defect ($n = 540-710$ transfected cells for each construct in three independent experiments). The remainder of the cells transfected with the S719R or D744N construct (70-75%) looked similar to those transfected with wt. Finally, the construct bearing the delK848 mutation was severely impaired in microvillar elongation (Fig. 2M and N). The delK848 construct caused only a 1.5-fold elongation, from $1.33 \pm 0.04 \mu m$ to $2.02 \pm 0.04 \mu m$ ($n = 136$ microvilli, 13 cells), compared to the nearly 5-fold elongation observed with wt. In fact, compared to the other constructs, the delK848 construct appeared to be less efficiently targeted to microvilli and commonly showed higher levels of accumulation in the nucleus. This trend, which was often noticeable as a green haze beneath the microvilli (Fig. 2N), was seen to better advantage in z-sections positioned below the microvilli (Fig. 2O). Western blots of the transfected cells labeled with affinity purified espin antibody showed a single major band at the expected molecular mass ($\sim 64$ kDa = $\sim 29$ kDa for GFP and linker + $\sim 35$ kDa for human espin 3A) for each construct and indicated that there were no major quantitative differences in the levels of the wild-type and mutated proteins in the transfected cells that could account for their differing effects on the microvilli (data not shown).

**Discussion**

Espins are associated with the parallel actin bundles of hair cell stereocilia and are the target of mutations that cause deafness and vestibular dysfunction in mice and nonsyndromic recessive hearing loss and vestibular areflexia in humans (2,3). Here, we report that mutations in ESPN also cause dominant forms of hearing loss without vestibular signs. Moreover, using the LLC-PK1-CL4 transfection model, we detect *in vivo* functional deficits for three of the new mutations that could affect the dimensions or organization of hair cell stereocilia.
All of the newly described mutations affect residues conserved across species and were never found in normal chromosomes. Three out of four of the mutations, D744N, R774Q and delK848, map to the distal COOH-terminal peptide that includes the actin-bundling module (5). This peptide, which is shared among all known espin isoforms (1), is necessary and sufficient for potent actin-bundling activity in vitro (5) and for microvillar elongation activity in vivo (4).

The delK848 mutation severely impairs microvillar elongation and causes abnormally high levels of espin protein to accumulate in the nucleus. This mutation is located in the COOH-terminal part of the actin-bundling module, in a region believed to encompass one of its two F-actin-binding sites (5). In fact, 13- and 19-amino acid COOH-terminal part of the protein that include the homologous lysine residue in rat espins are known to be required for actin-bundling (5) and microvillar elongation activity (4), respectively. Thus, our functional data on this mutated allele are in agreement with the results of previous studies. Moreover, the severity of the functional deficit noted with the delK848 mutation in the LLC-PK1-CL4 transfection model is strongly correlated with the severe phenotype of the corresponding patient, who experienced a bilateral severe sensorineural hearing loss with a very early onset in childhood. The basis for the increased nuclear accumulation of the delK848 construct is not clear, but this result raises the intriguing possibility that the dominant effect of this mutation is exerted in the nucleus.

Two of the mutated alleles, D744N and S719R, cause a novel defect in microvillar organization in the LLC-PK1-CL4 transfection model. The D744N mutation maps to the actin-bundling module, whereas the S719R mutation maps to an upstream location, which is currently without a known function. Although both of these mutated constructs appear capable of elongating microvilli like wt espin, they also cause a marked increase in the frequency of transfected cells showing long microvilli confined to small patches of apical surface. This peculiar behavior is noted only rarely in cells transfected with wt espins and has not been detected previously for espin constructs bearing a wide variety of different truncation or deletion mutations (4). This defect in microvillar organization could reflect aberrant espin targeting or a dominant-negative effect of the mutated espin on the other organizing elements at the apical end of the epithelial cell. Patients carrying these alleles present with a moderate to severe bilateral hearing loss.

Finally, the R774Q mutation causes no obvious problems in the LLC-PK1-CL4 transfection model. This mutation was detected in a sporadic case with a mild form of hearing loss and a late onset (over age 40). Some possible conclusions can be drawn from this case considering both functional and clinical data: a) the mutation is causative but its effect is too small to be detected in our functional assay, b) the mutated allele has a small contribution per se on the phenotype but interacts as a modifier with the genetic background contributing to the phenotype itself, c) the mutation, even if affecting an highly conserved residue and never being present in normal controls, is truly a neutral variant and not a causative allele.
In conclusion, the data presented here demonstrate an association between ESPN mutations and dominant forms of hearing loss. The detrimental effects of these mutations noted in the LLC-PK1-CL4 transfection model provide new insight into espin structure and function and suggest that the patients carrying these mutated alleles have defects in the elongation or organization of their hair cell stereocilia.

Acknowledgements
The work was supported by grants from: Telethon Foundation and CNR (to PG); the National Institutes of Health (R01 DC004314 to JRB); DURSI and the “Redes Tematica se Investigacion Cooperativa of the Fondo de Investigaciones Sanitarias” (G03/203) (to XE). E.B. is supported by a fellowship from the Generalitat de Catalunya (Departament d’Universitats, Recerca i Societat de la Informació, DURSI), 2003-FI066). We thank Dr. Patricia A. Loomis for her help on confocal microscopy, and Dr. Karen Avraham and Dr. Guy Van Camp for providing DNA samples and manuscript revision.

Figure Legends
Figure 1. Amino acid sequence alignments for the peptides that contain the newly identified ESPN mutations. Alignments were performed using ClustalX and WU-Blast 2. The mutated residues are shown in red. Note their conservation across species.

Figure 2. Functional comparisons of wt and mutated human espin 3A using the LLC-PK1-CL4 epithelial cell transfection model. Differentiated LLC-PK1-CL4 epithelial cells were transfected with GFP-wt human espin 3A or the designated mutated construct, labeled for F-actin with Texas Red-phalloidin and examined by confocal microscopy. Multiple examples are shown. A–C, wt. D–F, S719R. G–I, D744N. J–L, R774Q. M–O, delK848. All images are of the apical (microvillar) surface of the monolayer, except O, which is a z-section through the middle of the cells in N highlighting the nuclear accumulation of the delK848 construct. The F-actin-rich junctional complexes adjoining the apical-lateral boundaries of neighboring cells are often evident as red lines. Note that the wt construct (A–C) is colocalized (yellow) in microvilli that are much longer than the brush border microvilli (red) of surrounding control (untransfected) cells. The R774Q (J–L) is indistinguishable from wt. The S719R (D–F) and D744N (G–I) constructs are targeted to microvilli and cause microvillar elongation, but the long microvilli frequently appear in irregular patches that occupy only a small percentage of the apical surface. The delK848 construct (M–O) is severely impaired in microvillar elongation and shows abnormally high accumulation in the nucleus (O). The objects within the nucleus that exclude the GFP-delK848 construct are nucleoli. Bar, 5 µm.
References


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Table 1. Mutations and polymorphisms identified in *ESPN* (GenBank accession number NM_031475)

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