Mutations of *ESPN* cause autosomal recessive deafness and vestibular dysfunction

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We mapped a human deafness locus DFNB36 to chromosome 1p36.3 in two consanguineous families segregating recessively inherited deafness and vestibular areflexia. This phenotype co-segregates with either of two frameshift mutations, 1988delAGAG and 2469delGTCA, in *ESPN*, which encodes a calcium-insensitive actin-bundling protein called espin. A recessive mutation of *ESPN* is known to cause hearing loss and vestibular dysfunction in the jerker mouse. *Espn* is predicted to encode an 854 amino acid protein known to cause deafness and vestibular dysfunction in the *jerker* mouse. We screened the coding sequence of *ESPN* (GenBank accession number AL136880) by sequencing PCR amplified *ESPN* exons from genomic DNA of affected individuals in the two families. However, the sequencing analysis of *ESPN* was complicated due to the presence of a second *ESPN*-like sequence on chromosome 1p36.13, annotated as LOC284729 (GenBank accession number AL035288). As we identified frequent frameshift mutations in LOC284729 disrupting its open reading frame (data not shown) and due to the absence of sequence encoding homologous residues that are important for actin-bundling activity of espin, LOC284729 appears to be an unprocessed pseudogene. Based upon our analyses of LOC284729, this putative pseudogene has been assigned the symbol *ESPNP*. Except for the absence of sequences comparable to exon 1 and exon 13, *ESPNP* has all of the other exons and introns of *ESPN* to which it shows 95% nucleotide sequence identity. *ESPNP* is 10 Mb away from *ESPN* in reverse orientation with respect to *ESPN* and is outside the DFNB36 linkage interval defined by families PKSN32 (fig 1) and PKSR5A (data not shown).

Intonic primers for amplification of exons 1 and 13 were unique for *ESPN* since *ESPNP* lacks homologous sequences, and intronic primers for amplification of exons 2, 6, 7, 8, 9, and 10 were specific for *ESPN* due to multiple mismatches with *ESPNP* sequence (table 1). However, intronic primers for amplification of other exons were either identical to *ESPN* and *ESPNP* (exons 11 and 12) or failed to discriminate between the mismatches of the two sequences (exons 3, 4, and 5). Therefore, we subcloned the PCR products from exons 3, 4, 5, 11, and 12 and sequenced individual clones. The nucleotide mismatches allowed us to unambiguously assign individual clones to either *ESPN* or *ESPNP*.

We detected two different mutations of *ESPN* segregating with the deafness phenotype in families PKSR5A and PKSN32. In family PKSR5A, affected individuals were homozygous for a 4 bp deletion, 1988delAGAG, in exon 9 (fig 2A), while in family PKSN32 all affected individuals were homozygous for a 4 bp deletion, 2469delGTCA, in exon 13 (fig 2B). The obligate carriers in both families were heterozygous and neither mutation was detected among 150 normal-hearing individuals from Pakistan. The1988delAGAG mutation in exon 9 of *ESPN* was not present in *ESPNP* in deaf individuals of family PKSR5A. *ESPNP* lacks the site of 2469delGTCA mutation in family PKSN32 (fig 2C).

*ESPN* is predicted to encode an 854 amino acid protein (fig 3) referred to as espin, a name derived from ectoplasmic specialisations (+ in) on the basis of its discovery and localisation in parallel F-actin bundles of ectoplasmic

Abbreviations: ABM, actin-bundling module; ENG, electroneystagmography; ERG, electroretinography; IRB, Institutional Review Board; RP, retinitis pigmentosa

**SHORT REPORT**

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Abbreviations: ABM, actin-bundling module; ENG, electroneystagmography; ERG, electroretinography; IRB, Institutional Review Board; RP, retinitis pigmentosa
specialisation in testes. Domain prediction algorithms of human espin sequence revealed eight ankyrin-like repeats at the N-terminus, two proline rich regions, a consensus site for ATP or GTP binding (P-loop), which is contained within an actin-binding WH2 motif (amino acids 651–668), and a coiled coil (amino acids 756–831) (figs 2D and 3). There are 66 residues (amino acids 739–804) that show 33% sequence identity to a domain in forked proteins, which are essential for formation of actin bundles in bristles of Drosophila melanogaster. Deletion mutagenesis experiments have suggested that espin contains three actin-binding sites (figs 2D and 3). Two of the actin-binding sites at the C-terminus constitute the ABM (figs 2D and 3) and are important for espin activity. Espn expression constructs lacking either one or both of these actin-binding sites are unable to cross-link actin filaments when transfected into BHK fibroblasts.

The two mutations of ESPN, segregating with hearing loss linked to DFNB36, cause frameshifts in the ESPN translation reading frame. If translated, 1988delAGAG is predicted to

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**Table 1** Primers for amplification of ESPN exons* and flanking intronic sequences

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
<th>Size (bp)</th>
<th>Exon(s) amplified</th>
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<tbody>
<tr>
<td>1F</td>
<td>AATGCGACCAATGTTTACGTG</td>
<td>1010</td>
<td>1</td>
</tr>
<tr>
<td>1R</td>
<td>CCACCACCTGCAAGACTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2F</td>
<td>AGGAAAGGCCCAGGAGACATC</td>
<td>859</td>
<td>2</td>
</tr>
<tr>
<td>2R</td>
<td>ATGCTGAGGGGAGGCCCTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3F</td>
<td>GAGGTCAGACACACAGGGGTG</td>
<td>1954</td>
<td>3–5</td>
</tr>
<tr>
<td>5R</td>
<td>AGCCTGAGGTCCACTTAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6F</td>
<td>GGTCCTGAGGGGCCCTTTGAC</td>
<td>552</td>
<td>6</td>
</tr>
<tr>
<td>6R</td>
<td>CTTCTCCCACTGTTTAAAGCTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7F</td>
<td>TACGATCGTCTGGACATCT</td>
<td>6909</td>
<td>7 and 8</td>
</tr>
<tr>
<td>7R</td>
<td>GCTGCCAGAGCGCTCCCTAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9F</td>
<td>CCATCAGACTGCTGTAAGGA</td>
<td>1954</td>
<td>3–5</td>
</tr>
<tr>
<td>9R</td>
<td>ACTGCTGAGGCTGACGCTGA</td>
<td>377</td>
<td>9</td>
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<tr>
<td>10F</td>
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<tr>
<td>10R</td>
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<td>AGCTGAGGCGGAGGCTTACGAC</td>
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<tr>
<td>12R</td>
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<tr>
<td>13F</td>
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<tr>
<td>13R</td>
<td>AATGCGACCAATGTTTACGTG</td>
<td>439</td>
<td>13</td>
</tr>
</tbody>
</table>

*Sequence corresponds to AL136880. Nucleotides in each primer mismatched to ESPNP sequence are underlined. Amplification conditions and sequences of additional primers for mutational analysis of exons 2, 3, 4, 5, 7, and 8 are available as supplemental material (http://jmg.bmjournals.com/supplemental/).
introduce one substituted amino acid after residue 662, followed by a stop codon at nucleotide 1990 and 2469delGTCA is predicted to introduce 27 substituted amino acids after residue 821, followed by a stop codon at nucleotide 2533 resulting in a truncated 844 amino acid protein with 821 correct and 23 substituted residues. These mutations are presumed to result in loss of function of espin. The resulting mutant proteins are predicted to have either no actin-bundling module, (1988delAGAG), or lack one of the C-terminal actin-binding sites (2469delGTCA), which is necessary for espin activity. There is evidence for multiple isoforms of Espn in the mouse and the same may be true in humans as well. However, the mutations associated with DFNB36 occur in Espn exons, which are known to be present in all reported isoforms of Espn in mice.

PCR analysis of human fetal inner ear cDNA revealed expression of Espn in the inner ear (data not shown). In both the cochlea and the vestibule of the mouse inner ear, espin is localised mostly to the stereocilia of hair cells. Stereocilia are specialised microvilli projecting from the apical surfaces of inner ear hair cells and are vital for transduction of sound and for detection of linear and angular acceleration. They contain a densely packed core of parallel bundles of actin filaments. The formation and ordered arrangement of these filaments into bundles requires different actin-binding proteins, which cross-link actin filaments. Multiple actin-binding proteins such as fimbrin, plastin, and espin, are expressed in the inner ear. Espin plays a crucial role in cross-linking parallel actin bundles and varying concentrations of espin may determine the extent of elongation and, consequently, the length of parallel actin bundles. Espin is absent from the stereocilia of jerker mice, and consequently by postnatal day 10 the stereocilia are shortened and have reduced stiffness. Within 3 months of birth, a degenerative process leads to the complete loss of all sensory hair cells in the jerker mouse. In addition to espin,
Figure 3  Espin sequence alignment. ClustalW multiple protein alignment of rat, mouse, and human ESPN. Dark shaded residues denote identical amino acids. Light grey shading represents conserved amino acid substitutions and ‘’-’’ indicates a gap in the alignment. Bars on top of amino acid residues at the N-terminus indicate the location of ankyrin repeats. Two proline rich regions are boxed. A dashed line indicates actin-binding amino acids (xAB). Asterisks mark the amino acids corresponding to the P-loop. Amino acids forming the WH2 domain are indicated by a bracket. Amino acids constituting the actin-bundling module, ABM, are underlined. The first amino acid affected by 1988delAGAG or 2469delGTCA is indicated by ‘’D’’. The amino acids shared by mouse and human espin proteins exhibit 83% identity and 88% similarity. Human and rat ESPN are 86% identical and 90% similar.

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there are many other cytoskeletal proteins that are necessary for the development and maintenance of stereocilia. Mutations of ACTG1 encoding γ-actin were recently reported to cause progressive hearing loss in humans. Both β- and γ-actin are present in the stereocilia of auditory hair cells in chicken, raising the possibility that espin interacts with either β-actin, γ-actin, or both in the stereocilia. Moreover, espin has multiple sites for protein–protein interactions, which may serve as a scaffold for assembly of macromolecular complexes important for structure and function of the stereocilia.

Our findings indicate that espin is essential for both hearing and balance in humans. The association of profound deafness and vestibular dysfunction in the absence of other associated phenotypes is unusual, although vestibular dysfunction is often not carefully evaluated or documented for most non-syndromic recessive deafness loci. Vestibular dysfunction has been excluded for 11 DFNB loci: B1, B6, B7/11, B12, B17, B18, B21, B23, B26, B29, and B30 (see Homepage Hereditary Hearing Loss Homepage for individual references: http://dnalab-www.uia.ac.be/dnalab/hhh/). Affected individuals with mutations of MYO7A linked to DFNB2 have a vestibular phenotype27 comparable to that associated with DFNB36 and affected individuals with mutations of MYO15A also exhibit signs and symptoms of vestibular dysfunction.28 Vestibular dysfunction was also reported in a few deaf individuals who have recessive mutations of MYO6.14 The abnormal vestibular phenotype associated with ESPN mutations is unusual and will be a useful clinical marker for refining the differential diagnosis of non-syndromic deafness.

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Authors’ affiliations

S Naz, S Riazuddin, E R Wilcox, T B Friedman, Section on Human Genetics, LMG, NIDCD, NIH, Rockville, MD 20850, USA
A J Griffith, Section on Gene Structure and Function, LMG, NIDCD, NIH, Rockville, MD, USA
A J Griffith, Hearing Section, NIDCD, NIH, Rockville, MD, USA
L L Hampton, J F Battery Jr, Section on G-protein Coupled Receptors, NINDS, NIH, Bethesda, MD, USA
S N Khan, S Riazuddin, National Center of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan

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Conflict of interest: none declared.

GenBank accession numbers: mouse Espn AF239886, rat Espn NM_019622.1, human ESPN AL136880, ESPNP (LOC284729) AL035288; ESPN genomic sequence AL031848 and AL158217; genomic sequence for ESPNP AL021920; EST for ESPNP CB987978.

Correspondence to: Dr T B Friedman, Laboratory of Molecular Genetics, NIDCD, NIH, Rockville, MD 20850, USA; friedman@niddc.nih.gov

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