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ORIGINAL ARTICLE

USH3A transcripts encode clarin-1, a four transmembrane-domain protein with a possible role in sensory synapses

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Abstract

Usher syndrome type 3 (USH3) is an autosomal recessive disorder characterised by the association of post-lingual progressive hearing loss, progressive visual loss due to retinitis pigmentosa and variable presence of vestibular dysfunction. Because the previously defined transcripts do not account for all USH3 cases, we performed further analysis and revealed the presence of additional exons embedded in longer human and mouse USH3A transcripts and three novel USH3A mutations. Expression of Ush3a transcripts was localised by whole mount in situ hybridisation to cochlear hair cells and spiral ganglion cells. The full length USH3A transcript encodes clarin-1, a four-transmembrane-domain protein, which defines a novel vertebrate-specific family of three paralogues. Limited sequence homology to stargazin, a cerebellar synapse four-transmembrane-domain protein, suggests a role for clarin-1 in hair cell and photoreceptor cell synapses, as well as a common pathophysiological pathway for different Usher syndromes.

INTRODUCTION

Combined deafness and blindness in adults is most frequently caused by Usher syndromes (USH). These account for more than half of the dual sensory deficit, with a prevalence of 1/10 000 in the age group of 30 –50.[1] Sensorineural hearing loss and retinitis pigmentosa (RP) characterise this group of autosomal recessive hereditary disorders. Three USH types are distinguished clinically. Postlingual progressive hearing loss and variable vestibular function characterise USH3 (MIM 276902) [2] Progressive RP with variable age of onset occurs in all USH types. While USH1 and USH2 map to at least 10 distinct loci ,[3-11] only one locus for USH3 has been reported so far.[12,13] Four of the USH1 and the USH2A genes have been identified. Mutations in the unconventional myosin MYO7A cause USH1B (MIM276903)[14] and in two unique cases also atypical USH phenotype similar to that of USH3.[15] Mutations in the PDZ-domain-containing protein, harmonin, cause USH1C (MIM 276904)[16,17]. PDZ-domains are protein interaction modules that allow the binding to and clustering of specific membrane-associated proteins, such as receptors and ion channels.[18] Cadherin23 and protocadherin15 mutations have recently been shown to underlie USH1D (MIM 601067) and USH1F (MIM 602083), respectively.[19 – 22] Most cadherins are integral membrane glycoproteins that mediate the calcium-dependent formation of cell adhesion,[23] while protocadherins are thought to be involved in a variety of functions, including neural development, neural circuit formation and formation of the synapse.[24] Finally, mutations in Usherin, a gene that encodes a basement membrane protein, cause USH2A (MIM276901)[25].

Joensuu et al.[26] have recently identified three mutations (a missense, a nonsense and a 3 bp deletion) in exons 2 and 3 of a newly identified gene that maps to 3q24, approximately 100 kb proximal to the previously defined USH3A linkage interval in

3q25.1. These mutations established this gene as the USH3A gene. Northern blot analysis and reverse-transcription PCR indicated the expression of the USH3A gene in several tissues, including retina. Two predicted transmembrane domains were identified in its deduced protein product, which at the time did not show similarity to any known protein.[26]

In this study, we characterise new human and mouse USH3A transcripts (AF495717; AF495718; AF495719 and AF495720), identify three additional USH3 mutations, analyse the USH3A expression pattern and by in situ hybridisation localise transcripts of this gene to mouse cochlear hair cells and spiral ganglion cells. We redefine the USH3A protein, name it clarin-1 and affiliate it to a new four transmembrane domain (4TM) vertebrate-specific protein family. Furthermore, based on sequence similarities to stargazin, a well-studied member of this hyperfamily, we suggest a role for clarin-1 in the hair cells synaptic junctions.

MATERIALS AND METHODS

USH3 families and controls

Blood samples were drawn by venipuncture after obtaining informed consent in accordance with the guidelines of the Tel Aviv University Helsinki Committee. Three of the Jewish Ashkenazi USH3 families included in this study, were referred to us through the Center for Deaf – Blind Persons in Tel Aviv as part of a larger study on the genetics of Usher syndromes in Israel. The fourth Jewish Ashkenazi USH3 family living in the US has one affected and two unaffected siblings whose grandparents originate from Eastern Europe. The family was ascertained by one of us (JGF). The

USH3 Jewish Yemenite and the Spanish families have been described elsewhere.[27,28] Clinical diagnosis of affected members in these families is compatible with the USH3 phenotype and haplotype segregation analysis does not exclude linkage to the USH3A locus. Control DNA samples from unrelated Jewish Ashkenazi and Yemenite individuals, were provided by the National Laboratory for the Genetics of Israeli Populations. A total of 51 grandparents in families from the Centre d'Etude du Polymorphisme Humain (CEPH) were also studied as controls.

RNA expression

The expression of Ush3a was determined by in situ hybridisation and reverse-transcription (RT) – PCR in mice at embryonic day (E) 16, postnatal day (P)0, 5, 10, 15, 20, and 30. For RT – PCR, cochleae were dissected out of the temporal bones. Total RNA was prepared from cochleae with TRI Reagent (Sigma). Genomic DNA was removed from all RNA samples using DNA-free (Ambion). Total RNA was purified using phenol-chloroform extraction (PCI; Gibco) and phase lock gel tubes (Eppendorf), followed by an isopropanol precipitation. RT reactions were performed using Expand Reverse Transcriptase (Roche) with Homo-Oligomeric DNA d(T)12-18 and Random Hexamer (Amersham Pharmacia Biotech). cDNA was amplified from the RT reactions using primers (F) 5'-GGTCCAAGCCATCCCCGTA-3' AND (R) 5'-CTCCTGCTTCTGTTATTTTCC-3'. As a control, primers spanning the last intron of Myo6 (F) 5'-CTGGTGGTATGCCCATTTTGA-3' and (R) 5'-TCGCTTTGCATAAGGCATTTCTA-3' were used.[29]

For in situ hybridisation, cochleae were dissected from surrounding tissue and fixation as performed by immersion in 4% paraformaldehyde in PBS for 12 – 16 h at 48C. The samples were processed without decalcification until P10. From P10 onwards, the

samples were dehydrated in graded concentrations of methanol, from 25 – 100%, followed by decalcification (to facilitate dissection) in 8% formic acid in 100% methanol for up to 4 days. We generated digoxygenin-labelled cRNA probes in a standard transcription reaction (Roche Molecular Biochemicals). We performed whole mount in situ hybridisation as described,30 with modifications. Finally, we visualised in situ reaction product by cryoprotecting whole cochleae with sucrose, embedding the tissue in OCT, and cryostat sectioning (10 mm). Antisense and sense probes were in vitro transcribed from a linearised vector, pPCR-Script AMP SK(+) cloning vector (Stratagene), containing a partial mouse Ush3a cDNA (corresponding to exons 2 and 3) by using either T3 or T7 RNA polymerase. No in situ hybridisation signal was detected with the control sense RNA probe. Our use of animals was approved by the Tel Aviv University Animal Care and Use Committee (11-00-65).

Mutation detection and analysis

Using the primers detailed in Table 2 we amplified fragments encompassing USH3A exons from genomic DNA templates of USH3 patients and their family members. PCR products were gel-purified, sequenced using dye terminators of Big-Dyes kits (Perkin-Elmer/Applied Biosystems) and analysed on an ABI 3700 sequencer (Perkin-Elmer/ Applied Biosystems). Sequence comparisons were performed by the use of Sequencher 4.1 software from GeneCodes Corporation. The Jewish Ashkenazi N48K mutation was screened by StuI digestion (16 h at 378C) followed by agarose (2%) gel analysis. The 23 bp deletion (found in patients of Yemenite origin) was screened by PCR amplification of a 227 bp fragment followed by FMC's MetaPhor gel (4%) electrophoresis. The Y63X mutation (found in a patient of Spanish origin) was screened

by SSCP; a 207-bp fragment of exon 0 was PCR amplified by primers ex0F2 (5-ATCAAAGCCACTGTCCTCTG-3) and ex0R (5-CTGGGAAGAGTCTGCCTAAA-3), and the alleles were separated on 0.76MDE gels, for 18 h at 5 W, at room temperature and were visualised by silver staining.[31]

Computational analyses and sequence annotation

Genomic sequences were retrieved from four NCBI (National Center for Biotechnology Information) Human clones: AC020636, AC036109, AC011103, AC078816. Fragments were assembled by Sequencher 4.1 software from GeneCodes Corporation. Complete sequence annotation was performed using the GESTALT workbench.[32] Sequence similarity searches performed by the use of the BLAST[33] and Blimps34 programs. Sequences were aligned using the BlockMaker34 and MACAW[35] programs; PHYLIP ProtDist[36] PHYLIP – Phylogeny Inference Package (Version 3.2 Cladistics 5: 164 – 166) and CLUSTAL W programs[37] were used to compute trees and their significance (bootstrap) values. TM regions were predicted using the PHDhtm[38] and TMHMM programs.[39] Primers were designed using the Oligo primer analysis software (http://medprobe.com/is/oligo/html).

RESULTS

Genomic analysis and transcripts

Since no mutations were identified within the described USH3A transcripts26 in patients from other USH3 families, we assumed that there might be additional uncharacterised exons of this gene. Therefore, we assembled and reconstructed a 500 kb genomic interval spanning the published USH3A gene and the previous USH3A linkage

interval, defined by D3S3413 and D3S1279 (Figure 1A). Based on gene predictions and alignment with human and mouse ESTs, we designed primers that allowed the amplification of a new human USH3A transcript. This longer transcript begins with a newly identified exon 0, continues with exon 2, has exons 3 and 4 transcribed together with their intervening intron, but does not include the previously identified exons 1 and 1b[26] (Figure 1B).

The USH3A gene shows partial exon overlap (but no protein overlap) with the 5' end of the UCRP pseudogene[26] in an opposite polarity, and in the same polarity with the 5' end of a newly characterised gene, USHARF (USH3A Alternative Reading Frame, ms in preparation) (Figure 1C). By EST assembly and PCR amplification from mouse inner ear cDNA library we also defined three alternative transcripts of the Ush3A orthologue (Figure 1D). One of these transcripts (AF495719) has the same exon-intron structure as the longest human mRNA, and its protein product shares 88% identity with the product of the corresponding human transcript (AF495717). A longer mouse transcript (AF495718) has an extra exon coding for additional 18 amino acids (Figure 3C).

Expression patterns

By PCR amplification from cDNA or cDNA libraries we confirmed the previously reported expression of USH3A in retina and skeletal muscle. In addition, we have amplified USH3A transcripts from human testis and olfactory epithelium cDNA. The later observation might be related to previously reported expression of two other Usher genes, myosin VIIA and harmonin, in the olfactory tissue.[40-43] Yet, despite the observation of anomalies in olfactory cilia, no chemosensory deficits have been confirmed in Usher patients.[44]

By PCR amplification of mouse inner ear cDNA and by whole mount in situ hybridisation on mouse cochleae we also demonstrate the expression of Ush3a transcripts in the auditory sensory organ. A developmental expression profile of Ush3a was performed both by PCR amplification of mouse inner ear cDNA and whole mount in situ hybridisation on mouse cochleae. Ush3a transcripts were detected as early as E16, the earliest age tested, and at all postnatal stages examined (P0, P5, and P10) (Figure 2A,B). Hybridisation with an Ush3a RNA probe on whole cochleae detected mRNA expression in the sensory epithelium at E16 (Figure 2C,D), P0, P5, P10, P15, P20 (n=4 for each age) (data not shown). Sectioning through these cochleae revealed specific hybridisation in the inner and outer hair cells of the organ of Corti (Figure 2E,G). No hybridisation was detected in the supporting cells of the organ of Corti, including the Deiters' cells, the pillar cells, and the Hensen cells. The only other hybridisation found in the cochlea was in the spiral ganglion cells (Figure 2F) containing the primary neurons that innervate the cochlear sensory epithelia.

Gene product and protein structure

The open reading frame of the newly defined USH3A transcript is expected to encode a 232 amino acid protein with four transmembrane domains, which we named clarin-1, after the clarity of sensory perception allowed by the intact protein (Figure 3C). Sequence database searches by BLAST33 identified two additional clarin-1 human paralogues, clarin-2 and -3 (Figure 3A). The clarins were found to have respective orthologues in mouse as well as in fish (Takifugu rubripes [Fugu] and Tetraodon nigroviridis), all encoding small 224 – 284 amino acid proteins (Figure 3A). A partial clarin-1 mRNA was also identified in chicken. No orthologues were identified in the genomes of prokaryotes, yeast, plants, nematodes and insects. Four transmembrane-

domains (TMs), conserved sequence motifs and a single glycosylation consensus site between TM1 and TM2 characterise the clarin family members (Figure 3C).

In a wider context the clarins appear to belong to a large hyperfamily of small integral membrane glycoproteins with four transmembrane domains. These include the tetraspannins 45 (InterPro families PR00218 and PS00930) as well as the PMP22/EMP/MP20 and claudin family (InterPro family IPR000729) that perform diverse membrane transport, transduction and cell – cell interaction and scaffolding functions. Yet, BLAST searches did not provide a sequence similarity basis for a relationship between clarin-1 and any members of this hyperfamily. To examine this question further, it was necessary to employ more sensitive search routines, capable of detecting subtle sequence similarities. First, we asked whether any of the clarins contained protein motifs already included in the BLOCKS database, but the results were negative. Therefore, using the newly discovered group of 12 clarin sequences, we defined novel clarin-specific Blocks motifs. These were subsequently analysed by the Local Alignment of Multiple Alignments (LAMA) method,[46] looking for remote but statistically significant similarities between the clarin Blocks and all others. Two of the clarin Blocks, in and around the first and fourth putative TM domains generated a hit with two of the 4TM proteins Blocks (Figure 3B,C). Pairwise Smith-Waterman alignments 47 then allowed us to delineate the broad similarity relations between the clarins and representative members of several human 4TM families (Figure 5).

In order to find out which of the 4TM proteins is most highly related to the clarins, we performed further pairwise alignments with accurate statistical testing (Figure 6). This highlighted the calcium channel gamma subunit proteins (CACNG) as best clarin sequence matches. Of these CACNG2 (stargazin)[48,49] was the only protein showing significant matches (P50.001) to all three clarins. It is noteworthy that like in some

USH3 cases,[2] stargazin mutation appears to affect also the inner ear vestibular function.[48,49]

USH3A mutations

Three novel USH3 mutations were identified among 10 affected individuals from six USH3A families (Table 1 and Figure 4). A 143T4G substitution expected to cause a N48K missense mutation was found in six affected individuals from four unrelated families of Eastern European Jewish origin (Figure 4A). Shared microsatellite and SNP haplotypes on carrier chromosomes (data not shown) suggest the existence of a founder effect for this mutation. A 189C4A substitution, expected to cause a Y63X nonsense mutation, was found in a homozygous state in three affected individuals from a non-consanguineous Spanish family (Figure 4B; Table 1). A 23 bp deletion, spanning nucleotides 187 – 209 downstream from the first methionine codon, was found in a homozygous state in two affected individuals from a non-consanguineous family of Yemenite Jewish origin (Figure 4C).

DISCUSSION

Affiliation of clarin-1 to the newly defined clarin family and the definition of several motifs characterizing all members of this family indicate that the full-length coding sequence of the clarin-1 has now been determined. The additional 18 aa encoded by the longest mouse transcript as well as additional protein segments encoded by the long transcripts of Trclarin-1 and Hsclarin-2 elongate the putative first extracellular loop (Figure 3C). On the other hand, Trclarin-3 and Tnclarin-3 seem to be missing the C-terminal TM domain, indicated by a dashed blue line in Figure 3C. The fact that no

orthologues were identified in the genomes of prokaryotes, yeast, plants, nematodes and insects, suggests that this gene family is limited to vertebrates.

The single missense USH3A mutation newly identified in this study, N48K, disrupts the molecule's only N-glycosylation consensus site (Figure 3C), and may render clarin-1 incapable of proper intracellular trafficking and plasma membrane insertion.50 Both other newly identified mutations, the Y63X and the 187 – 209del23 bp mutations, are expected to result in truncated proteins. Thus, these three mutations likely render clarin-1 functionally inactive, and account for the disease in most studied USH3A patients (Figure 4). Of the previously identified missense mutations, [26] one (M120K under the new enumeration) leads to the replacement of a hydrophobic methionine residue by a charged lysine inside or very near to the border of TM2, and another (IL153,154M) results in the shortening of TM3 by one residue. Because the four TMs are rather well conserved, such mutations may also be functionally deleterious.

USH3 is progressive, i.e. at birth both vision and audition are much less severely impaired than later in life. This implies that the role played by clarin-1 in the hair cell and retina may display at least a measure of functional redundancy. The best candidates for serving as substitute for clarin-1 may be its two orthologues, clarin-2 and clarin-3. However, it is also possible that other 4TM proteins might subserve this function. Future studies will be needed to clarify the mechanism of time-dependent loss of such potential redundancy. Other small 4TM proteins have previously been shown to underlie deafness and retinal diseases. Mutations in four different connexins (26, 30, 31, and 43) and in claudin-14 underlie several forms of deafness (Hereditary Hearing Loss Homepage: http://www.uia.ac.be/dnalab/hhh/). Mutations in connexin 50 cause congenital cataracts[51] and mutations in human peripherin/RDS and mouse ROM were shown to disrupt photoreceptor morphogenesis, leading to retinitis pigmentosa.45 Also,

mouse mutations in Cacng2 (stargazin) cause defects not only in cerebellum but also in inner ear.[52] Yet, such information cannot help define more accurately the underlying mechanism for USH3, because the above listed genes participate in diverse cellular pathways, including the assembly and maintenance of gap junctions, tight junctions and synaptic junctions.[53 – 55]

Because stargazin has been shown to play a key role in the shaping and maintenance of cerebellar synapses,[55] our protein sequence analysis is suggestive of a synaptic role for clarin-1 as well. The case is strengthened by corroborative evidence: (a) Stargazin has been shown to interact with PDZ domain-containing proteins of the Membrane Associated Guanylate Kinase type (MAGUK), serving as intracellular anchors essential for the integrity of synaptic densities.[55] Intriguingly, one form of the Usher syndromes (USH1C) is caused by mutations in another PDZ protein harmonin. This may indicate that clarin-1 and harmonin are part of the same synapse-formation pathway, despite the absence of a clear PDZ-binding consensus[56] shared by all the clarins (Figure 3C). (b) Stargazin was shown to be involved in controlling the expression and mobilisation of amino-hydroxyl-methyl-isoxazole propionate (AMPA) glutamate receptors to the post-synaptic cleft in cerebellar granule cells. That cochlear hair cells also utilize glutamate (or a highly similar compound) as a rapid excitatory neurotransmitter 57 seems relevant. (c) Stargazin and other members of the CACNG family have been suggested to also play a role of forming protein – protein contacts across the synaptic cleft.58 In bridging two cellular membranes they resemble other 4TM proteins such as the gap junction forming connexins and the tight junction-forming claudins. That clarin-1 is expressed in inner ear, both pre- and post-synaptically, is consistent with possible clarin – clarin homophylic interactions, which might work in the hair cell synapse. Thus, we would like to propose that clarin-1 has a role in the excitatory ribbon synapse junctions

between hair cells and cochlear ganglion cells,59 and presumably also in analogous synapses within the retina.

We have previously reported a possible epistatic interaction between the USH3A locus and the MYO7A gene,[27] whereby two USH3A haploidentical patients of a Yemenite family showed different USH phenotypes, the patient with the more severe, USH1-like phenotype was also found to be a carrier for a complex rearrangement (3260T4C and 3266delG) in the tail of myosin VIIA.27 Both patients are shown here to be homozygous for the 23 bp deletion in USH3A exon 0 (Figure 4C). While the double heterozygous mother and siblings are healthy, in the context of a homozygous USH3A null mutation the presence of one null mutation in the MYO7A tail (the out of phase one base deletion in the complex rearrangement) mimics haploinsufficiency, and illustrates a departure from the monogenic model.

Since MYO7A expression in the inner ear is restricted to sensory hair cells,[60 – 62] including the sensory synaptic region,[62] the present observation that Ush3a is expressed in mouse sensory hair cells, and the implication of a synaptic role, are compatible with a clarin-1/MYO7A interaction. Furthermore, while myosin VIIA was localised only in presynaptic cells, MyRIP, a most recently defined MYO7A-interacting protein,63 is present in both pre- and post-synaptic cells, similar to clarin-1. The proposed clarin-1/MYO7A interaction is also consistent with a pathophysiological continuum between USH1 and USH3A, and is relevant to their treatment. Access to Ush3 mutant models should allow testing of these hypotheses and contributing to a better understanding of the role of clarin-1 in the retina and inner ear.

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