ONLINE MUTATION REPORT

A novel *COCH* mutation, V104del, impairs folding of the LCCL domain of cochlin and causes progressive hearing loss

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Recently the causative gene of autosomal dominant sensorineural nonsyndromic late onset hearing loss (DFNA9) has been identified as the *COCH* gene, which lies in the DFNA9 region of human chromosome 14 (gene map locus 14q12–q13). Molecular analysis of cases of DFNA9 have identified several families with five different mutations in this gene.[1–5]

The cochlin protein encoded by *COCH* is an extracellular matrix protein that contains an LCCL domain and two von Willebrand type A domains.[1] Interestingly, all mutations causing DFNA9 type deafness disorder affect the LCCL domain of cochlin. In view of the central role of this domain in the DFNA9 disorder, we initiated studies on it to explore the molecular basis of the disease.[6] We found that most DFNA9 mutations affected conserved structural elements of the LCCL fold and disrupted the proper folding of this domain; recombinant mutant LCCL domains expressed in *Escherichia coli* failed to adopt the wild type fold and instead formed insoluble aggregates.[7] It is noteworthy that insoluble deposits are detected in temporal bone sections of individuals affected by DFNA9.[8] These results support the notion that the DFNA9 mutations may act through a gain of function mechanism; it is the presence of the abnormal protein that causes the disease. According to this view, accumulation of deposits in vestibular and cochlear nerve channels leads to strangulation and progressive degeneration of the dendrites, and loss of cochlear and vestibular neurones.

Our present investigation was aimed at identifying novel mutations affecting the LCCL domain of cochlin in order to gain more insight into the pathomechanism of DFNA9. Hungarian patients selected according to the diagnostic criteria of DFNA9 were screened for the presence of mutations affecting the LCCL domain. These studies identified one person heterozygous for a novel mutation in the coding region of *COCH*. The mutation has resulted in the deletion of Val104, a residue conserved in the human, mouse, and chicken cochlin sequences. It affects a critical β strand of the LCCL domain and was found to prevent refolding of recombinant LCCL domain expressed in *E. coli*.

Key points

- Autosomal dominant nonsyndromic sensorineural deafness 9 (DFNA9, OMIM #601369) is a rare, late onset hearing loss. In DFNA9 patients, hearing loss usually begins in the third decade of life as a high frequency loss with concomitant vestibular dysfunction.
- DFNA9 is caused by mutations in the *COCH* gene, which encodes cochlin, an extracellular matrix protein that contains an LCCL domain and two von Willebrand type A domains.
- Molecular analysis of cases of DFNA9 has identified five different mutations in this gene. Interestingly, all mutations causing DFNA9-type deafness disorder affect the LCCL domain of cochlin.
- Here we describe a novel *COCH* mutation in a Hungarian patient, which results in the deletion of Val104, a residue conserved in the human, mouse, and chicken cochlin sequences. The deletion affects a critical β strand of the LCCL domain and was found to prevent refolding of a recombinant LCCL domain expressed in *Escherichia coli*.

MATERIALS AND METHODS

Selection of patients and gene analysis

Patients were selected from the cochlear implantation list of the Department of Otorhinolaryngology of Semmelweis University according to diagnostic criteria of the DFNA9 disorder (OMIM #603196): progressive hearing loss associated with vestibular dysfunction, the age of onset (20–56 years) and probable familial origin. In the selected patients, audiometrically documented hearing loss initially affected only higher frequencies with later progression to lower frequencies. Many patients reported episodes of vertigo.

DNA from 17 hearing impaired persons from 14 different families was used for genetic analysis, and compared with DNA from 50 unrelated controls. A DNA sample was also collected from a relative of the proband identified in the present study. Genomic DNA (20 ng) was isolated from peripheral blood cells using the Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN, USA).

Mutation screening was performed only for exons 4 and 5 of *COCH* (NM 004086), which encode the LCCL domain of the protein. Intronic PCR primers (sense: 5′-CCACTATGCCCC AAGAAGTCCTAAGAATGC-3′, antisense: 5′-GGGTGGGAGA ATATTACAGAATGAGAAC-3′) were used to amplify the genomic region encompassing exon 4, intron 4, and exon 5 of *COCH*. PCR was performed with an initial denaturation step for 2 minutes at 95℃ followed by 35 cycles of 94℃ for 30 seconds, 65℃ for 30 secconds and 68℃ for 2 minutes. Amplification products were purified using the NucleoSpin Extract Kit (Macherey-Nagel Gmb, Düren, Germany).

Dideoxy sequencing of the PCR products for exons 4 and 5 was carried out using the primers 5′-CGTCTGCATT TCTCTCCCACCCTGGTG-3′, flanking exon 4, and 5′-TGA AAAAGTGTGGATAGCATCTCAGCTGC-3′ flanking exon 5. To verify the sequence of the genomic DNA of the patient carrying a mutation in exon 5 of *COCH*, PCR primers (sense: 5′-CTGAAAAAGTGTGGATAGCATCTCAGCTGC-3′, antisense: 5′-GGGTGGGAGAATATTACAGAATGAGAAC-3′) were used to amplify only the affected exon 5. The PCR product was blunt end ligated into *Sma*I digested M13mp19 vector, and phages carrying the mutant or wild type alleles were sequenced. Restriction enzymes and the M13 sequencing kit used for dideoxy sequencing of cloned DNA were

purchased from Biolabs (Beverly, MA, USA). *E. coli* strain JM-109 was used to propagate the phage.

Expression and characterisation of the recombinant V104del LCCL domain

The DNA fragment encoding the LCCL module of the V104del mutant protein of the proband was produced by a two step PCR strategy. Firstly, we amplified exon 4 and 5 of the mutant *COCH* gene separately by using 5′-GCGGGATCCGTCGACCGCTCCCATTGCTATCACATG-3′ (sense) and 5′-GAGTTGCTGATTACTCCCCTGTGGACAGCAGCCCCA-3′ (antisense) primers for exon 4, and 5′-TGGGGCTGCT GTCCACAGGGGAGTAATCAGCAACTC-3′(sense) and 5′-GCGAAGCTTACTCGAGAGTTACTGTGAAAGAAGCAG-3′ (antisense) primers for exon 5. For exon 4, the template was the genomic DNA of the proband, while for the mutant exon 5 we used the M13mp19 clone containing the mutant allele of exon 5. The two cDNA fragments were linked in a second reaction with 5′-GCGGGATCCGTCGACCGCT CCCATTGCTATCACATG-3′ (sense), 5′-GCGAAGCTTACTC GAGAGTTACTGTGAAAGAAGCAG-3′ (antisense).

The fragment was cut with *Hin*cII and *Hin*dII, and ligated in a *Pvu*II/*Hin*dIII digested pmed23 expression vector. This construct encodes a fusion protein in which the first 35 amino acids of β galactosidase are fused to the mutant LCCL domain. The *E. coli* strain JM-109 was used for plasmid propagation and protein expression.

Expression, refolding, and characterisation of the recombinant V104del mutant LCCL domain was performed using the same protocol as that used for the wild type protein and several other mutants of the LCCL domain.[6 7] Expression of the recombinant proteins was induced with 100 µmol/l IPTG and inclusion bodies containing recombinant protein were dissolved in 0.1 mol/l Tris-HCl, 8 mol/l urea, 10 mmol/l EDTA, and 0.1 mol/l dithiothreitol, pH 8.0. The mixtures were incubated at 25℃ for 60 minutes with constant stirring and the solubilised proteins were chromatographed on a Sephacryl S-300 column equilibrated with 100 mmol/l Tris-HCl, 8 mol/l urea, 10 mmol/l EDTA, and 0.1% 2-mercaptoethanol. Fractions containing pure fusion proteins were identified by SDS-PAGE, pooled and refolded by dialysis against 0.1 mol/l Tris-HCl and 10 mmol/l EDTA, pH 8.0 at 25℃.

RESULTS AND DISCUSSION

Sequence analysis of the region encoding the LCCL domain of *COCH* of one hearing impaired patient indicated that the patient was heterozygous for a deletion in this region. To determine the precise location and size of the deletion we cloned the affected region into M13mp19 in order to separate the mutant and wild type alleles. Single strand sequencing of phages confirmed that one of the alleles had the wild type sequence, whereas the mutant allele carried a deletion of nucleotides 367–369 in exon 5. The deletion of three nucleotides results in the deletion of a Val104 residue without causing a frameshift. No mutation in the LCCL domain was detected in the other 16 patients with hearing loss or in the 50 control individuals.

The hearing loss of the proband (born in 1943) was first observed at the age of 32 years to have severe vertigo, nausea, vomiting, and perceptive hearing impairment of the left ear. After 4 years, severe hearing loss appeared in the right ear as well. Severe to profound hearing loss across all frequencies was developed by the fifth decade. The patient received a cochlear implant at the age of 46 years but it did not improve his hearing. The latest examinations (at the age of 60 years) showed total cochleovestibular areflexia on both ears and the patient feels weak vertigo in the dark. He has no brothers or sisters. His father died in 1944 at the age of 30 years, his mother is 93 years old without any hearing problems. He has only one daughter (29 years old), who has no hearing

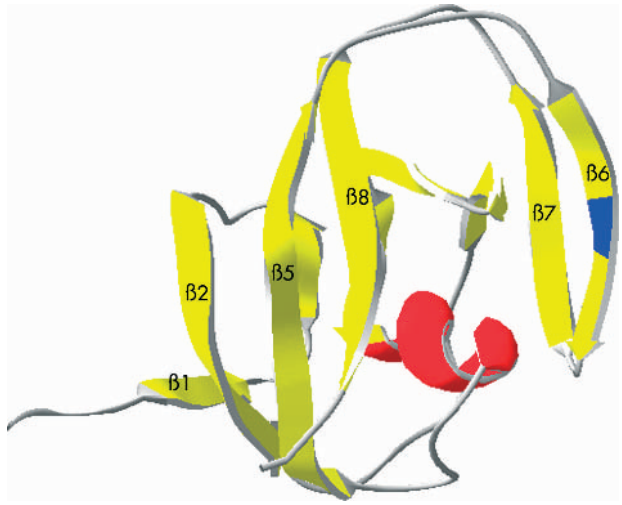

Figure 2 Ribbon representation of the structure of the LCCL domain of human cochlin.[7] The coordinates of the LCCL domain (1JBI) were taken from the Protein Data Bank. The β strands (yellow) are numbered as in fig 1, the single α helix is shown in red. The position of Val104 (deleted in the novel V104del mutant) is shown as a blue segment of the β6 strand.

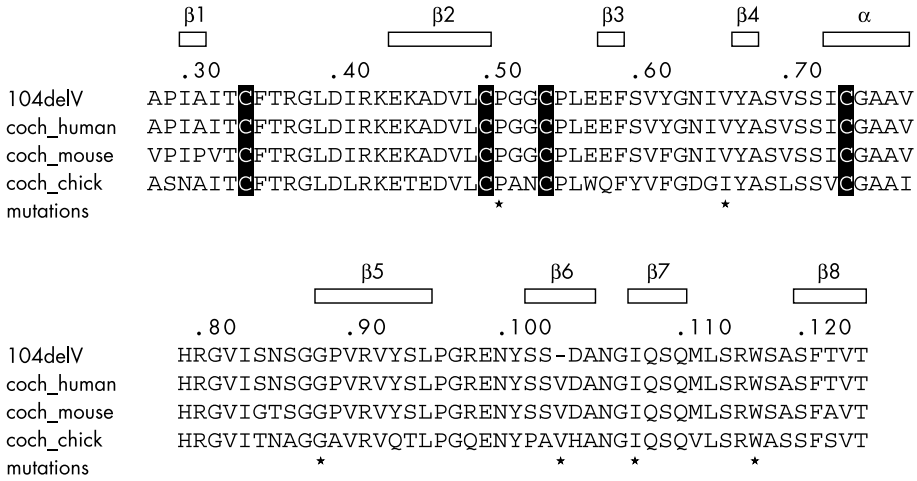

Figure 1 Alignment of the amino acid sequences of LCCL domains of cochlins. The locations of the β strands and the α helix in the LCCL domain of human cochlin[7] are shown at the top. The conserved cysteines of the LCCL domains of human (coch_human, residues 28–124), mouse (coch_mouse, residues 30–126), and chicken cochlin (coch_chicken, residues 24–120) are shaded. The top sequence (V104del) represents the sequence of the mutant protein identified in the present study. In the bottom line, asterisks mark the locations of residues where mutations in the human cochlin protein have been found to result in the DFNA9 disorder.

Table 1 Comparison of the V104del mutation with other DFNA9 mutations

Mutation	Genotype	Age of onset	Refolding of LCCL domain
V104del*	Heterozygous	4th decade	Impaired*
I109N[5]	Heterozygous	2nd–3rd decade	–
V66G[2]	Heterozygous	2nd–3rd decade	Impaired[7]
G88E[2]	Heterozygous	5th decade	Impaired[7]
W117R[2]	Heterozygous	5th decade	Preserved[7]
P51S[3]	Heterozygous	4th decade	Impaired[7]

*Present work.

problem and her genetic analysis did not identify a mutation in the LCCL domain of *COCH*. Although the possibility that the proband's father carried the mutation cannot be ruled out, it is also possible that the mutation arose de novo.

In order to examine the effect of the mutation on the structure of the LCCL domain we expressed the mutant domain using the same protocol as for the wild type protein.[6 7] Whereas in the case of the wild type recombinant LCCL domain, this protocol yields a soluble protein that adopts a unique three dimensional structure,[7] the same refolding protocol did not yield any soluble protein for the V104del mutant LCCL domain, and dialysis led to complete precipitation of the recombinant protein. As the deletion of Val104 affects a conserved residue of the β6 strand structure of the LCCL domain (figs 1 and 2), it seems likely that this deletion affects this essential structural element of the LCCL fold, impairing its ability to form the native structure. In this respect, the V104del mutation is similar to most other DFNA9 causing mutations identified so far, which also impair refolding of recombinant LCCL domain (table 1).

In our opinion, this novel mutation provides additional support for the notion that the characteristic eosinophilic deposits in DFNA9 affected inner ear structures could be the result of accumulation and aggregation of aberrant, mutated cochlins. Recent studies have indeed shown that DFNA9 mutations do not impair secretion and post-translational processing of cochlin produced by mammalian cells.[9] It seems likely that in vivo the mutations may lead to a slow aggregation of cochlin over a longer time course, consistent with the late onset and progressive nature of this disorder.

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