Histopathology of the Human Inner Ear in the p.L114P \textit{COCH} Mutation (DFNA9)

Barbara J. Burgess$^1$, Jennifer T. O’Malley$^1$, Takefumi Kamakura$^{1,5}$, Kris Kristiansen$^1$, Nahid G. Robertson$^2$, Cynthia C. Morton$^{2,3,4,5}$, and Joseph B. Nadol Jr.$^{1,5}$

$^1$Human Otopathology Laboratory, Department of Otolaryngology, Massachusetts Eye and Ear Infirmary, Boston, MA USA
$^2$Departments of Obstetrics, Gynecology, and Reproductive Biology, and of Pathology, Brigham and Women’s Hospital, Boston, MA USA
$^3$Broad Institute of MIT and Harvard, Cambridge, MA
$^4$School of Psychological Sciences, University of Manchester, UK
$^5$Harvard Medical School, Boston, MA USA

Abstract

The histopathology of the inner ear in a patient with hearing loss caused by the p.L114P \textit{COCH} mutation and correlation with the clinical phenotype are presented. To date, 23 \textit{COCH} mutations causative of DFNA9 autosomal dominant sensorineural hearing loss and vestibular disorder have been reported, and the histopathology of the human inner ear has been described in four of these. The p.L114P \textit{COCH} mutation was first described by Choi et al. [2013] in a Korean family. We have identified the same mutation in a family of non-Asian ancestry in the United States, and the temporal bone histopathology and clinical findings are presented herein. The histopathology found in the inner ear was similar to that shown in the four other \textit{COCH} mutations and included degeneration of the spiral ligament with deposition of an eosinophilic acellular material, which was also found in the distal osseous spiral lamina, at the base of the spiral limbus and in mesenchymal tissue at the base of the vestibular neuroepithelium. This is the first description of human otopathology of the \textit{COCH} p.L114P mutation. In addition, it is the only case with otopathology characterization in an individual with any \textit{COCH} mutation and with residual hearing, therefore allowing assessment of primary histopathological events in DFNA9, before progression to more profound hearing loss. A quantitative cytologic analysis of atrophy in this specimen and immunostaining using anti-neurofilament and anti-myelin protein zero antibodies confirmed that the principal histopathologic correlate of hearing loss was degeneration of the dendritic fibers of spiral ganglion cells in the osseous spiral lamina. The implications for cochlear implantation in this disorder are discussed.
INTRODUCTION

Mutations in \textit{COCH}, encoding the secreted protein cochlin, cause autosomal dominant nonsyndromic sensorineural hearing loss (DFNA9). To date, 23 \textit{COCH} mutations (21 missense and 2 in-frame deletions) have been reported [Bae et al. 2014; Tsukada et al. 2015]. The common clinical phenotype includes adult onset progressive, bilaterally symmetric sensorineural hearing loss with reduced word recognition scores. The histopathology of the human inner ear has been described in four of these mutations (Table 1). In all four cases, the sensorineural loss was severe to profound at the time of death, and in all four cases there was a clinical history of vestibular dysfunction [Bae et al. 2014]. The histopathology that has been described includes degeneration of the spiral ligament with loss of cellularity and deposition of an eosinophilic acellular material, which was also found in the distal osseous spiral lamina and limbus. The amorphous material has been shown by immunohistochemistry to be consistent with mutated cochlin [Robertson et al. 2001, Robertson et al. 2006]. The organ of Corti demonstrated loss of some outer and inner hair cells. However, the principal histopathologic correlate of the sensorineural loss appeared to be degeneration of the dendritic processes of the spiral ganglion cells, greater in degree than the loss of spiral ganglion cell bodies [Merchant and Nadol, 2010]. In addition to the cochlear findings, there was severe degeneration of the stroma of both the cristae and maculae with loss of cellularity and deposition of eosinophilic acellular material similar to that described in the cochlea. Severe degeneration of peripheral processes of Scarpa’s ganglion has also been described [Merchant et al. 2000; Robertson et al. 2006].

The \textit{p.L114P COCH} mutation was first identified by Choi et al. [2013] in a Korean family. However, no otopathology or detailed clinical history were presented. We have identified the same mutation in a family in the United States, and herein present and discuss temporal bone findings of L114P mutations, representing the fifth \textit{COCH} mutation for which human otopathology is currently available. However, in this case, unlike previous ones with characterized histopathology, there was considerable retained hearing in a 42-year old man, providing an opportunity to study the degenerative process of the inner ear prior to progression to profound deafness.

The physiologic role of cochlin is not fully understood. \textit{COCH} is expressed, not only in cochlear and vestibular organs but also in eye, spleen, thymus, brain, and cerebellum, all at much lower levels than in the inner ear [Bhattacharya, 2006]. Cochlin is a secreted protein with three functional domains: an N-terminal factor C homology (FCH), also known as the LCCL domain; and two von Willebrand factor A domains (vWFA1, vWFA2). There is mounting evidence to suggest that the LCCL domain may be involved in host defense mechanisms. Py et al. [2003] have reported expression of \textit{Coch} by follicular dendritic cells in mouse spleen and lymph nodes, and demonstrated involvement of cochlin in systemic...
innate immune response, with mechanisms, yet to be fully characterized. The von
Willebrand domains are thought to associate with extracellular matrix proteins, typically
fibrillar collagens, but further possible function of these domains has not been characterized.

The mechanism by which mutated cochlin is involved in disease processes is likewise not
fully understood. It has been suggested that mutated cochlin may aggregate [Robertson et al.
2001, Robertson et al. 2003, Bhattacharya, 2006] and interfere with extracellular matrix
protein interactions. In the cochlea, mutated cochlin may have a “toxic effect” on the
dendritic processes of spiral ganglion cells and perhaps vestibular neurons [Merchant et al.
2000, Robertson et al. 2006] as a primary effect or secondary to disruption of ionic cycling
particularly in the spiral ligament. It is thought that the pathologic effect of mutated cochlin
is due to a “gain of function” rather than haploinsufficiency [Robertson et al. 2006].
Interestingly cochlin aggregates may have a role in causing glaucoma through its deposition
in the trabecular meshwork of the eye [Bhattacharya, 2006].

MATERIALS AND METHODS

Temporal Bone Acquisition

Temporal bone specimens were obtained in compliance with Human Subject Assurance
Number FWA00006221 (exemption 4) from the Massachusetts Eye and Ear Infirmary.

Temporal Bone Preparation and Light Microscopy

Temporal bones were removed for further study approximately 48 hours after death fixed in
10% formalin, decalcified with ethylene diamine tetracetic acid (EDTA), dehydrated in
graded alcohols, and embedded in celloidin. Serial sections were cut at a thickness of 20
micrometers. Every tenth section was stained with hematoxylin and eosin and placed on
glass slides. The right temporal bone was sectioned in a vertical plane because of the
radiologic evidence during life of a dehiscence of the superior semicircular canal. The left
temporal bone was sectioned in the conventional axial (horizontal) plane and reconstructed
in two dimensions for quantification of cellular elements.

Neurofilament and Myelin Protein Immunostaining

After removal of the celloidin embedding medium [O’Malley J et al. 2009], sections were
incubated with primary antibodies for 14 hours in a humid chamber and rinsed in three
washes of PBS. Secondary antibodies diluted 1:200 were applied and incubated for one
hour. Following three rinses in PBS avidin biotin horseradish peroxidase (standard ABC kit)
(Vectora Labs, Burlingame, CA) was applied to the sections, incubated for one hour, and
followed by three washes in PBS. Colorization was performed using 0.01% Diaminobenzodine and 0.01% H2O2 for 5 to 10 minutes, rinsed, dehydrated, and covered
slipped as described by O’Malley [O’Malley J et al. 2009]. Selected sections were stained
using anti-myelin protein zero antibody (Abcam, City, ST), or anti-neurofilament antibody
(Boehringer Mannheim, City, ST).
DNA Extraction and Sequencing

Sanger sequencing was employed to evaluate COCH exons 4 and 5 (encoding the LCCL domain, which harbors the majority of known COCH mutations) in the following manner. Ten milligrams of muscle obtained at autopsy and preserved at −80°C was used for DNA extraction, using the Gentra Puregene tissue kit (Qiagen, Redwood City, CA), in accordance with the manufacturer’s protocol, yielding approximately 5 micrograms of genomic DNA. Approximately 25 nanograms of this genomic DNA was used as template for PCR amplification, using Platinum PCR Supermix (Invitrogen, Waltham, MA) with COCH primers flanking exon 4 (forward: 5’cttaaatctcacactgtagtc3’, reverse: 5’aagagaaataatctgctgc3’) and exon 5(forward: 5’tcttgatgactctgtgag3’, reverse: 5’tcacaggtttcccataaggta3’. After PCR amplification, primers were removed using the Qiagen Mini Elute PCR Purification Kit, followed by Sanger sequencing of PCR products at the Massachusetts General Hospital DNA Sequencing Core Facility. Trace sequences were read using Snap Gene Viewer software version 2.7.2 (GSL Biotech LLC, Chicago, IL).

RESULTS

Clinical History and Findings

This 42-year old male had a history of progressive, bilateral, down-sloping sensorineural hearing loss first noted at approximately age 30 years. Hearing loss was also present in his mother, two sisters and one of two brothers, suggesting a genetic etiology (Fig. 1). His mother was said to have done well with a cochlear implant as treatment of her sensorineural loss. Over the 10 years prior to his death a progressive down sloping sensorineural loss with decrement in speech discrimination scores was noted. The last audiogram was performed two weeks prior to death and is shown in Figure 2. A CT scan of the temporal bones was done at age 42. This demonstrated a questionable boney dehiscence of the right superior semicircular canal but no other abnormality. There was no history of vestibular complaints. The patient’s past medical history included anaplastic T-cell non-Hodgkin’s lymphoma diagnosed six years prior to death and treated with full body radiation, an allogenic stem cell transplant, and chemotherapy. The treatment was complicated by graft-versus-host disease. Postmortem examination showed no evidence of residual lymphoma. There was evidence of gastro-intestinal graft-versus-host disease and healing pneumonitis of both lungs.

Histopathologic Findings

Left ear—Prominent throughout the cochlea was a diffuse loss of cellularity and deposit of amorphous material in the spiral ligament, distal end of the osseous spiral lamina, and base of the limbus (Fig. 4) consistent with DFNA9 as the underlying disorder (Merchant et al. 2000). Although there was significant postmortem artifact, there was preservation of the stria vascularis and some inner and outer hair cells in all three turns of the cochlea. A cytocochleogram is shown in Figure 5, which illustrates the preservation and degeneration of elements of the cochlea.

Cochlear Neurons: The total spiral ganglion cell count was 14,279, which represented 53% of normal age-matched controls. The dendritic processes between the spiral ganglion cells and the organ of Corti demonstrated more severe degeneration compared to the spiral
ganglion cell bodies (Fig. 5), best illustrated using immunostaining for myelin protein and neurofilament (Figs. 6, 7).

**Vestibular system:** There was evidence of mild to moderate atrophy of the neuroepithelium of the three semicircular canals and the macula utriculi and macula sacculi. There was an amorphous deposit in the mesenchymal tissue between the cribrose area of the vestibular end organs and the neuro-epithelium (Fig. 8). The total Scarpa’s ganglion cell count was 11,288, which represented 56% of normal age-match controls. Degeneration of distal vestibular dendritic fibers at the base of the neuroepithelium was demonstrated using anti-neurofilament immunostaining (Fig. 9). There was deposition of a mixture of basophilic and eosinophilic amorphous material in the incudomalleal joint (Fig. 10) and in the incudostapedial joint as well as near the umbo of the tympanic membrane. The stapediovestibular joint was normal.

**Right ear—** There was a small dehiscence of the bony superior semicircular canal. Otherwise the findings in the cochlea, including an amorphous deposit in the spiral ligament, osseous spiral lamina and limbus, were similar to that described in the left ear. Likewise the density of spiral ganglion cells was similar to that of the left ear, and the numbers of dendritic processes of the spiral ganglion cells were significantly diminished throughout the cochlea. The vestibular end organs demonstrated moderate atrophy of the neuroepithelium and the presence of amorphous deposits in the mesenchymal tissue between the cribrose area and neuroepithelium, similar to that described on the left.

**Sequence Analysis**

Given that our analysis of the temporal bone sections by light microscopy revealed histopathology characteristic of DFNA9 [Khetarpal et al. 1991; Robertson et al. 1998, Merchant et al. 2000], we proceeded with sequencing of COC, with mutations that are causative of DFNA9. Sequence analysis of genomic DNA isolated from the subject’s post-mortem muscle biopsy, revealed a c.T341C missense mutation, resulting in a p.leu114pro (L114P) amino acid change. This mutation has been described previously in a Korean family (Choi et al. 2013), through larger scale mutation screening of families with sensorineural hearing loss, but with no further detailed clinical phenotypic or histological studies.

**DISCUSSION**

This 42-year old male had a bilateral progressive sensorineural hearing loss with marked reduction in speech discrimination scores and our finding of a missense mutation (p.L114P) in COCH (DFNA9). This is the first description of the histopathology of the inner ear in this specific mutation. The histopathologic findings were similar to those previously described in other kinships (Table 1), both in the inner ear and in the extracochlear locations as previously described [McCall et al. 2011; Robertson et al. 2014]. Within the cochlea, the histopathologic findings include the diffuse loss of cellularity of the spiral ligament and an accumulation of an extracellular deposit in the spiral ligament, osseous spiral lamina and the limbus and the subepithelial mesenchymal tissue of the vestibular neuroepithelium.
In addition to the fact that this is the first description of the otopathology in this specific mutation, there was considerable residual hearing at the time of death which offered the opportunity to study further the pathogenesis of hearing loss in DFNA9 given that all four previous histopathologic descriptions were of individuals who were profoundly deaf at the time of death.

Although there was degeneration of multiple cytologic elements of the cochlea (Fig. 5), degeneration of the dendritic processes of spiral ganglion cells as seen in the osseous spiral lamina was more advanced than that seen in other cytologic elements, suggesting a primary cochlear neuronal degeneration. Previous published descriptions of the histologic correlate of sensorineural hearing loss in DFNA9 also reported loss of dendritic fibers of the spiral ganglion cells [Merchant et al. 2000] and suggested that this selective loss of dendritic fibers was perhaps due to dysfunction of the spiral ligament or a direct “toxic effect” of the deposited material on the dendritic fibers in the osseous spiral lamina. Khetarpal et al. [1991] also suggested a direct deleterious effect on dendritic processes by the deposition of extracellular material in the outer osseous spiral lamina. Robertson et al. [2006] have demonstrated that the extracellular deposits are consistent with mutant cochlin and that obstruction of the channels of the osseous spiral lamina by aggregates of this mutant cochlin may be involved in the pathogenesis of the neural degeneration.

The case presented herein confirms the previous histological findings of other COCH mutations and in addition, given the presence of some preserved hearing at the time of death, supports the conclusion that loss of dendritic fibers and of spiral ganglion cells maybe a primary event rather than secondary to degeneration of other cytological elements. As shown in Figure 5, of all cytologic elements quantified, the dendritic fibers showed the most severe degeneration and in particular more degeneration than that of inner hair cells and of the spiral ganglion cell bodies. This suggests that the loss of dendrites is not secondary to degeneration of inner hair cells or the spiral ganglion cell bodies.

Despite the evidence of degeneration of the neuroepithelium of the vestibular system, the presence of an amorphous deposit in the mesenchymal tissue at the base of the neuroepithelium and partial atrophy of Scarpa’s ganglion cells and degeneration of vestibular dendritic fibers as seen with anti-neurofilament immunostaining, there was no clinical history of balance or vestibular complaints. This was presumed to be due to a slow degenerative process allowing compensation for partial loss of vestibular function.

The physiologic role of cochlin in the normal ear is unknown. The location of expression of COCH in the human, mouse, and chicken inner ear is identical to that described in patients affected with DFNA9, namely the osseous spiral lamina, spiral ligament, limbus, and stroma of the maculae and cristae [Robertson et al. 1998, Robertson et al. 2006]. It has been hypothesized that normal cochlin is involved in antibody independent host defense mechanisms [Bhattacharyya 2006]. Its expression by follicular dendritic cells of the secondary lymphoid organs and also selective localization in the extracellular network in the spleen and lymph nodes [Py et al. 2013] also suggest a role of cochlin as a modulator of the innate immune response to bacteria.
The role of mutant cochlin in the pathogenesis of disease is equally not fully understood. In DFNA9, it appears that the COCH mutations result in aggregation of mutated cochlin as part of the extracellular matrix [Robertson et al. 2006] which may play a role in altering extracellular matrix protein interactions [Bhattacharyya 2006] possibly interfering with ionic homeostasis. Similarly a possible role for cochlin in the causation of glaucoma by aggregated deposits in the trabecular meshwork of the eye has been hypothesized [Bhattacharya 2006].

A “toxic effect” on the dendritic processes of spiral ganglion cells has been previously suggested by Merchant et al. [2000] and Robertson et al. [2006]. The aggregates of mutated cochlin in the inner ear have been suggested as a possible cause of dendritic degeneration and disruption of ionic cycling [Robertson et al. 2006]. The von Willebrand domains have been suggested as playing a role in increased shear induced platelet aggregation [Bhattacharya 2006], possibly resulting in vascular disease and secondarily to degenerative disease. The pathogenic genetic mechanism underlying aggregates of mutated cochlin is thought to represent a “gain of function” rather than COCH haploinsufficiency [Makishima et al. 2005, Robertson et al. 2006, Jones et al. 2011].

Cochlear Implantation in DFNA9

Given the primary neuronal degeneration, at least at the dendritic level in the organ of Corti in DFNA9, it is somewhat surprising that patients treated with cochlear implants for hearing loss due to DFNA9 do at least as well as other cochlear implant patients with other progressive hearing losses as judged by postoperative word recognition scores [Vermeire et al. 2005]. This implies that at least in hearing loss caused by DFNA9, electrical stimulation of the auditory nerve does not require the presence of a normal population of dendritic fibers and may actually occur at a more proximal level, that is, by simulation of the spiral ganglion cell bodies or their central axons.

Acknowledgments

Supported by grants 5R01DC152 (JBN) and 5R01DC03402 (CCM) from the National Institute on Deafness and Other Communication Disorders (NIH).

REFERENCES


Audiol Neurootol. Author manuscript; available in PMC 2017 March 30.
Fig. 1.
Pedigree with propositus marked with red arrow.
Fig. 2.
Audiogram of patient obtained two weeks prior to death.
Fig. 3.
Sanger sequencing chromatograms, showing a heterozygous c.T341C missense mutation (resulting in a p.L114P amino acid change) in COCH exon 5. Overlapping peaks of nucleotides T and C, in the sense strand (top panel), and complementary nucleotides A and G in the anti-sense strand (bottom panel), indicated by arrows, show presence of both the normal and mutated allele.
Fig. 4.
A. Midmodiolar section of left cochlea. The boxed area is shown at higher magnification in B.
B. Section of the organ of Corti (OC) of the middle turn of the left ear. An amorphous deposit (Dep) is seen in the spiral ligament (SL), distal end of the osseous spiral lamina (OSL), and at the base of the limbus (L).
Fig. 5.
Cytocochleogram of the left ear. Missing cytological elements are shown in black. There were missing inner and outer hair cells and some atrophy of the stria vascularis. There was loss of approximately 50 percent of the spiral ganglion cell neurons. However, the dendritic processes between the spiral ganglion cells and organ of Corti showed the most severe degeneration.
Fig. 6. 
MPZ (myelin protein zero) immunostaining of the cochlea.
A. Mid-modiolar section of control right cochlea from patient with no otologic disease.
B. Mid-modiolar section of left cochlea, subject patient.
C. Control right ear, basal turn.
D. Subject patient, left ear, basal turn.
In the control ear (A,C) immunostaining for MPZ was positive for axons (Ax), spiral ganglion (SPG) and dendritic fibers (Den) within the osseous spiral lamina. In the left
cochlea of subject patient (B, D), there was marked reduction of immunostaining of the dendritic fibers (Den).
Fig. 7.
Anti-NF (neurofilament) immunostaining of the cochlea.
A. Midmodiolar section of right cochlea in the control human.
B. Midmodiolar section of the left cochlea in the subject patient.
C. Basal turn of cochlea in the control right ear.
D. Basal turn of cochlea in the left ear of subject patient.
Anti-neurofilament immunostained axons (Ax), spiral ganglion cells (SPG), and dendritic fibers (Den).
Immunostaining of the dendritic fibers was markedly reduced in the cochlea of the subject patient (B,D) as compared to the normal control (A,C).
Fig. 8.
Utricle of left ear of subject patient (H&E). There was a deposit of amorphous material (Dep) in the mesenchymal tissue between the cribrose area and the neuroepithelium of the utricle.
Fig. 9.
Anti-neurofilament immunostaining of the macula sacculi in a control ear (A) and in the subject patient (B). There was a reduction of staining of the dendritic fibers (Den) innervating the saccular neuroepithelium (SN) of the subject patient as compared to the control ear.
Fig. 10.
Incudomallear joint of subject ear. (H&E). A mixture of basophilic and eosinophilic deposits (D) of amorphous material was seen within the joint space.
# Table 1

Published Human Cochlear Histopathology of DFNA9

<table>
<thead>
<tr>
<th>KINDRED</th>
<th>MUTATION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>W117R</td>
<td>Robertson et al. 1998 Merchant et al. 2000</td>
</tr>
<tr>
<td>4</td>
<td>P51S</td>
<td>Robertson et al. 2006</td>
</tr>
</tbody>
</table>