Published in final edited form as:

Otol Neurotol. 2015 June ; 36(5): 923–931. doi:10.1097/MAO.0000000000000680.

Identification of target proteins involved in cochlear Otosclerosis

Céline Richard, MD, PhD^{*}, Joni K. Doherty, MD, PhD^{**}, Jose N. Fayad, MD[∞], Ana Cordero^{**}, and Fred H. Linthicum Jr, MD^{**}

^{*} Department of Otolaryngology, CHU of St Etienne, University of Saint – Etienne, France

^{**} House Temporal Bone Laboratory of UCLA, Los Angeles, United States

[∞]House Clinic, Los Angeles, United States

Introduction

Otosclerosis is a bone disorder characterized by abnormal remodeling of the endochondral layer of the otic capsule.¹ Clinical and histological otosclerosis can be identified as separate entities. Clinical otosclerosis is one of the most common etiologies of acquired hearing loss in the Caucasian population, affecting 0.5%. Clinical otosclerosis manifests as a progressive conductive hearing loss. Otosclerosis may eventually evolve into a mixed hearing impairment if the lesion extends to the cochlear endosteum and involves the spiral ligament (SL), resulting in hyalinization.^{1–4} Clinical progression of the disease is unpredictable and varies, probably due to hereditary factors.⁵ Conversely, histological otosclerosis is ten times more common than clinical otosclerosis worldwide, affecting up to 12.5% in some populations. It is rare in African blacks and Native Americans, but present in up to 2.5% of the Caucasian population.⁶ In Asian populations, histological otosclerosis rarely results in clinical otosclerosis as foci do not occur in the fistula ante fenestrum, or anterior oval window area, resulting in stapes fixation, as is common in Caucasians. Histological otosclerosis is diagnosed on postmortem histological examination and can be divided into three different forms, as the cellularity and bone remodeling features fluctuate: spongiosis, sclerosis, and spongio-sclerosis.

Some authors have suggested an inflammatory etiology for otosclerosis due to persistent measles virus infection of the otic capsule^{7, 8} and have suggested a hereditary predisposition.^{9–11} Heritability suggests an autosomal dominant mode with incomplete penetrance, as 40–50% of cases are familial.⁹ However, differences in inheritance modality were revealed between stapes fixation and cochlear involvement. Genetic linkage analyses have revealed several loci to be involved in otosclerosis, with variable phenotypic expression, emphasizing the extreme heterogeneity of the disease, and the probable interplay of genetic and environmental factors.^{12, 13}

Genome wide association studies (GWAS) and mRNA expression profiling (transcriptome studies) that permit a better understanding of biological processes are underway. However, due to the complexity of gene expression (splicing variants, post-translational modifications, etc.) such techniques can only provide limited insight into the dynamic processes involved.¹⁴ Despite numerous genetic studies, implication of identified genes in the otosclerotic/otospongiosis process remains elusive.¹⁵

Most likely, genetics and environmental factors interplay. It is also possible that different factors are involved in each stage of otosclerosis from spongiosis to sclerosis. Analyzing the expression and functions of proteins is the complement of genomic and transcriptome studies to reveal the components involved in the dynamic process of otosclerosis.¹⁶ Since biopsies of otosclerotic lesions are unrealistic on living human subjects and no adequate murine models have been characterized, analysis of post-mortem human temporal bones can provide clues to the process. Formalin fixed celloidin embedded (FFCE) temporal bone collections provide invaluable data for proteomic analysis via identification of cellular components and enzymes involved in the otosclerosis process. Such studies may, potentially, facilitate genetic associations. Recently, a reliable method for liquid chromatography-mass spectrometry (LC-MS) analysis on formalin fixed celloidin embedded tissues was developed.^{17, 18} We employed this method to study otosclerotic lesions in our FFCE collection, in order to identify proteins involved in the otosclerotic process.

Material and Methods

The St. Vincent Medical Center Internal Review Board approved this protocol. Proteomic analysis was performed on two human temporal bones (hTB), one from a patient with severe otosclerosis and the other from a patient without prior history of otological disease, which served as the case control.

hTB processing

Removal of the temporal bones was performed in both cases within 8 hours post-mortem time and immediately fixed into 10% formalin, then processed routinely via decalcification in ethylenediaminetetraacetic acid (EDTA) followed by celloidin embedding, as previously described.¹⁹ Celloidin blocks were serially cut in 20µm sections and every tenth section was stained with Hematoxylin and Eosin (H&E), while the intervening sections were stored in 80% ethanol. Figure 1 shows the otosclerosis infiltrating the otic capsule on an H&E-stained midmodiolar section from the patient with otosclerosis. Histological analysis of the clinically documented otosclerotic bone revealed minor post-mortem degeneration, oval window otosclerosis and several peri-cochlear foci. Examination of the control bone showed minor post-mortem degeneration and confirmed the absence of any other inner ear anomaly.

For each protein sample, nine serial sections were selected in the midmodiolar location. Sections were pre-treated as previously described,¹⁷ using a bibulous paper soaked in formalin to stick onto Superfrost® Plus slides (Thermo Fisher Scientific, Waltham, MA). Celloidin was removed with acetone for 30 minutes²⁰ and then hydrated with decreasing concentrations of ethanol.¹⁷ Otosclerotic lesions were microdissected using scalpel under an operating microscope from the hTB with otosclerosis, and similar areas of otic capsule were

dissected from the control case, in order to eliminate other structures (e.g., Organ of Corti, modiolus, and nonotic capsule surrounding bone) from the analysis. Microdissected specimens likely contained some adjacent spiral ligament tissue, including hyalinized spiral ligament in some cases of otosclerosis, since the spiral ligament was closely adherent to adjacent otic capsule endosteum. Nine dissected areas of interest were pooled for each sample and transferred to a collection tube to be treated using the modified protocol previously described by Aarnisolo et al.¹⁷ Briefly, extraction buffer from the Liquid Tissue kit® (MS Protein Prep Kit, Expression Pathology, Rockville, MD) was added and samples were incubated at 95°C for 90 minutes, with 5-seconds centrifugations every 20 minutes. Then the samples were centrifuged at 10,000 rcf for 1 minute and cooled on ice before being processed as described in the modified protocol of Aarnisolo et al.¹⁷

Proteomic analysis

Shotgun mass spectrometry (MS) analysis was performed in the proteomics core facility at the University of Southern California (USC; Dr. Ebrahim Zandi, in collaboration), via tandem 2D chromatography MS/MS using an LTQXL Orbitrap (Thermo Fischer) as described in Wang et al.²¹ The MS/MS data analysis and peptide matching to FASTA human databases was done using SEQUEST and proteome discoverer software.

Each sample was desalted using C18 ziptips (Thermo Scientific). Samples were analyzed using an LC/MS system consisting of an Eksigent NanoLC Ultra 2D (Dublin, CA) and Thermo Fisher Scientific LTQ Orbitrap XL (San Jose, CA). Samples were differentially labeled with Cy3 (otosclerosis) and Cy5 (control). Peptides were separated on a 10 cm fuse silica column (75 µm inner diameter) packed in-house with 5 µm C18 beads on an Eksigent NanoLC Ultra 2D system using a binary gradient of buffer A (0.1 % formic acid) and buffer B (0.1% formic acid and 80% ACN). The peptides were loaded directly without any trapping column in buffer A at a flow rate of 300 nL/min. Elution was carried out at a flow rate of 250 nL/min, with a linear gradient of 10% to 35% buffer B for 95 min followed by 50% B for 15 min. At the end of the gradient, the column was washed with 90% B and equilibrated with 5% B for 10 min. The eluted peptides were sprayed into the LTQ Orbitrap XL.²² The source was operated at 2.1-2.25 kV, with no sheath gas flow, with the ion transfer tube at 250°C. MS spectra in the range of m/z 350–2000 were acquired in the orbitrap at a FWHM resolution of 60,000 after accumulation to an AGC target value of 500,000 in the linear ion trap with 1 microscan.

The data-dependent acquisition mode was enabled, and each FTMS MS1 scan (60,000 resolution) was followed by 10 MS2 scans. For all sequencing events, dynamic exclusion was enabled to minimize repeated sequencing. Peaks selected for fragmentation more than once within 60 s were excluded from selection (10 ppm window).

Data Processing and Analysis

Proteome Discoverer 1.3 (Thermo Fisher Scientific) was used for protein identification using Sequest algorithms. The following criteria were followed. For MS/MS spectra, the variable modification of cysteine, carbamidomethylation of cysteine and oxidation of methionine with a maximum of four modifications was allowed. Searches were conducted

against Uniprot or in-house prepared database with PDI sequence. Up to two missed cleavages were allowed for protease digestion and peptide had to be fully tryptic. MS1 tolerance was 10 ppm and MS2 tolerance was set at 0.8 Da. Peptides reported via search engine were accepted only if they met the false discovery rate of 1%. There is no fixed cutoff score threshold, but instead spectra are accepted until the 1% FDR rate is reached. Only peptides with a minimum of six amino acid lengths were considered for identification. We also validated the identifications by manual inspection of the mass spectra. Data comparison was made by Venn diagram software.²³

Immunohistochemistry

Immunostaining was performed as described.²⁴ Briefly, sections were floated onto cigarette paper into a dish filled with 80% ethanol, before being placed face down onto a subbed slide, covered with a piece of bibulous paper, and flatten with a roller. Then, the first bibulous paper was removed and replaced by another one soaked into 10% formalin, wood blocks and 500g lead weights were added over the bibulous paper. Sections were left to dry in air for 1 hour. A fresh preparation of sodium methoxide (50 gm NaOH pellets dissolved in 50 ml of methanol and then diluted 1:2 in methanol) was added twice for 15 minutes with a 100% methanol rinse in between. Sections were placed into a bath of absolute methanol for 3 minutes, then twice into a bath of absolute ethanol before rehydrating the sections into decreasing baths of ethanol (95%, 80%, 70% and 50%) for 3 minutes each. The sections were washed with DiH₂O and then 3 times in Tween TBS for 5 minutes before blocking the sections with 10% normal goat serum (Invitrogen®) for 60 minutes. Primary antibodies were incubated with sections overnight at 4° C. The following primary antibodies were used at dilutions indicated in parentheses (listed in Table 4): alpha-actinin 4 (ACTN4; Epitomics, Abcam, CA; 1:100); alpha-actinin 1 (ACTN1; Novus Biologicals®, CO; 1:200), sialyltransferase (ST6 Gal2; R&D Systems; 1:200), heat shock protein 90 kDa alpha, class B member 1 (Abgent; 1:50); heat shock protein beta 1 (Biossenses; 1:500), keratinocyte proline-rich protein (KPRP; Abcam; 1:200), Ribophorin II (Abcam; 1:350), superoxide dismutase (SOD; Abcam; 1:500), transforming growth factor beta 1 (TGFβ1) monoclonal antibody (p Tyr369, Novus Biologicals®, CO; 1:100), Ubiquitin (10C2-2; Novus Biologicals®, CO; 1:50 and 1:100), collagen type VI alpha 3 (COL6A3; Sigma; 1:100), and Pigmented-epithelium derived factor (PEDF; Millipore; 1:500). The following day, sections were washed 3 times with Tween TBS for 5 minutes each and goat-anti-mouse (for mouse monoclonal primary antibodies; Table 4), goat-anti-rabbit (for rabbit primary antibodies; Table 4), or goat-anti-sheep (for sialyltransferase primary antibody only) secondary antibody (Biocare®) was added for 60 minutes. Between 2 steps of 3 Tween TBS washes of 5 minutes each, sections were floated into AP Strept-avidin (Biocare®) for 60 minutes. Vulcan Fast Red (Biocare®) was used to reveal the staining, then a few drops of Hematoxylin counterstain was added and the sections were mounted. Immunostaining with each antibody was performed on two control sections and two otosclerotic sections from the infiltrated otic capsule region of hTB#834.

After verification of protein expression, several more hTB specimens were selected for analyses based on the presence of cochlear otosclerosis, SL hyalinization, and low post-mortem degeneration (i.e., bones that were harvested within a short period after death and,

therefore, with relatively good preservation of proteins). We selected fourteen additional cases and performed immunostaining for TGF β 1 directed against the extra-cellular matrix, TGF β 1, Ribophorin II, sialyltransferase, superoxide dismutase, HSP90, COL6A3, Ubiquitin, and Alpha-actinin 1, since these antibodies demonstrated reactivity in hTB#834.

Results

Proteomic analysis

Samples were analyzed in duplicate. Figure 2 displays the results of each experiment and the combined results, showing that 21 proteins were found (i.e., overlapping) in both normal otic capsule bone (hTB#773) and otosclerosis-infiltrated otic capsule (hTB#834), and 48 unique proteins were identified in otosclerosis only. Tables 1-3 list the proteomic analysis results. Proteins (Table 1) or predicted cDNA of identified peptides that are uncharacterized (Table 2) known to be involved in cell death, in cell proliferation or in both pathways were discovered in the otosclerosis bone and not in the control. In the otosclerotic bone, the most abundant protein identified was XTP3TPA-transactivated protein, an enzyme that hydrolyzes deoxynucleoside triphosphates (dNTPs) to the corresponding nucleoside, whereas tubulin alpha 1b (TUBA1B) protein was predominant in the control bone, although these two were present in both samples. Cochlin was also identified in both bones, as expected based on its abundant expression within the inner ear. Table 3 shows proteins that were found in the normal bone and not in the otosclerosis. Table 4 lists proteins of interest unique to the otosclerosis sample that were selected for further analysis. The selection was based on availability of antibodies directed against human isoforms and useful in immunohistochemistry (i.e., tested on paraffin-embedded human tissues, in most cases).

Immunostaining

Among the proteins of interest identified in the otosclerotic lesion and not in the control were TGF β 1, Ribophorin II, sialyltransferase, superoxide dismutase, and HSP90. Ubiquitin and COL6A3 stained diffusely throughout the otosclerosis and normal bone (not shown); however, the staining was weak. Alpha-actinin 1 stained strongly positive in otosclerotic lesions and in several foci within surrounding 'unaffected' bone (not shown). We did not achieve positive immunoreactivity with any of the other antibodies studied (not shown). The otosclerotic lesion and also hyalinized spiral ligament displayed reactivity with TGF β 1 and Ribophorin II, as well as sialyltransferase, whereas normal bone showed very little reactivity (Figure 3). Table 5 displays the results of the immunostaining panel on each of the ten selected otosclerosis cases. Notably, TGF β 1 was significantly positive in nine out of 15 hTB cases (60%) and also reacted with hyalinized SL in five or one-third of cases (33%). Ribophorin II also showed abundant expression via immunohistochemistry in nine of 15 hTBs (60%), but the expression did not always coincide with TGF β 1 expression (see Table 5).

Discussion

Previous studies have described efficient methods to achieve proteomic analysis from formalin fixed, paraffin embedded,^{25, 26} and celloidin-embedded tissues.¹⁷ However, it

appears that the protein identification rate is lowered by factors inherent in the use of celloidin-embedded tissues that increase sample complexity. Particularly, the methods required to remove celloidin can lead to protein degradation and/or modification.¹⁷ In addition, the use of the mass spectrometer is limited in detecting low-abundance peptides in a sample.²⁷

Aware of this potential bias, we performed proteomic analysis on our collection samples to reveal proteins involved in the otosclerotic lesion and realized further analysis of protein expression using immunohistochemistry on selected proteins with available antibodies. After verifying selective expression within the otosclerotic lesion and not in normal or uninvolved bone, we screened several other cases from our temporal bone collection via immunohistochemistry. This analysis revealed TGF β 1 to be consistently expressed in a majority of the otosclerotic foci and hyalinized spiral ligament and not in the corresponding areas of control samples. TGF β 1 is known to be one of the major osteogenic cytokines involved in bone mass and bone matrix regulation in long bones²⁸ and has been implicated in the pathogenesis of otosclerosis by other investigators.^{13, 29} TGF β 1 is expressed as a secreted cytokine in its active form. It is secreted by osteoblasts and stored within the bone matrix of the otic capsule, as well as other skeletal bones.²⁸ Our results suggest that TGF β 1 is a major factor in the development and/or progression of otosclerosis.

Several major studies attempted to identify genetic factors in otosclerosis. However, although many different genetic loci have been identified, no causative gene reliably mapped to the disease across populations. A possible role of TGF β 1 was identified in a study of proteins involved in progression of the otosclerotic process.^{13, 29}

The hormonally active peptide TGF β 1, is known to induce several different actions in connective tissues, such as the formation of collagen and fibronectin in mesenchymal cells.^{30, 31} TGF β 1 is involved in connective tissue synthesis by increasing the promoter activity of fibronectin and collagen type I genes.^{32, 33} Conversely, TGF β 1 is involved in connective tissue degradation, as well.³⁴ TGF β 1 overexpression could affect SL fibrocytes, interfering with potassium circulation, as described by Weber et al,³⁵ and, at least partially, explain the sensorineural hearing loss observed in cases involving the endosteum.⁴ Similar functions have been observed during otic capsule chondrogenesis: TGF β 1 regulates otic capsule growth and remodeling, by enhancing chondrogenesis of the mesenchyme and promoting otic capsule growth in early stages of inner ear development, whereas inhibition of TGF β 1 is required for perilymphatic space formation.³⁶ Residual cartilage rests within the dense otic capsule bone, termed globbuli interossei, are unique to the inner ear and have been speculated to be the target of an immune reaction, resulting in otosclerosis. Recently, a genetic study suggested the implication of TGF β 1 in otosclerosis, some variants of the TGF β 1 gene being involved in an increase of the patient's susceptibility to otosclerosis.³⁷ Evidence of a possible association of a TGF β 1 polymorphism and otosclerosis involving stapes ankylosis has been shown³⁸ with the implication of bone morphogenetic proteins (BMP) 2, 4, 5, and 7, which are members of the TGF β superfamily.^{29, 39} BMP 5 and 7 may play a predominant role in the active part of the stapedia involvement with otosclerosis, its pattern evolving with the disease.³⁹ Although otosclerotic stapes ankylosis can be limited to the oval window area, without any cochlear extension, these lesions likely share similar

pathophysiology as the present study suggests. Furthermore, a recent case-controlled genetic study revealed that the TGF β 1 polymorphism c.-509C> provides a disease-causing effect in the Indian population, and mRNA analysis demonstrated increased expression of TGF β 1 in ankylotic stapes specimens compared with controls.³⁹ Mechanisms involved in cochlear extension of the otosclerotic lesion are still unclear, but the implication of TGF β 1 is supported by the present proteomic data and confirmed by our immunostaining results as well. No previous studies of which we are aware have shown the implication of TGF β 1 in the cochlear otosclerotic lesion with SL hyalinization. The established role of TGF β 1 in the chondrogenesis process supports the theory of an immunogenic reaction targeting the globulae interossei within the otic capsule.

Interestingly, Ribophorin II, which was identified in otosclerosis via proteomics also appeared to be expressed in some of the otosclerotic hTBs and in hyalinized SL. Ribophorin II is a type I integral membrane protein that plays a role in proteasome function as a component of the RAMP N-oligosaccharyl transferase (OST) complex that is ubiquitous in eukaryotic cells.⁴⁰ Ribophorin II also participates in glycosylation of nascent proteins as part of the OST.⁴¹ While the role of Ribophorin II in otosclerosis is not known, it has been identified as a target gene of TGF β 1.⁴² Additionally, Ribophorin II has been implicated in pulmonary fibrosis and has recently been identified for molecular-targeted therapy in the treatment of lung cancer.⁴³

Sialyltransferase also showed positive staining, particularly in hyalinized SL (Figure 3D). Sialyltransferase is a ubiquitous cytosolic enzyme involved in glycosylation of cellular proteins, and it has been identified in several epithelial cancers, including breast, due to its overexpression.⁴⁴ Thus, it may play a role in cochlear endosteal invasion with otosclerosis.

The presence of both heat shock protein 90 (hsp90) and superoxide dismutase (SOD) suggest an inflammatory process. Heat shock protein is upregulated in inflammatory diseases, and SOD catalyzes the breakdown of superoxide radicals to oxygen and hydrogen peroxide. It has also been identified in some cancers, where it is upregulated and has been recently implicated in promoting metastasis.⁴⁴ Hsp90 and SOD are ubiquitous and expressed in the cytosol of all cell types; SOD is localized within mitochondria.⁴⁵

Aside from the identified proteins that were analyzed, many cDNAs of uncharacterized proteins were predicted via proteomic analysis, with implications in cell death and/or proliferation (see Table 2), suggesting a possible interaction with otosclerotic bone remodeling. While this work is preliminary towards understanding the dynamic processes dictating otosclerosis pathophysiology, integration of proteomic and genetic data may help us reveal specific processes involved in otosclerosis development and progression.

Conclusion

The otosclerotic process remains elusive and is likely multi-factorial, involving the interaction of complex autoimmune and genetic factors. The ability of proteomic methods to identify proteins involved in such a complex pathology will enable determination of their respective genes that may play a role in otosclerosis development. Through directed

proteomic analysis, we have identified TGF β 1 in otosclerosis with cochlear extension and spiral ligament hyalinization. This finding corroborates other evidence linking TGF β 1 to the otosclerotic process and provides further insight into its pathophysiological process. In addition, TGF β 1 may provide a potential therapeutic target to prevent the development and extension of otosclerosis in susceptible individuals.

Acknowledgments

Supported in part by NIH grant U24 DC008559 and grant “Perspectives ORL 2010, Sanofi-Aventis”. We graciously thank Jennifer O'Malley and Antti Aarnisalo for their expertise, support, and guidance regarding the protein extraction methods used on celloidin-embedded temporal bones.

Supported by NIH grant number: U 24 DC 011962 HO 1

Supported by grant “Perspectives ORL 2010, Sanofi-Aventis”

List of abbreviations

hTB	human temporal bone
FFCE	Formalin fixed celloidin embedded tissues collections
EDTA	Ethylenediaminetetraacetic acid
TGFβ1	transforming growth factor beta 1
SL	spiral ligament

References

1. Chole RA, McKenna M. Pathophysiology of otosclerosis. *Otol Neurotol*. 2001; 22(2):249–57. [PubMed: 11300278]
2. Kelemen G, Linthicum FH Jr. Labyrinthine otosclerosis. *Acta otolaryngol suppl*. 1969; 253:1–68. [PubMed: 5267215]
3. Töpsakal V, Fransén E, Schmerber S, et al. Audiometric analyses confirm a cochlear component, disproportional to age, in stapedial otosclerosis. *Otol Neurotol*. 2006; 27(6):781–7. [PubMed: 16885785]
4. Doherty JK, Linthicum FH Jr. Spiral ligament and stria vascularis changes in cochlear otosclerosis: effect on hearing level. *Otol Neurotol*. 2004; 25:457–464. [PubMed: 15241221]
5. Weegerink NJD, Schrauwen I, Huygen PM, et al. Phenotype of the first otosclerosis family linked to OTSC10. *Laryngoscope*. 2011; 121(4):838–45. [PubMed: 21433022]
6. Declau FM, Van Spaendonck JP, Timmermans L, et al. Prevalence of otosclerosis in an unselected series of temporal bones. *Otol Neurotol*. 2001; 22(5):596–602. [PubMed: 11568664]
7. McKenna MJ, Mills BG, Galey FR, Linthicum FH Jr. Filamentous structures morphologically similar to viral nucleocapsids in otosclerotic lesions in two patients. *Am J Otol*. 1986; 7(1):25–8. [PubMed: 3946578]
8. Arnold W, Niedermeyer HP, Lehn N, Neubert W, Höfler H. Measles virus in otosclerosis and the specific immune response of the inner ear. *Acta Otolaryngol*. 1996; 116(5):705–9. [PubMed: 8908246]
9. Van Den Bogaert KP, Govaerts J, De Leenheer EMR, et al. Otosclerosis: a genetically heterogeneous disease involving at least three different genes. *Bone*. 2002; 30(4):624–30. [PubMed: 11934656]
10. Thys M, Van Den Bogaert K, Iliadou V, et al. A seventh locus for otosclerosis, OTSC7, maps to chromosome 6q13-16.1. *Eur J Hum Gen: EJHG*. 2007; 15(3):362–8.

11. Insaf BHA, Thys M, Beltaief N, et al. A new locus for otosclerosis, OTSC8, maps to the pericentromeric region of chromosome 9. *Hum Gen.* 2008; 123(3):267–72.
12. Ealy M, Smith RJH. The genetics of otosclerosis. *Hear Research.* 2010; 266(1- 2):70–4.
13. Thys M, Schrauwen I, Vanderstraeten K, et al. Detection of rare nonsynonymous variants in TGFBI in otosclerosis patients. *Ann Hum Gen.* 2009; 73(2):171–5.
14. Zheng QY, Rozanas CR, Thalmann I, et al. Inner ear proteomics of mouse models for deafness, a discovery strategy. *Brain Res.* 2006; 1091(1):113–21. [PubMed: 16600193]
15. Markou K, Goudakos J. An overview of the etiology of otosclerosis. *Eur Arch Otorhinolaryngol.* Jan; 2009 266(1):25–35. [PubMed: 18704474]
16. Thalmann I. Inner ear proteomics: a fad or hear to stay. *Brain Res.* May 26; 2006 1091(1):103–12. [PubMed: 16540098]
17. Aarnisalo AA, Green KM, O'Malley J, et al. A method for MS(E) differential proteomic analysis of archival formalin-fixed celloidin-embedded human inner ear tissue. *Hear Res.* Dec 1; 2010 270(1-2):15–20. [PubMed: 20708670]
18. Makarem AO, Hoang TA, Lo WW, et al. Cavitating otosclerosis: clinical, radiologic, and histopathologic correlations. *Otol Neurotol.* 2010; 31:381–384. [PubMed: 20195188]
19. Schuknecht, HF. *Pathology of the Ear.* 2nd Edition.. Lea and Febiger; Philadelphia: 1993. p. 8-11.
20. Keithley EM, Horowitz S, Ruckenstein MJ. Na, K-ATPase in the cochlear lateral wall of human temporal bones with endolymphatic hydrops. *Ann. Otol. Rhinol. Laryngol.* 1995; 104:858–863. [PubMed: 8534024]
21. Wang LC, Okitsu CY, Kochounian H, et al. A simple and inexpensive oncolumn frit fabrication method for fused-silica capillaries for increased capacity and versatility in LC-MS/MS applications. *Proteomics.* May; 2008 8(9):1758–61. [PubMed: 18384106]
22. Zhou Y, Yi T, Park SS, et al. Rapid and enhanced proteolytic digestion using electric-field-oriented enzyme reactor. *J Proteomics.* Jun 10; 2011 74(7):1030–5. [PubMed: 21338726]
23. Oliveros, JC. VENNY.. An interactive tool for comparing lists with Venn Diagrams. 2007. Available at:<http://bioinfogp.cnb.csic.es/tools/venny/>
24. Tian Q, Linthicum FH Jr, Keithley EM. Application of labeling techniques to archival temporal bone sections. *Ann Otol Rhinol Laryngol.* 1999; 108(1):47–53. [PubMed: 9930540]
25. Hood BL, Darfler MM, Guiel TG, et al. Proteomic Analysis of Formalin-fixed Prostate Cancer Tissue. *Mol Cell Proteomics.* 2005; 4(11):1741–53. [PubMed: 16091476]
26. Palmer-Toy ED, Krastins B, Sarracino DA, Nadol JB Jr, Merchant SM. Efficient method for the proteomic analysis of fixed and embedded tissues. *J Proteome Res.* 2005; 4(6):2404–11. [PubMed: 16335994]
27. Prakash A, et al. Assessing bias in experiment design for large scale mass mass spectrometry-based quantitative proteomics. *Mol Cell Proteomics.* 2007; 6:741–8.
28. Balooch G, Balooch M, Nalla RK, Schilling S, Filvaroff EH, et al. TGFbeta regulates the mechanical properties and composition of bone matrix. *Proc Natl Acad Sci U S A.* 2005; 102:18813–18818. [PubMed: 16354837]
29. Schrauwen I, Thys M, Vanderstraeten K, et al. Association of bone morphogenetic proteins with otosclerosis. *J Bone Miner Res.* 2008; 23:507–16. [PubMed: 18021008]
30. Ignatz RA, Massagué J. Transforming Growth Factor-beta Stimulates the Expression of Fibronectin and Collagen and Their Incorporation into the Extracellular Matrix. *J Biol Chem.* 1986; 261(9):4337–45. [PubMed: 3456347]
31. Varga J, Jimenez SA. Stimulation of normal human fibroblast collagen production and processing by transforming growth factor-beta. *Biochemical and biophysical research communications.* 1986; 138(2):974–80. [PubMed: 3461787]
32. Rossi P, Karsenty G, Roberts AB, et al. A nuclear factor 1 binding site mediates the transcriptional activation of a type I collagen promoter by transforming growth factor-beta. *Cell.* 1988; 52(3): 405–14. [PubMed: 2830985]
33. Ritzenthaler JD, Goldstein RH, Fine A, et al. Transforming-growth-factor-beta activation elements in the distal promoter regions of the rat alpha 1 type I collagen gene. *Biochem J.* 1991; 280:157–62. [PubMed: 1741743]

34. Laiho M, Saksela O, Andreassen PA, Keski-Oja J. Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factorbeta. *J Cell Biol.* 1986; 103:2403–10. [PubMed: 3491081]
35. Weber PC, Cunningham CD 3rd, Schulte BA. Potassium recycling pathways in the human cochlea. *Laryngoscope.* 2001; 111(7):1156–65. [PubMed: 11568535]
36. Frenz DA, Van de Water TR, Galinović-Schwartz V. Transforming growth factor beta: does it direct otic capsule formation? *Ann Otol Rhinol Laryngol.* 1991; 100:301–7. [PubMed: 2018288]
37. Priyadarshi S, Ray CS, Panda KC, et al. Genetic association and gene expression profiles of TGFB1 and the contribution of TGFB1 to otosclerosis susceptibility. *JBone Miner Res.* Dec; 2013 28(12):2490–7. [PubMed: 23703862]
38. Thys M, Schrauwen I, Vanderstraeten K, et al. The coding polymorphism T263I in TGF-beta1 is associated with otosclerosis in two independent populations. *Hum Mol Gen.* 2007; 2007; 16(17): 2021–30. [PubMed: 17588962]
39. Csomor P, Liktó B, Szekanecz Z, et al. Expression of bone morphogenetic protein 2, 4, 5, and 7 correlates with histological activity of otosclerotic foci. *Acta oto-laryngologica.* 2012; 132(6):624–31. [PubMed: 22385409]
40. Dunn WA. Studies on the mechanism of autophagy: formation of the autophagic vacuole. *J Cell Biol.* 1990; 110:1923–33. [PubMed: 2351689]
41. Nabokikh A, Ilhan A, Bilban M, et al. Reduced TGFB1 expression and its target genes in human insulinomas. *Exp Clin Endocrinol Diabetes.* 2007; 115:674–82. [PubMed: 18058603]
42. Fujita Y, Takeshita F, Ochiya, et al. A novel platform to enable inhaled naked RNAi medicine for lung cancer. *Sci Rep.* 2013; 3:3325. [PubMed: 24270189]
43. Takahashi R. Ribophorin II regulates breast tumor initiation and metastasis through the functional suppression of GSK3β. *Sci. Rep.* 2013; 3:2474. [PubMed: 23959174]
44. Wang PH. Altered glycosylation in cancer: Sialic acid and sialyltransferases. *J Cancer Mol.* 2005; 1(2):73–81.
45. Kamarajugadda S, Cai Q, Chen H, et al. Manganese superoxide dismutase promotes anoikis resistance and tumor metastasis. *Cell Death Dis.* 2013; 4:e504. [PubMed: 23429290]

Figure 1.
Two representative H&E-stained sections from hTB#834 with cochlear otosclerosis infiltrating otic capsule (A, B).

Figure 2. FFCE human temporal bone data. Here are shown the number of proteins (unique and overlapping) obtained for the otosclerotic bone (OTO) and the control one (CONTROL).

Figure 3.

Representative sections showing immunohistochemistry results with otosclerotic hTB#834: control (A); TGF β 1 (B); HSP90 (C); sialyltransferase reacting with SL hyalinization (D, arrows); Ribophorin II (E, F); and Superoxide Dismutase (H, I, J). oto = otosclerotic bone; nl = normal bone.

Table 1

Proteins identified that were unique to the otosclerotic bone (i.e., not identified in the control otic capsule).

Accession	Protein Name	Molecular Function
Q96BG6	ACTN4 protein	metal ion binding, protein binding
Q5ZEZ4	Alpha-actinin 1	calcium ion binding
Q8NDV1	Alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	sialyltransferase activity
Q0QEN7	ATP synthase subunit beta	catalytic activity, metal ion binding, nucleotide binding, protein binding, receptor activity, signal transducer activity, transporter activity
B7ZMJ7	COL6A3 protein	enzyme regulator activity, protein binding
Q5T9W6	Heat shock protein	catalytic activity, enzyme regulator activity, nucleotide binding, protein binding
P04792	Heat shock protein beta-1	enzyme regulator activity, protein binding
Q16777	Histone H2A type 2-C	DNA binding
Q6B823	Histone H4	DNA binding
Q5TBF5	Osteoglycin (Fragment)	protein binding
P36955	Pigment epithelium-derived factor	enzyme regulator activity
O43610	Protein sprouty homolog 3	protein binding
A8MWG3	Putative uncharacterized protein DCN	protein binding
Q5CZ99	Putative uncharacterized protein DKFZp686I1370	catalytic activity, protein binding, structural molecule activity
Q5JYR4	Ribophorin II	catalytic activity, protein binding
P62314	Small nuclear ribonucleoprotein Sm D1	protein binding, RNA binding
Q14916	Sodium-dependent phosphate transport protein 1	metal ion binding, transporter activity
B2R9V7	Superoxide dismutase	antioxidant activity, catalytic activity, metal ion binding, protein binding
Q5PY19	Transforming growth factor beta 1	protein binding, transcription regulator activity
Q71U36	Tubulin alpha-1A chain	GTP binding
P62988	Ubiquitin	catalytic activity, protein binding, transcription regulator activity
B0YJC4	Vimentin variant 3	catalytic activity, motor activity, protein binding, structural molecule activity
Q1KSF8	XTP3TPA-transactivated protein 1	structural molecule activity

Table 2

Predicted cDNA identified as peptides in the otosclerotic bone (i.e., proteins not characterized).

Accession	cDNA and related protein	Molecular Function
B3KPF0	cDNA FLJ31712 fis, clone NT2RI2006445, highly similar to Insulin-like growth factor-binding protein 3	enzyme regulator activity, metal ion binding, protein binding
B3KRQ7	cDNA FLJ34709 fis, clone MESAN2003101, highly similar to Growth-arrest-specific protein 6	metal ion binding, protein binding, signal transducer activity
B3KSE6	cDNA FLJ36080 fis, clone TESTI2019872, highly similar to CLUSTERIN	catalytic activity, protein binding
B7Z6G1	cDNA FLJ50671, highly similar to Periostin	-
B4DNG6	cDNA FLJ52002, highly similar to Annexin A5	enzyme regulator activity, metal ion binding, protein binding
B4DTB1	cDNA FLJ52936, weakly similar to Tropomyosin alpha-4 chain	metal ion binding, protein binding, structural molecule activity
B4DMJ5	cDNA FLJ53012, highly similar to Tubulin beta-7 chain	catalytic activity, nucleotide binding, protein binding, structural molecule activity
B4E304	cDNA FLJ53962, highly similar to AP-2 complex subunit mu-1	-
B4DGW3	cDNA FLJ54625, highly similar to ATP synthase subunit alpha, mitochondrial	catalytic activity, metal ion binding, nucleotide binding, protein binding, transporter activity
B4DQH5	cDNA FLJ57806, highly similar to Sushi repeat-containing protein SRPX	protein binding
B4DNL4	cDNA FLJ59360, moderately similar to Biglycan	protein binding, structural molecule activity
B4E1W8	cDNA FLJ60213, highly similar to Tenascin	protein binding
B4DN61	cDNA FLJ60723, highly similar to Olfactomedin-like protein 1	protein binding
A8K7Q2	cDNA FLJ77848	catalytic activity, nucleotide binding, protein binding, transcription regulator activity

Table 3

Proteins identified in normal bone (i.e., not identified in otosclerosis).

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides
B0YJC4	Vimentin variant 3	40,46	17,87	8	7
P04004	Vitronectin	35,40	10,88	2	4
P00734	Prothrombin	29,82	6,27	5	3
D3DTX7	Collagen, type I, alpha 1, isoform CRA	29,09	2,49	1	2
P08123	Collagen alpha-2(I) chain	15,66	2,42	1	2
C0IMJ4	Periostin isoform thy8	15,51	5,13	9	2
Q3L3Q5	Truncated beta-globin	15,07	72,50	50	2

Table 4

Selected List of proteins for further investigation: verification of expression via immunohistochemistry.

Protein	acronym	antibody	manufacturer	dilution
Alpha-actinin 4	ACTN4	Polyclonal	Epitomics, Abcam	1:100
Alpha-actinin 1	ACTN1	monoclonal	Novus Biologicals	1:200
Sialyltransferase	ST6 Gal2	Polyclonal (sheep)	R&D Systems	1:200
Heat shock 90 AB1	HSP90	polyclonal	Abgent	1:50
Heat shock beta 1	HSP β 1	polyclonal	Biosenses	1:500
Keratinocyte proline-rich	KPRP	polyclonal	Abcam	1:200
Ribophorin II	RBII	polyclonal	Abcam	1:350
Superoxide dismutase	SOD	polyclonal	Abcam	1:500
Transforming growth factor beta 1	TGF β 1	polyclonal	Novus Biologicals	1:100
Ubiquitin	UB	monoclonal	Novus Biologicals	1:50
Collagen type VI alpha 3	COL6A3	polyclonal	Sigma	1:100
Pigment-epithelium derived factor	PEDF	monoclonal	Millipore	1:500

Table 5

Immunohistochemistry results using a panel of antibodies on hTBs that demonstrated cochlear otosclerosis and spiral ligament hyalinization.

	<i>Subcellular Location</i>	TGFB1	RBII	HSP90	SOD	COL6A3	ST6Gal2	ACTN1	UB	PEDF
		Extracellular Matrix	Membrane of the rough Endoplasmic Reticulum	•Cytoplasmic in interphase cells •Translocates to the nucleus during heat shock	Mitochondria	Extracellular Matrix	Golgi Apparatus	Cytoskeleton	Cytoplasm	Melanosome
	<i>Cell/Tissue</i>	Osteoblast	ubiquitous	•Inner and Outer Hair Cells •Osteoblasts	All tissues of the inner ear		Bone marrow	•Abundant in Outer hair Cells, •Sparse in Inner Hair cells	Found in almost all tissues	•Basilar membrane •Lateral wall •Bone cells during endochondral bone formation
hTB	834	+++	+++	+	+	NS	+	+	NS	NS
	798	+++	++	NS	-	NS			NS	NS
	734	NS	-	NS	-				-	NS
	673	++	++	NS	NS	NS			NS	NS
	648	+	-		-	NS			NS	NS
	577		+							
	556	NS	-		-		++			NS
	515	NS	++							NS
	425	+	++					+		
	411	NS	++					+		
	361	+	+							
	347	NS	+			NS		+		NS
	331	++					++			
	312	+								
296	++	-						+		

NS = nonspecific staining (i.e., uninvolved bone also positive or uninvolved bone positive with less intense staining in otosclerotic lesion); - = nonreactive; + = otosclerotic bone stained positively; ++ = otosclerotic bone lesion and SL stained positively; +++ = otospongiosis (active lesion), otosclerosis, and SL stained positively. TGFB1 = transforming growth factor beta 1; RBII = ribophorin II; HSP90 = heat shock protein 90kD; SOD = superoxide dismutase; COL6A3 = collagen VI alpha 3; ST6Gal2 = sialyltransferase; ACTN1 = alpha actinin 1; UB = ubiquitin; PEDF = pigmented epithelium-derived factor.