Downregulation of Calreticulin and Annexin A2 in Cholesteatoma by 2-DE Analysis

Kuen-Yao Ho

Kaohsiung Medical University

Kuo-Feng Hung

Yu Jun Biotechnology Co. Ltd.

Chen-Yu Chien

Kaohsiung Medical University

Chee-Yin Chai

Kaohsiung Medical University

Wan-Tzu Chen

Kaohsiung Medical University Chung Ho Memorial Hospital

Hsun-Mo Wang

Kaohsiung Municipal Siaogang Hospital

Shih-Meng Tsai

Kaohsiung Medical University

Leong-Peng Chan

Kaohsiung Municipal Ta-Tung Hospital

Ning-Chia Chang (✉ aka@ms.kmuh.org.tw)

Kaohsiung Medical University <https://orcid.org/0000-0002-3191-154X>

Yu-Jen Wu

Meiho University

Research

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Abstract

Background: Many factors are thought to be associated with the development of cholesteatoma, while the mechanisms of its formation remain unclear. This study aimed to identify the potential mechanisms of the proliferation and growth of cholesteatoma by analysis of the differential expressions of proteins in cholesteatoma and retroauricular skin tissue collected from patients. **Methods:** Comparative proteomics analyses using two-dimensional gel electrophoresis (2-DE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), in addition to immunohistochemical analysis, were conducted to identify differentially-expressed proteins in cholesteatoma tissue as compared with retroauricular skin tissue. Western blotting was also employed to verify the expression patterns of the specific proteins identified by 2-DE and to measure the changes in potential modulators related to cholesteatoma proliferation and growth. **Results:** Calreticulin (CRT) and annexin A2 (AnxA2) were identified as being differentially-expressed in cholesteatoma by 2-DE and LC-MS/MS, the results of which were in agreement with the results of immunohistochemical analysis and western blotting. Downregulation of CRT and AnxA2 were observed in cholesteatoma. **Conclusion:** Our data suggested that CRT and AnxA2 were associated with cholesteatoma. We speculated that the reduced expression of CRT and the persistent inflammatory response play important roles in the epithelial proliferation of cholesteatoma.

Background

Cholesteatoma is an epidermal inclusion cyst formed by the squamous epithelium in the middle ear, characterized by accumulation of exfoliated keratin. Cholesteatoma is histologically composed of an epithelial matrix and a connective tissue prematrix, with persisting inflammation. In normal tissue, a state of dynamic equilibrium is maintained by cell proliferation and apoptosis; however, this homeostasis is impaired in cholesteatoma, resulting in keratin accumulation(1).

Several characteristics that are normally associated with neoplastic cells, including invasion, migration, altered differentiation, aggressiveness, and potential recurrence, are also known features of cholesteatoma(2, 3) Patients with cholesteatoma may develop hearing loss, vestibular dysfunction, facial paralysis and terminal intracranial complications(4–6). Over recent decades, many theories regarding cholesteatoma development have been proposed, enabling otologists to improve treatment. However, some processes are still unclear. Recent studies of the pathogenesis of acquired cholesteatoma that focused on epithelial hyperproliferation, altered differentiation, apoptosis mechanisms and inflammation provided more detail at the cellular and molecular levels regarding the pathogenesis of cholesteatoma(4, 6–10).

Several studies proposed that cholesteatoma is linked to osteoclast activation and a number of cellular responses(11–13). Multiple theories are currently acceptable models for cholesteatoma formation, but the surfeit of possible processes render the pathogenesis of cholesteatoma a molecular and cellular puzzle. Prevention of complications and the recurrence of cholesteatoma are the main issues of concern for otologists today. Identifying differentially-expressed proteins in cholesteatoma may uncover the mechanisms of cholesteatoma formation, and could therefore benefit the development of new treatments for the disease.

At present, there are still few studies on Cholesteatomas using proteomic analysis methods. Previous studies using 2D electrophoresis, MALDI-TOF/MS analysis and Immunohistochemical staining found that FKH 5–3 and titin are more abundant in congenital cholesteatoma tissue. It is speculated that congenital cholesteatoma origins may differ from those of acquired cholesteatomas, which originate from retraction pocket Epithelia(14). Randall et al. used mass-based spectrometry proteomic approach to assess relative changes between proteins found in the middle ear mucosa and postauricular skin relative to the cholesteatoma stroma. In addition, the inclusion of the middle ear mucosa increases the

amount of novel protein in the matrix compared to postauricular skin. Many of the potential biomarkers found in the study were used to assess residual or recurrent disease, particularly BLMH, TYMP, FLBP5, FLG / FLG2 and CKAP4(15).

The authors' results of extensive bioinformatics analysis of the first large-scale proteomics study of cholesteatoma. Proteomics studies were implicated suggest several altered biological processes associated with the pathology of cholesteatoma. Down-regulation of several extracellular matrices and basement membrane proteins (such as COL18A1 and NID2) observed may have a major impact on tissue integrity. The up-regulation of ELANE and pro-inflammatory S100 proteins (eg S100A7A and S100A7) is mainly regulated biological areas in cholesteatoma(16).

In the present study, we used a comparative proteomics approach to distinguish proteins that were differentially-expressed in cholesteatoma tissue in comparison with retroauricular skin tissue obtained from patients.

In the current study, we employed 2-DE and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to investigate proteins differentially-expressed in cholesteatoma as compared with normal retroauricular skin, and elucidated their pathophysiological significance, with the aim of identifying new targets for cholesteatoma treatment.

Materials And Methods

Sample collection

Tissue samples from five patients (one male and four females) with a mean age of 56.8 years (range, 40–65 years) were analyzed in this study (Table 1). Tissues were collected between May 2009 and July 2011 at the Department of Otorhinolaryngology, Kaohsiung Medical University Hospital. Informed consent was obtained from all participants. The surgically-removed cholesteatoma specimens were resected from all patients during surgery, and retroauricular skin samples of the patients were also collected as controls.

Protein extraction and two-dimensional gel electrophoresis

Each sample (1 mm × 1 mm × 1 mm) was homogenized with 50 mM Tris-HCl buffer (pH 8.0, 10 mM EDTA and 10 mM protease cocktail) and centrifuged at 15,000 × g for 30 min. The proteins in the sample were then precipitated from the supernatant by adding 3 × volume of 10% TCA/acetone solution (containing 20 mM DTT) overnight at -20 °C. After centrifugation at 7000 × g for 30 min at 4 °C, the pellet was washed with cold acetone (containing 20 mM DTT), followed by air-drying using a Speed Vac (Branstead, Ipswich, England). The protein sample was then resuspended in sample buffer (6 M urea, 2 M thiourea, 0.5% CHAPS, 0.5% IPG buffer, 20 mM DTT, 0.002% bromophenol blue) at 4 °C overnight, and the protein concentrations measured using a 2-D Quant Kit (GE Healthcare, Chicago, IL, USA).

First-dimension electrophoresis was carried out using a GE Healthcare Ettan IPGphor 3, as previously described(17). Proteins (250 µg) extracted from cholesteatoma and retroauricular skin tissues were loaded on 11-cm IPG strips (pI 4–7 and pI 3–11, Immobiline DryStrip) for first-dimension electrophoresis, followed by separation on sodium dodecyl sulphate polyacrylamide electrophoresis gel (12.5%) using an SE 600 Ruby (Hoeffer) at 150 V for 6.5 h.

Protein spot identification by LC-MS/MS

Protein spots on the 2-DE gel were visualized after CBR staining. Spots of interest were then excised into a piece (1 mm × 1 mm) from the 2-DE gel and digested by trypsin. The in-gel digested sample was identified by LC-MS/MS using an AB SCIEX QTRAP 5500Q mass spectrometer (Applied Biosystems, CA, USA). The detailed procedure was described in a previous study(17).

Western blot analysis

1-DE or 2-DE PAGE samples were transferred to a PVDF membrane (Millipore) under 0.4 A for 2 h using Transphor TE 62 (GE Healthcare, Chicago, IL, USA). The membrane was first incubated with rabbit anti-human CRT and AnxA2 (ProteinTech Group, Chicago, IL, USA) or rabbit anti-human β -actin antibodies (Sigma, St. Louis, MO, USA), then with goat anti-rabbit or rabbit anti-mouse horseradish peroxidase-conjugated IgG (1/5,000 dilution, Millipore, Bellerica, MA, USA) at room temperature for 2 h. After washing with PBST three times, the protein concentrations were determined from the chemiluminescence intensity using Pierce™ ECL Western blotting reagents (Thermo Fisher Scientific, Waltham, MA, USA).

Immunohistochemical staining and immunostaining evaluation of calreticulin

The tissue samples of the five patients analyzed in the proteomics study were also used for immunohistochemical analysis. A cholesteatoma specimen and a retroauricular skin specimen of each patient were prepared as 4- μ m-thick paraffin sections and subjected to immunohistochemical analysis. After de-paraffinization, rehydration and autoclave treatment at 121 °C for 10 min in DAKO Target Retrieval Solution, pH 9.0 (DAKO, Glostrup, Denmark) to induce antigen retrieval, the paraffin sections of all samples were incubated with 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity. The sections were then stained with CRT or AnxA2 polyclonal antibodies (1:50; ProteinTech Group, Chicago, IL, USA) at room temperature for 1 h, and a DAKO REAL EnVision Detection kit (DAKO, Glostrup, Denmark) was then used as the secondary antibody. After incubation in 3'3-diaminobenzidine for 5 min, the sections were counterstained with Mayer's hematoxylin and mounted. Negative controls were prepared by replacing the primary antibody with non-immune serum of the same species. They were assigned a score number according to the following rules: a score of 0 for 0% epithelium cells positive, a score of 1 for 1–24% epithelium cells positive, a score of 2 for 25–49% epithelium cells positive, a score of 3 for 50–74% epithelium cells positive and score of 4 for 75–100% epithelium cells positive. The intensity of cellular staining was also assigned a score number: a score of 0 for zero intensity, a score of 1 for weak intensity, a score of 2 for moderate intensity and a score of 3 for strong intensity(17). A staining score was obtained by multiplying the percentage score with the intensity score, with a maximum score of 12. Statistical evaluations were performed using the paired t-test. A difference considered statistically significance is 0.05.

Results

Downregulation of calreticulin and annexin A2 proteins in cholesteatoma tissue determined by 2-DE analysis.

Representative results of 2-DE analysis of the samples of cholesteatoma tissue and retroauricular skin of the five patients are shown in Fig. 1. 2-DE was run with a loading of 250 μ g protein, and protein spots were selected using PDQuest 2D image analysis software. Among the identified spots, calreticulin (CRT) and annexin A2 (AnxA2) were found to be downregulated in cholesteatoma as compared with the control tissue (Figs. 1 and 2). The increased levels of CRT and AnxA2 in retroauricular skin samples were successfully identified by LC-MS/MS analysis and a MASCOT search, as shown in Table 2.

Expressions of CRT and AnxA2 determined by 2-DE and 1-DE western blotting analysis

2-DE western blotting analysis of CRT and AnxA2 of cholesteatoma and retroauricular skin tissues of the patients was performed, and representative results are shown in Fig. 3. The results demonstrated that CRT and AnxA2 had higher expression levels in retroauricular skin, which was in agreement with the results presented in Fig. 1 and Fig. 2. Next, we compared the expression levels of CRT and AnxA2 in cholesteatoma and retroauricular skin tissues of five patients by 1-DE western blotting. Side-by-side comparison of the expressions of CRT and AnxA2 for each individual patient showed that the expressions of both proteins were obviously higher in retroauricular skin than in cholesteatoma (Fig. 4), which was in accordance with the results of proteomic analysis.

Immunohistochemical distribution of CRT in cholesteatoma and retroauricular skin

CRT immunoreactivity in retroauricular skin showed a greater presence and a higher intensity than that in cholesteatoma tissue (Fig. 5). CRT immunoreactivity in retroauricular skin was clearly stronger than that in cholesteatoma. The results supported the findings of 1-DE and 2-DE western blot analysis.

Discussion

In this study, we found that CRT and AnxA2 were downregulated in cholesteatoma tissue as compared with their expressions in retroauricular skin tissue of the same patient. CRT is a 46-kDa protein with high-capacity Ca^{2+} -binding and a multifunctional protein that has been shown to regulate several important cellular processes. It is mainly located in the endoplasmic reticulum and binds to newly-synthesized glycoproteins, which can prevent protein aggregation and improve correct folding of proteins(18). CRT is important in the MHC class I antigen assembly and many cellular processes(19), including cell proliferation, cell migration, and integrin-dependent Ca^{2+} signaling(20). Other studies have demonstrated that CRT plays essential roles in the regulation of gene expression(21) and wound-healing(22).

Overexpression of CRT in tumors in relation to normal tissue has been reported in human breast carcinoma(23–26), pancreatic cancer(27), prostate cancer(28), bladder cancer(29), hepatocellular carcinoma(30), gastric cancer(31), and oral cancer(32). CRT can also promote cell proliferation, cell differentiation and invasion in acute myeloid leukemia cells(33), and cell proliferation and migration in Schwann cells(34).

Previous research revealed that GRP75, GRP78 and GRP94 were upregulated in cholesteatoma, which may act against stress in the endoplasmic reticulum, avoiding cell apoptosis and protein unfolding and leading to increased growth of cholesteatoma(17). CRT is also an endoplasmic reticulum chaperone and is involved in endoplasmic reticulum stress to prevent protein unfolding, but is downregulated in cholesteatoma. Fischer et al.(35) reported that CRT inhibited lipopolysaccharide-induced inflammatory osteoclastogenesis and bone resorption. With the lytic enzymes and cytokines produced in cholesteatoma, the inflammatory condition may further promote epithelial proliferation in cholesteatoma. We speculated that the reduced expression of CRT and the inability to inhibit the persistent inflammatory response induces epithelial proliferation in cholesteatoma.

AnxA2 is a 36-kDa calcium-dependent phospholipid-binding protein. It is involved in several biological processes, such as immune responses, anti-inflammatory effects, Ca^{2+} transport, Ca^{2+} -dependent exocytosis, and phospholipase A2 regulation(36). It also plays roles in the regulation of cellular growth, cell division and signal transduction pathways(37, 38). AnxA2 has been shown to interfere with multiple cellular processes, and particularly in cancer progression. AnxA2 is overexpressed in numerous types of cancer, including colorectal cancer, hepatocellular cancer, esophageal cancer, oral cancer, gastric cancer and pancreatic cancer(39–44). It is also a serum marker for hepatocellular carcinoma, and has been suggested to have an important role in liver cancer progression. Downregulation of AnxA2 in hepatocellular carcinoma cells has been shown to inhibit cell migration and cell invasive potential, and interfere with cytoskeleton establishment in tumor cells(40). It has been reported to be a useful biomarker for oral cancer(39). Previous study demonstrated AnxA2 participation in cell proliferation in orbital fat tissue of thyroid orbitopathy patients(45). Comprehensive research showed that AnxA2 plays an important role in cell proliferation. Kim et al.(46) described the expression of AnxA2 in cholesteatoma and the possible physiologic role of AnxA2 in keratinocyte cell hyperproliferation during the development of human cholesteatoma. According to our results, AnxA2 is expressed more highly in retroauricular skin as compared with cholesteatoma tissue. The results are contrary to previous study.

Conclusions

AnxA1 is the first protein family member of the annexin family that binds to the cell membrane in a calcium-dependent manner. ANXA1 may be induced by glucocorticoids in inflammatory cells and share many anti-inflammatory effects with

these drugs(47). AnxA2 also have anti-inflammatory effects. It is speculated that the reduced expression of AnxA2 and inhibited the inflammatory reaction, thereby inducing epithelial proliferation of cholesteatoma.

Abbreviations

2-DE: two-dimensional gel electrophoresis

LC-MS/MS: liquid chromatography-tandem mass spectrometry

CRT: Calreticulin

AnxA2: annexin A2

Declarations

Ethics approval and consent to participate

The protocol for the use of human specimens in this study was approved by the Institutional Review Board (IRB) of the hospital (approval no KMUH-IRB-980046).

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HKY was the program instructor and was a major contributor in writing the manuscript. HKF, CCY, WHM and CLP contributed in the collection of samples and data. CCY, CWT and TSM contributed in the sample and data analyses. CNC and WYJ critically revised the draft and approved the final manuscript.

Acknowledgments

Not applicable

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Tables

Table 1. Statistical characteristics of patients with cholesteatoma and the staining scores of calreticulin for the five patients.

Patient No	Sex	Age	Calreticum staining score [†]	
			Cholesteatoma	Retroauricular skin
1	Male	63	3	8
2	Female	65	4	8
3	Female	40	3	9
4	Female	53	3	9
5	Female	63	4	8
Mean		56.8	3.4±0.55	8.4±0.55

†: Paired t- test, p < 0.001.

Table 2. Differentially-expressed proteins identified by LC-MS/MS

Spot number	Protein name	Accession No.	Mr/pI	Peptide matched	Sequence covered (%)	MASCOT score
Spot 1	Calreticulin precursor (CRP55)	P27797	48.112/4.29	45	37	397
Spot 2	Annexin A2 (AnxA2)	P07355	38.58/7.57	28	37	505
Spot 3	Annexin A2 (AnxA2)	P07355	38.58/7.57	21	35	401

Figures

Figure 1

Representative 2-DE results of analysis of cholesteatoma and retroauricular tissue samples from patients 1 and 2. (A, C) retroauricular skin tissue (pI 4–7). (B, D) cholesteatoma tissue (pI 4–7). Spot 1 protein was identified by LCMS/MS.

Figure 2

Representative 2-DE results of analysis of cholesteatoma and retroauricular tissue samples from patients 3 and 4. (A, C) retroauricular skin tissue (pI 3–10). (B, D) cholesteatoma tissue (pI 3–10). Spots 2 and 3 proteins were identified by LCMS/MS.

Figure 3

Side-by-side comparison of 2-DE western blot images of CRT and AnxA2 in cholesteatoma tissue and retroauricular skin with pI 4–7 and pI 3–10, respectively. Images in A are for CRT; images in B are for AnxA2.

Figure 4

Validation of CRT and AnxA2 by western blotting analysis. Proteins extracted from retroauricular skin and cholesteatoma tissues from all six patients were analyzed. S: retroauricular skin tissue; C: cholesteatoma tissue. β-actin was used as the loading control for normalization.

A **B**

Figure 5

Immunoreactive staining of CRT in patient 4. Brown staining at various intensities indicated a heterogeneously-positive level in all layers of the cholesteatoma epithelium (A, arrow). Stronger and more homogenous positive staining of a brown color in retroauricular skin tissue was observed (B, arrow). Immunoreactivity was limited to the cytoplasm of keratinocytes. Magnification: (A and B) $\times 200$.