Upregulation of Bcl-2 in nasal polyps from patients with cystic fibrosis

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Background: Nasal polyps in patients with cystic fibrosis (CF) are believed to be phenotypically different than polyps affecting non-CF patients. The aim of this study was to investigate differences in cell cycle regulatory mechanisms between these 2 groups. In this prospective study at a tertiary care academic medical center, multiple techniques were used to confirm the upregulation of antiapoptotic Bcl-2 family proteins in CF polyps.

Methods: Nasal polyps were prospectively obtained from CF and non-CF patients. The Sigma Panorama Protein Microarray for Cell Signaling was used to identify differences in protein expression between the 2 polyp groups. Western blot analysis confirmed altered expression of a subset of these proteins. Immunohistochemical staining was performed on archived tissue to further investigate B-cell lymphoma 2 protein (Bcl-2) expression. Following review by a pathologist, slides were digitized using an Aperio[TM] ScanScope XT system and staining intensity was quantified with the Positive Pixel Count algorithm. The mean staining intensity for each polyp group was compared.

Results: The protein microarray suggested a greater than 2-fold upregulation of Bcl-xl in CF polyps relative to non-CF polyps. Western blot analysis confirmed the upregulation in CF polyps of Bcl-2, a more commonly studied protein analog of Bcl-xl. The CF polyp group was noted to have a higher quantitative intensity of immunohistochemical staining for Bcl-2 compared to the non-CF group ($p < 0.05$).

Conclusion: Through multiple modalities of protein investigation, we have demonstrated an upregulation of Bcl-2 family proteins in CF polyps relative to polyps from non-CF patients. © 2013 ARS-AAOA, LLC.

Key Words:
nasal polyps; chronic rhinosinusitis; Bcl-2; cystic fibrosis; protein array

Nasal polyposis is a common chronic inflammatory disorder that affects up to 4% of the general population; however, its pathogenesis is not completely understood.[1,2] Polyps in patients with cystic fibrosis (CF) appear to have a different phenotype than polyps from non-CF patients. Chronic rhinosinusitis is common among CF patients, and roughly 6% to 48% of these patients suffer from nasal polyposis.[3,4] After sinus surgery, CF patients tend to have poor outcomes compared to non-CF patients.[5,6] The histopathology is different as well. Polyps from CF patients feature a thinner basement membrane, abundant acid mucin, less submucosal hyalinization, and less eosinophilic infiltrate.[7] Defects in ion channel transport of the respiratory epithelial cells are thought to contribute to these differences.[8,9] These alterations in ion transport lead to dehydration of mucus and impaired mucociliary clearance. Further study of cell cycle regulatory mechanisms of CF polyps may ultimately contribute to an improved understanding of disease pathogenesis.

A number of established and reliable assays exist to investigate gene regulation and transcription. However, multiple regulatory steps occur from gene transcription to protein expression and function. A novel platform for profiling protein expression has been created, allowing for a molecular fingerprint of pathologic tissue. The Sigma Panorama[TM] Ab Microarray for Cell Signaling (CSAA1; Sigma-Aldrich, St. Louis, MO) is a proprietary assay designed for the study of relative levels of protein expression in either cell or tissue extracts. This particular array contains 224 different antibodies, representing 6 groups of biological pathways: apoptosis, cell cycle, neurobiology, cytoskeleton, signal transduction, and nuclear signaling proteins. Protein profiling can be performed with small amounts of tissue—approximately 1 g of tissue or 1 mg of protein extract is

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TABLE 1. Six patients were prospectively enrolled in this study for microarray analysis: age, sex, CF genotype, and CF bacterial colonization state are listed

Patient	Age (years)	Sex	Genotype	Colonization
CF1	46	M	ΔF508/ΔF508	S. aureus, P. aeruginosa
CF2	36	F	ΔF508/L206W/R668C	S. aureus, P. aeruginosa
CF3	33	M	ΔF508/ΔF508	S. aureus, P. aeruginosa
Non-CF1	38	F	n/a	n/a
Non-CF2	46	M	n/a	n/a
Non-CF3	63	F	n/a	n/a

CF = cystic fibrosis; n/a = not applicable.

the minimum amount necessary for the established protocol. Use of this protein microarray was recently described in the comparison of nasal polyps from aspirin-sensitive and aspirin-tolerant patients with chronic rhinosinusitis.[10] In this study, we used this array to compare the translational protein profile of nasal polyps from patients with and without CF. Our initial results suggested an upregulation of Bcl-xl in CF polyps relative to non-CF polyps.

To confirm protein microarray findings we performed immunohistochemical staining for B-cell lymphoma 2 protein (Bcl-2) on archived, formalin-fixed sinonasal polyps from patients with and without CF. Historically, the interpretation of immunohistochemistry has typically been a time-intensive, manually performed process. Assessing the degree of tissue staining positivity can also be variable. Multiple scoring systems have been introduced to provide semiquantitative analysis of immunohistochemical staining. Recent advances in computer automated image analysis systems, however, have allowed accurate and reliable quantification of immunohistochemical staining.[11] Automated image analysis has already been proposed as a reliable method for quantifying estrogen receptor immunohistochemical staining in the field of breast cancer research.[12] In this study, we utilized an automated image analysis protocol from Aperio™ to quantify immunohistochemical staining intensity.

Patients and methods

Patient characteristics

This study was approved by the institutional review boards of National Jewish Health (Denver, CO) and the University of Colorado Denver (Denver, CO). A total of 6 patients were prospectively enrolled in this study (Table 1). The experimental group consisted of 3 patients with known CF as documented by genotype testing and nasal polyposis visualized on nasal endoscopy. All 3 of the patients with CF had prior sinus or bronchoalveolar lavage cultures demonstrating colonization with both *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The comparison group consisted of 3 patients without CF, who presented with aspirin-tolerant nasal polyposis diagnosed on history, physical examination, and nasal endoscopy. All 6 patients had been treated with a standard regimen of daily nasal steroids and nasal saline irrigations prior to biopsy. Oral steroids were not used in any patient for at least one month prior to biopsy. Diagnosis of benign inflammatory polyp was confirmed by hematoxylin and eosin (H&E) stain.

Tissue harvest

The biopsy specimens were obtained and processed in the following manner. Patients were biopsied either in the office under local anesthesia or at the onset of definitive surgical treatment under general anesthesia in the operating room. After topical application of oxymetazoline and lidocaine, polypoid tissue emanating from the middle meatus was grasped with smooth, atraumatic forceps and placed in a cold bath of normal saline. Approximately 1 to 2 g of tissue was obtained from each patient.

Protein array

The specimen was processed according to the guidelines specified by the Sigma Panorama™ Antibody Microarray protocol (CSAA1). All chemical reagents and solvents were obtained from Sigma as part of the Panorama™ Antibody Microarray kit for cell signaling. Approximately 1 mg of protein extract from each specimen was collected and labeled with either Cy3 or Cy5 dye according to the standard procedure. Excess dye was removed from the solution by use of the SigmaSpin Post-Reaction Clean-up Columns (Sigma-Aldrich). Equal amounts of protein from each specimen were combined to form 2 group specimens. Each grid on the array slide contains 7 antibody duplicates plus a Cy3- and Cy5-conjugated bovine serum albumin (BSA) positive control and a non-labeled BSA negative control resulting in a total of 512 spots. Each spotted antibody in the array has been previously validated for its ability to bind proteins in the array assay using samples from human, mouse, and rat. The technical aspects of this assay have been improved and verified in various mouse tissues.[13] Incubation on the array was performed for 30 minutes in accordance with the standard technique using the provided buffers and phosphate-buffered saline (PBS)-Tween 0.05% for washes. The slide was scanned on a microarray scanner and the raw intensity values were recorded.

Protein array data analysis

Raw intensity values were normalized to the median to allow comparison between slides. Values were highly repeatable, both when Cy3 and Cy5 were reversed, as well as across samples. Statistical significance of differentially regulated proteins was established by analysis of variance (ANOVA).

Western immunoblot

To validate our results, Western blot analysis for Bcl-2 was performed. Equal amounts of protein extract (40 μg per lane) from each biopsy specimen were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a nitrocellulose membrane. Three additional well-documented CF patients were enrolled in our study and underwent tissue collection (as described above in Tissue Harvest) subsequent to the protein microarray work. Therefore, 3 additional CF biopsy specimens were available for Western blot analysis in addition to the original 6 specimens described. The proteins were probed with monoclonal antibody and visualized using chemiluminescence.

Immunohistochemistry

After receiving approval from the institutional review board of the University of Colorado Denver, archived, formalin-fixed tissue blocks of nasal polyp specimens from non-CF and CF patients were obtained from the University of Colorado Hospital surgical pathology database. Ten new non-CF and 10 new CF biopsy specimens were selected for inclusion in this arm of the study. All 20 patients had nasal polyposis and patients ranged in age from 26 to 63 years. Patient records were checked for preoperative oral and topical nasal steroid use. Tissue sections (5 μm) were mounted on charged glass slides (Superfrost Plus; Fisher Scientific, Waltham, MA) and baked overnight at 60°C. A monoclonal mouse anti-human Bcl-2 antibody was obtained for immunohistochemical staining (Dako, Carpinteria, CA). Slides were deparaffinized with xylene and rehydrated with a graded alcohol series. Endogenous peroxidase activity was blocked with 3.0% hydrogen peroxide for 15 minutes. A decloaking chamber (Biocare Medical, Concord, CA) with citrate buffer (20 mmol/L) was used for antigen retrieval at 120°C for 15 minutes. An autostainer (Dako) was used to perform the staining. The Envision+ Dual Link HRP (Dako) detection kit was used according to the product's protocol. The final Bcl-2 antibody dilution was 1:400. Slides were incubated at room temperature for 30 minutes with this antibody. Positive control slides with sections of normal human tonsillar tissue (Dako) were utilized. Negative control staining was performed on all sections using plain antibody diluent (Dako) in place of the Bcl-2 antibody. Bcl-2 staining was visualized by development with 3,3′-diaminobenzidine (Dako), counterstained with hematoxylin, and dehydrated with graded alcohols. Coverslips were placed. Slides were then inspected by an attending pathologist to confirm the appropriateness of the staining.

Immunohistochemical data analysis

Slides were digitized at an original magnification of ×40 using an Aperio™ ScanScope XT scanner (Aperio, Vista, CA). These scanned images were then imported in the Aperio™ ImageScope viewer. The Aperio™ positive pixel count algorithm (version 9.0) was then used to calculate
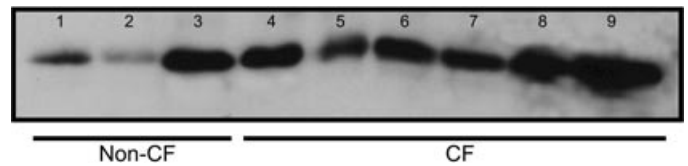

FIGURE 1. Bcl-2 Western blot showing that all 6 CF specimens have significantly higher intensity than all but 1 of the 3 non-CF specimens.

the total tissue positivity index of each specimen, a value ranging from 0 to 1. The entirety of each sectioned polyp specimen was included in this analysis. Regions of artifact and nonspecific staining outside of each polyp were excluded. The mean total tissue positivity index for each experimental group was calculated. Using these means, as well as the respective standard deviations, the non-CF and CF groups were compared. A t test was used for statistical analysis and calculation of a 1-tailed p value.

Results

Protein microarray

Significant dysregulation of Bcl-xl was discovered in the CF group. Analysis of these data revealed 2-fold upregulation of the pro cell-cycle protein Bcl-xl in polyps of patients with CF ($p < 0.05$). Cyclin D1, heat shock protein 90 (HSP90), and tyrosine hydroxylase were downregulated in the CF polyp group.

Western immunoblot

Confirmation of findings was achieved by immunoblot. Western blot analysis suggests qualitative upregulation of Bcl-2, an analog of Bcl-xl, in CF tissue (Fig. 1). All 6 CF specimens included in this blot demonstrate significantly more intensity than all but 1 of the 3 non-CF specimens.

Immunohistochemistry

The mean total tissue positivity indices of the non-CF group and CF group were 0.238 and 0.344, respectively. These data confirm a higher quantitative staining intensity in the CF group relative to the non-CF group (1-tailed $p < 0.05$).

Discussion

We have used a novel technology to screen for protein translational differences between nasal polyps of patients with and without CF. Using a single Sigma Panorama™ Antibody Microarray, the expression of 224 proteins related to the cell cytoskeleton, cell signaling, cell cycle progression, nuclear signaling, apoptosis, and cell neurobiology was simultaneously investigated. This same array technology has been used effectively in the search for markers for early detection and targets for therapeutic intervention in breast cancer research.[14] Our work represents high-throughput protein profiling of nasal polyp tissue.

Although the vast majority of cell cycle proteins demonstrated no significant difference, we did note a significant

upregulation of Bcl-xl in CF nasal polyp tissue relative to non-CF polyp tissue. To confirm this finding, we used another novel technology initially described in breast cancer research: automated image analysis. This computer-based analysis allowed for the rapid and reproducible quantification of immunohistochemical staining intensity of Bcl-2, a more commonly studied analog of Bcl-xl. Our immunohistochemical data suggest an upregulation of Bcl-2 within CF nasal polyp tissue relative to non-CF polyp tissue.

Prior studies have demonstrated an increase in epithelial cell proliferation in nasal polyps, as evidenced by higher proportions of polyp epithelial cells in the S-phase of mitosis. Additionally, these studies have shown an even higher proportion of cells in S-phase in the CF polyp subtype, initiating the belief that there were molecular differences in nasal polyps of CF patients.[15,16] The Bcl family of proteins, which regulates the apoptosis pathway, is known to interact with a multitude of other cell regulatory proteins, including Map 1b, cyclin D1, and several cyclin-dependent kinases. These proteins all have a role in cell-cycle progression. Bcl-xl is considered the dominant regulator of apoptosis. It is known to regulate interleukin-1beta (IL-1β) production in macrophages, and has been found in higher amounts in granulocytes of patients with CF. In rat models, Bcl-xl interacts with coronavirus to prevent apoptosis in epithelial cells, causing acute infection to become persistent.[17] Furthermore, the Bcl family of proteins is closely tied to p53 dysregulation, which has been recently associated with increased lymphocytic infiltrates in nasal polyps.[18]

Upregulation of the anti-apoptotic Bcl-2 protein may interfere with the normal clearance of cells and tissue homeostasis in CF patients, thus contributing to refractory sinonasal disease and polyps in this group.

A number of limitations exist with the use of this protein microarray. First, with this array protocol it is not possible to separate the individual contributions of the epithelial, fibroblast, and lymphocytic populations within the overall protein extract. As a result, with this technology, we cannot conclude which cell type is dysregulated. Second, pathologic differences may not be innate to the disease process, but may be effects of the local microenvironment of the polyp biopsy. Perhaps chronic inflammation resulting from *S. aureus* or *P. aeruginosa* contributes to these changes in protein expression. Third, patient to patient variability may become a factor when grouping 3 samples together. For instance, if a single patient highly expresses a particular protein, that protein may appear to be dysregulated in the overall group. Last, one must consider that our results demonstrate roughly 2-fold dysregulation; from anecdotal

experience, 10-fold or even higher dysregulation may be discovered with use of this array. Hence, it is somewhat arbitrary what level one considers suggestive of functionally significant dysregulation based on array results.

Interpretation of immunohistochemical staining with an automated image analysis protocol is an emerging technology. This computer-based analysis carries with it multiple limitations as well. First, many programs designed to quantify the intensity of staining of a specific chromogen, such as the algorithm we used in this study, will not differentiate between specific and nonspecific background staining. In attempt to overcome this limitation, we reviewed our slides with an attending pathologist to ensure our staining was specific and that an insignificant degree of nonspecific background staining was present. Second, a trained pathologist will be superior to a computer program at distinguishing specific cell types to determine which populations might be associated with higher intensities of staining. Our algorithm did not distinguish cell types but rather quantified the average staining intensity of the entire polyp. Last, our chromogen (diaminobenzidine) demonstrates a linear relationship between antigen concentration and staining intensity only with certain antigen concentration ranges.[11] Consequently, any potential nonlinear relationship between antigen concentration and staining intensity may potentially result in inaccurate results outputted from the automated image analysis program.

Conclusion

In this study we demonstrated increased expression of Bcl-2 family proteins in nasal polyp tissue harvested from patients with CF. Three techniques for studying protein expression were used: protein microarray, Western immunoblot, and immunohistochemical staining. Protein microarrays may be used as a tool for mass analysis of changes in protein expression in cultured cells or fresh tissue. This technique is rapid, easy to use, and can detect protein levels as low as a few nanograms per milliliter. Automated image analysis of immunohistochemical staining is another rapid technique which can reduce the arbitrary nature of traditional scoring systems. Our use of these emerging technologies in the study of CF nasal polyps suggests an upregulation of Bcl-2 family proteins in these tissues relative to non-CF nasal polyps. Dysregulations of the cell cycle and apoptotic pathways may have a contributory role in the pathophysiology of polyp formation and progression in patients with CF. Further study is necessary to confirm and apply these findings. ❸

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