

Protein microarray analysis of nasal polyps from aspirin-sensitive and aspirin-tolerant patients with chronic rhinosinusitis

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ABSTRACT

Background: The purpose of this study was to apply protein microarray technology to the study of sinonasal tissue and to identify differential protein expression in nasal polyps from aspirin-sensitive (AS) versus aspirin-tolerant (AT) patients with chronic rhinosinusitis (CRS) and CRS with nasal polyps (CRSwNPs).

Methods: Nasal polyp specimens were prospectively obtained from two groups of patients with CRSwNP. The test group (AS) consisted of five patients that were diagnosed with CRSwNP and intolerance to aspirin based on medical history and physical exam. The control group (AT) consisted of four AT patients with CRSwNP. Protein was extracted and labeled from harvested polyps and the Sigma Panorama Antibody Microarray–Cell Signaling Kit was used to identify differences in protein expression between the two polyp groups. Western blot analysis was used to validate the results of the protein microarray.

Results: The protein microarray showed a greater than twofold change in expression of both beta-adaptin and heat shock protein 70 (HSP70). Western blot analysis confirmed up-regulation of beta-adaptin and HSP70 in nasal polyp tissue from AS patients.

Conclusion: Pooled samples of AS and AT nasal polyps evaluated by protein microarray show distinct protein expression profiles in the stress response and receptor-mediated endocytosis pathways. This study establishes the successful application of protein microarray technology to study nasal polyposis, which in turn can be validated by Western blot analysis.

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Key words: Aspirin sensitive, beta-adaptin, heat shock protein 70, microarray, nasal polyps, protein, rhinosinusitis

Sinonasal polyposis is a multifactorial chronic inflammatory process that affects 1–4% of the general adult population.¹ Chronic inflammation of the nasal and paranasal sinus mucosa leads to polyp formation, which is characterized by edematous masses of inflamed mucosa prolapsing into the nose, leading to nasal obstruction, abnormal secretions, loss of smell, headache, and a reduced quality of life. These edematous mucous membranes release inflammatory mediators and large numbers of eosinophils and lymphocytes gather in the lamina propria. This inflammatory cell infiltrate sets up an extensive network of cellular interactions, which are thought to propagate nasal polyp proliferation.² However, the pathophysiology of nasal polyposis remains relatively unclear on a molecular level. The aspirin-sensitive (AS) subgroup of patients with nasal polyps appears to be a particularly challenging group to manage and understand.¹ When compared with the aspirin-tolerant (AT) group, the AS patients tend to have

more severe disease, worse asthma, and are prone to treatment failure.

Microarray technology can be used to quickly and efficiently analyze the expression of many proteins and genes from specific tissues. Several recent publications have described the application of gene microarray technology in the genetic analysis of nasal polyp tissue. For example, Figueiredo *et al.* used a microarray cDNA technique confirmed by reverse transcription–polymerase chain reaction to evaluate the expression of 96 inflammatory genes. They found a higher expression of transforming growth factor 1 in inflammatory mucosa when compared with adjacent nasal polyp tissue.³ A genome-wide transcriptional profiling of nasal polyp tissue from patients with chronic rhinosinusitis (CRS) indicated that the met proto-oncogene (MET) was up-regulated compared with controls.⁴ MET encodes a receptor tyrosine kinase, an important regulator of cell proliferation and differentiation. These authors used gene microarray technology. Thus, when interpreting the results of these studies one must keep in mind that mRNA undergoes a number of alternative processing steps before and after translation. There can be a poor correlation between mRNA and actual protein expression.⁵ Ultimately, it is the protein that acts as the functional unit that alters cell physiology; thus, analyzing protein expression in tissue is required to fully understand these molecular mechanisms. To date, the application of protein microarray technology in the study of sinonasal disease is limited. One recently published study used protein microarray technology to analyze the peripheral blood of nasal polyp patients. Vascular cell adhesion molecule 1, E-selectin and L-selectin, proteins

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that regulate eosinophil migration showed significantly higher expression when compared with healthy controls.⁶

The use of protein microarray technology in the direct evaluation of nasal polyp tissue has not been previously reported. The current study uses protein microarray technology to directly compare the expression levels of hundreds of target proteins in nasal polyp tissue from two distinct patient populations with nasal polyposis. The commercially available Sigma Panorama Antibody Microarray–Cell Signaling Kit (Sigma-Aldrich Biotechnology, St. Louis, MO) was used because it has previously been shown to provide accurate and robust protein expression profiling of biological pathways that are integral to cell regulation.⁷ Protein microarray technology was used to directly compare cell signaling protein expression in AS and AT patients with nasal polyps. The objectives of this study were to (1) apply protein microarray technology to the study of sinonasal tissue and (2) identify dysregulated proteins that might be associated with the development of nasal polyps in AS patients.

METHODS

Patient Characteristics

Nasal polyp specimens were prospectively obtained from two groups of patients with CRS and CRS with nasal polyps (CRSwNPs). All study patients had been scheduled for endoscopic sinus surgery to address symptoms related to advanced nasal polyposis. The test group (AS) consisted of five patients that were diagnosed with AS respiratory disease based on medical history and physical exam. The AS patients had experienced a significant adverse reaction to aspirin or nonsteroidal anti-inflammatory drugs including wheezing, dyspnea, and/or an acute asthma attack. None of the AS patients had undergone aspirin desensitization at the time of this study. The control group (AT) consisted of four patients with CRSwNP that were aspirin/nonsteroidal anti-inflammatory drug tolerant. All of the patients had significant manifestations of the disease that were refractory to medical therapy and required surgical intervention. All patients were >18 years old and none of them had a prior history of cystic fibrosis, systemic granulomatous disease, or sinonasal neoplasm. Lund-Mackay scores were calculated using each patient's preoperative CT scan.

Institutional Review Board approval of this protocol was obtained from National Jewish Health and the University of Colorado Denver. Preoperative steroid usage was recorded for all patients. All specimens were obtained in the operating room under general anesthesia while the patients were undergoing endoscopic sinus surgery and nasal polypectomy. Polyp tissue from the middle meatus or ethmoid sinus was grasped with smooth atraumatic forceps under direct visualization. The specimen was then placed in saline and kept on ice while being transported to the laboratory for immediate processing. Additional tissue from the same location was submitted for routine histological analysis.

Tissue Processing and Labeling

The tissue specimen was carefully examined and cut into small fragments and bony elements and mucous were removed. The tissue pieces (0.05–0.15 g) were then transferred

into a buffer solution (buffer A) with protease and phosphatase inhibitors. The solution was then homogenized at 13,000 rpm at 4°C for 15 minutes and spun down in the centrifuge at 10,000 rpm. The protein concentration of the supernatant was then determined using the Bradford method. The extract was then diluted to 1 mg/mL in buffer A.

The protein sample was then labeled with either Cy3 or Cy5 Monofunctional Reactive dye (Amersham Biosciences PA23001/PA25001, GE Healthcare, Little Chalfont, UK) by mixing 1 mL of extract in the dye vial and incubating for 30 minutes in the dark. The Cy3 dye was used to label AS specimens and Cy5 was used for AT specimens. Excess dye was removed using a SigmaSpin column. Once again, the protein concentration was calculated using the Bradford method. At this point the dye/protein molar ratio was then calculated. All samples had a dye/protein of >2, which was necessary to eliminate excess background.

Sample Incubation on the Array

The 6.25- μ g of each AT protein sample were pooled and added to 5 mL of array incubation buffer containing 5 μ g of each of the AS protein samples. The Panorama microarray slide containing 224 different antibodies to cell signaling proteins was then placed in the solution and incubated for 30 minutes while protecting it from light exposure. The slide comes pretreated with a proprietary blocking buffer to reduce nonspecific background staining and to maximize the signal-to-noise ratio. Next, the slide was then washed with buffer (PBS-Tween 0.05%) and water and allowed to completely dry. The slide was protected from light exposure at all times during preparation.

Scanning the Antibody Array

A microarray scanner (Perkin Elmer, Boston, MA) using ScanArray Express software was used to scan the slides. The scanner measured the fluorescence of the Cy3- and Cy5-labeled proteins bound to the slide and generated a raw intensity value. These raw intensity values were normalized to the median to allow comparison between the AT and AS groups. The normalized values were then used to determine the total fold change of protein expression between groups. As established by the Sigma Panorama Microarray protocol, significant change in protein expression was indicated by a greater than twofold change in fluorescence signal. Each of the 224 antibodies was spotted in duplicate on the array and the signal intensities for the proteins that showed a greater than twofold change were in agreement, showing good reproducibility.

Western Immunoblot Validation

To validate the protein microarray results, Western blot analysis for two dysregulated proteins, β -adaptn and heat shock protein 70 (HSP70), were performed. Protein extract (50 μ g) from three AT and five AS samples and the designated positive control was separated by SDS-PAGE and blotted onto nitrocellulose membranes. One of the AT samples did not have sufficient protein to be added to the immunoblot analysis; thus, three samples were used. The proteins were then probed with the corresponding monoclonal antibodies β -adaptn (sc-58226; Santa Cruz Biotechnology, Inc., Santa

RESULTS

The ages of patients sampled ranged from 34 to 68 years. One patient in the AT group did not have asthma. Lund-Mackay scores were elevated in both groups ranging from 17 to 24 out of 24 total. Three of the four AT patients and four of the five AS patients were treated with prednisone preoperatively (Table 1).

Significant dysregulation of two proteins was discovered between the AT and AS groups. There was a negative fold change of greater than two for both β -adaplin and HSP70 (Table 2.) This degree of negative fold change was interpreted as an increase in the expression of these proteins in the AS group when compared with the AT samples.

Confirmation of the protein array findings was achieved by immunoblot. Western blot analysis confirmed up-regulation of β -adaplin and HSP70 in AS tissues. All five of the AS specimens showed a strong expression of HSP70 while two of the three AT specimens showed a marked decrease in expression. Four of five AS specimens stained strongly for β -adaplin while all three AT specimens only moderately expressed this protein (Fig. 1).

Caspase 8 and caspase 9 are key proteins in the apoptosis pathway; however, they only had a 1.1- and 1.3-fold change in expression on the protein array. Western immunoblots were performed using anti-caspase 8 (Cell Signaling Technology no. 9746) and anti-caspase 9 (Cell Signaling Technology no. 9501) antibodies. The AS and AT specimens equally expressed these proteins.

DISCUSSION

This study used a novel protein array analysis to examine the expression of 224 proteins related to cell signaling, cell cycle progression, nuclear signaling, apoptosis, and cell neurobiology. This array technology has the ability to reliably and quickly screen nasal polyp tissue for alterations in these pathways that might help expand our understanding of the pathophysiology of this process. When our protein array detected a greater than twofold change in the expression of a particular protein we were able to validate this finding using Western blot. On the contrary, when the fold change was less

than two, as was the case for caspase 8 and caspase 9, the immunoblot showed similar protein expression between the AS and AT groups. Thus, the Western blot appears to validate the protein microarray method. Similar microarray technology is being used in the study of neoplastic processes such as pituitary adenomas and breast cancer with the goal of finding markers for early detection and targets for therapeutic intervention.^{8,9} To our knowledge, this study represents the first description of high throughput protein profiling of nasal polyp tissue in patients with CRS and nasal polyposis.

Our protein array showed a 2.6-fold increased expression of HSP70 in the AS group and this was confirmed with a Western blot. A variety of environmental stressors cause eukaryotic cells to produce several closely related 70-kDa proteins. The HSP70 family of proteins has long been recognized to have cytoprotective effects. When a cell undergoes environmental disruptions its proteins unfold; thus, HSP70 is produced to act as a chaperone that protects these proteins from degradation. When this process is ineffective or down-regulated, the misfolded proteins accumulate and trigger apoptosis.¹⁰ HSP70 has been shown to inhibit apoptotic signaling at multiple points and to up-regulate survival signaling; thus, it ultimately has a net prosurvival effect on cells.¹¹

Although several studies have analyzed the role of HSP70 in the olfactory epithelium's response to toxins and hyperthermia,^{12,13} its expression in sinonasal mucosa or nasal polyp tissue has not previously been studied. An in depth discussion of the pathophysiology of this finding is beyond the scope of this article; however, one could argue that the higher expression of HSP70 in the AS polyp tissue represents a higher degree environmental stress (bacterial infection, inflammation, and free radicals) in the sinonasal environment in AS patients. Increased levels of HSP70 in the AS patients could indicate a greater degree of tissue damage or a more robust stress response. The increased expression of HSP70 may represent a cytoprotective adaptive response and result in altered cell regulation. This, in turn, may contribute to cellular proliferation and ultimately a more aggressive form of nasal polyposis refractory to treatment characteristically seen in AS patients. Finally, HSP70 levels could also serve as a biomarker to help predict the severity of disease or a patient's risk of recurrence but would require further study.

In addition, the protein array also showed a 3.9-fold decrease in the expression of β -adaplin in AT polyp tissue.

Table 1 Patient information and preoperative steroid usage

Group	Gender	Age (yr)	Asthma	Lund-McKay Score	Preoperative Oral Steroid Usage
AT	M	68	Yes	24	Yes
AT	M	37	No	17	Yes
AT	M	56	Yes	20	No
AT	M	35	Yes	21	Yes
AS	M	41	Yes	20	Yes
AS	F	59	Yes	18	Yes
AS	F	57	Yes	17	No
AS	M	34	Yes	19	Yes
AS	F	65	Yes	20	Yes

AT = aspirin tolerant; AS = aspirin sensitive.

Table 2 Protein microarray results

Protein	Median Fluorescence Intensity (Cy3 or AS)	Median Fluorescence Intensity (Cy5 or AT)	Fold Change
Adaptin	3060	92	−3.9
HSP70	2775	83	−2.6
τ-pSer199/202	2965	2571	2.5
Tyrosine hydroxylase	2984	2711	−2.5
Cdc27	5937	3709	2.3
Bcl-xl	4837	3603	1.8
Caspase 8	4264	3426	1.3
Caspase 9	3520	3111	−1.1

Cy5 monofunctional reactive dye (Amersham Biosciences PA25001) was used to label the AT protein samples.

Cy3 monofunctional reactive dye (Amersham Biosciences PA23001) was used to label AS protein samples.

AT = aspirin tolerant; AS = aspirin sensitive; HSP70 = heat shock protein 70.

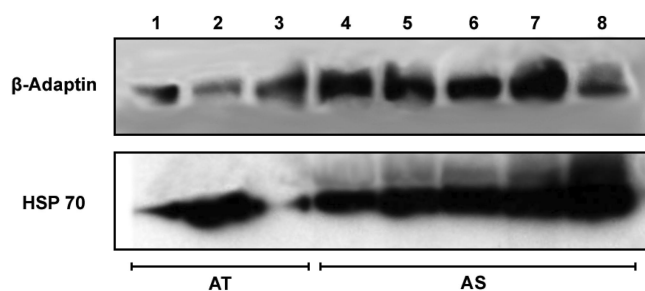


Figure 1. Western blot results for β -adaptin and heat shock protein 70 (HSP70). All five of the aspirin-sensitive (AS) specimens showed a strong expression of HSP70 while two of the three aspirin-tolerant (AT) specimens showed a marked decrease in expression. Four out of five AS specimens stained strongly for β -adaptin while all three AT specimens only moderately expressed this protein.

Western blot confirmed that the AS group had a much stronger expression of this protein. β -Adaptin is one of many proteins that form complexes in clathrin-coated pits and vesicles during receptor-mediated endocytosis. The function of receptor-mediated endocytosis is very diverse among cells ranging from taking up low-density lipoprotein or iron into the cell, to transducing signals from the cell periphery to the nucleus to internalizing activated receptors into cell endosomes. The role of this protein in nasal polyp physiology also has not been studied or reported. One could hypothesize the importance of this mechanism in local immune response, intercellular protein transport, or, perhaps, targeted drug delivery *via* specific protein receptors. Targeted drug delivery has been studied extensively in cancer cells using folate receptor-mediated endocytosis to deliver toxic chemotherapeutic agents into targeted cells.¹⁴

Several study limitations should be addressed. The protein array technique is a semiquantitative analysis of pooled samples and our sample size was small. Thus, overexpression or underexpression of protein from a single sample could significantly alter the group's median raw intensity value on the array. In an effort to correct for this potential factor, the Western blot technique was used to analyze individual specimen's protein expression.

An additional potential limitation is the impact of oral steroid use on our results. Because a preoperative burst of oral steroids is often required to optimize patients for surgery and to help avoid an asthma exacerbation, steroid usage was not controlled for in this clinical study. Nevertheless, steroid usage was similar between groups with all but one patient in each group receiving preoperative prednisone. It is assumed that the impact on protein expression is mitigated by the roughly equal steroid use in each patient group. Use of intra-nasal and inhaled steroids was also equivalent across patient groups.

CONCLUSION

Protein microarray technology has great potential for screening nasal polyp tissue for alterations in cell signaling pathways. Proteins with a greater than twofold change on the array do indeed show differential expression as confirmed by Western blot analysis. In this study, we found AS patients with nasal polyps appear to have up-regulated stress responses and receptor-mediated endocytosis. Additional study of these molecular pathways in nasal polyp patients may provide a better understanding of the disease process and how to design targeted therapies. This study shows the successful application of protein microarray technology to the study of nasal polyposis. This technique appears to be validated by Western blot analysis and should represent a powerful tool in the future investigation of the molecular mechanisms of sinonasal inflammation.

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