

Gene Expression Profiling of Nasal Polyps Associated With Chronic Sinusitis and Aspirin-Sensitive Asthma

Konstantina M. Stankovic, MD, PhD; Hernan Goldsztein, MD; Douglas D. Reh, MD; Michael P. Platt, MD; Ralph Metson, MD

Objective: To identify genes whose expression is most characteristic of chronic rhinosinusitis and aspirin-sensitive asthma through genome-wide transcriptional profiling of nasal polyp tissue.

Study Design: Prospective, controlled study conducted at a tertiary care institution.

Methods: Thirty genome-wide expression microarrays were used to compare nasal polyp tissue from patients with chronic rhinosinusitis alone (CRS, n = 10) or chronic rhinosinusitis and a history of aspirin-sensitive asthma (ASA, n = 10) to normal sinonasal mucosa from patients who underwent surgery for non-sinus related conditions (controls, n = 10). Genes found to be most characteristic of each polyp phenotype, as determined from bioinformatic analyses, were validated using real-time quantitative polymerase chain reaction (RT-PCR) and immunohistochemistry in different patient sets.

Results: The transcriptional signature of the control mucosa was distinctly different from that of either polyp phenotype. Genes most characteristic of the CRS phenotype included two upregulated genes—met proto-oncogene (MET) and protein phosphatase 1 regulatory subunit 9B (PPP1R9B)—and two downregulated genes—prolactin-induced protein (PIP) and zinc alpha2-glycoprotein (AZGP1). The gene most characteristic of the ASA phenotype was periostin (POSTN), which was upregulated relative to controls. Differences between the CRS and ASA phenotypes were associated with alterations in the 6p22, 22q13, and 1q23 chromosomal regions.

Conclusions: Nasal polyps appear to have characteristic transcriptional signatures compared to normal sinonasal mucosa. The five genes identified in this study likely play roles in the pathogenesis of polyps associated with CRS and ASA, and are therefore attractive targets for novel medical therapies for these common debilitating diseases.

Key Words: Sinonasal polyposis, chronic rhinosinusitis, sinusitis, asthma, triad asthma, aspirin-sensitive asthma, microarray, periostin, POSTN, c-Met, met proto-oncogene, prolactin-induced protein, zinc alpha2-glycoprotein, protein phosphatase 1.

Laryngoscope, 118:●●●–●●●, 2008

INTRODUCTION

Sinusitis is one of the most commonly diagnosed diseases in the United States, affecting an estimated 37 million people each year.¹ Studies have demonstrated the major economic impact of this disorder, which can dramatically reduce workplace productivity and quality of life in affected individuals.² Patients with chronic sinusitis whose symptoms are most refractory to treatment regimens often develop nasal polyps. Growth of these polyps leads to obstruction of the sinonasal passages, requiring repeated courses of antibiotics to treat underlying infections and steroid therapy to reduce polyp load. In advanced cases, surgery may be necessary to remove the polyps and restore sinus ventilation. Histologically, sinonasal polyps are characterized by proliferation and thickening of mucosal epithelium with focal squamous metaplasia, glandular hyperplasia, subepithelial fibrosis, and stromal edema with numerous blood vessels.³ These findings suggest that activation of multiple genes is responsible for the resulting histologic and clinical phenotype.

Most patients who develop nasal polyps have chronic rhinosinusitis. A subset of these patients are diagnosed with aspirin-sensitive asthma, also known as triad asthma and Samter's triad, characterized by the presence of nasal polyps, asthma, and aspirin allergy. When individuals with this disorder take aspirin (or nonsteroidal antiinflammatory agents, in many cases) immediate and

From the Department of Otolaryngology (K.M.S., H.G., D.D.R., M.P.P., R.M.), Massachusetts Eye and Ear Infirmary, and the Department of Otolaryngology (K.M.S., R.M.), Harvard Medical School, Boston, Massachusetts, U.S.A.

Editor's Note: This Manuscript was accepted for publication January 29, 2008.

Funding for this study was provided by a Foundation Research Endowment Grant from the Massachusetts Eye and Ear Infirmary.

Send correspondence to Ralph Metson, MD, Zero Emerson Place, Boston, MA 02114. E-mail: ralph_metsen@meei.harvard.edu

DOI: 10.1097/MLG.0b013e31816b4b6f

severe bronchospasm results, often necessitating treatment in the emergency room. It is hypothesized that a common stimulus is causing inflammation of both the sinonasal and bronchopulmonary mucosa.⁴

Microarray technology has revolutionized the field of genetic analysis, making it possible to quantify the simultaneous expression of thousands of genes. Prior studies that used this technology to analyze tissue from patients with sinusitis and asthma focused on limited gene subsets and patient populations.^{5–12} The purpose of this study was to screen expression of the entire human genome in an attempt to identify the genes most characteristic of sinonasal polyposis in patients with chronic rhinosinusitis and aspirin-sensitive asthma.

MATERIALS AND METHODS

Study Population and Tissue Collection

Sinonasal tissue was collected from 57 patients with three distinct phenotypes: 1) patients with chronic rhinosinusitis and sinonasal polyposis without a history of aspirin allergy (CRS group); 2) patients with chronic rhinosinusitis and sinonasal polyposis with a history of asthma and aspirin allergy (ASA group); and 3) patients with no history or clinical evidence of sinusitis, asthma, or aspirin allergy (control group).

Microarray analyses were performed in 30 subjects, 10 in each of the three phenotypic groups. Results were corroborated with real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) and immunohistochemistry using tissue from a separate set of subjects (Table I).

All patients underwent nasal endoscopy and sinus computed tomography (CT) to determine the extent and location of their polyps. To ensure a study population with a phenotype for severe sinus disease, enrollment of polyp patients required a CT stage of three or higher, indicating bilateral disease with frontal and/or sphenoid involvement, according to a previously described staging system.¹³ The refractory nature of the polyp disease in study subjects was also reflected by the finding that at least one

previous sinus surgery for removal of polyps had been performed in half of the CRS patients and all of the ASA patients (Table I). Exclusion criteria included age less than 18 years, a history of cigarette smoking within 1 year of surgery, or use of oral steroids within 1 month of surgery.

Study specimens were obtained under general anesthesia at the start of the patient's surgical procedure. Polyps were harvested from the anterior nasal cavity or ethmoid sinus for patients in the CRS and ASA groups. For control patients, mucosa was obtained from the inferior turbinate (n = 3) or ethmoid sinus (n = 7) during the performance of non-sinus surgery, including septoplasty (n = 4), dacryocystorhinostomy (n = 5), and orbital decompression (n = 1). All tissue specimens collected for microarray and RT-qPCR analyses were rinsed in normal saline to remove blood and mucous, placed immediately in RNeasy (Ambion, Austin, TX, AM7021), and stored at –80°C until the time of ribonucleic acid (RNA) extraction.

RNA Extraction

Total RNA was purified using RNeasy spin-columns (Qiagen, Valencia, CA) according to the manufacturer's protocol and a modification for hypocellular, dense connective tissues¹⁴ as previously described.¹⁵ Quantification and quality assessment of the RNA was performed using Agilent 2100 Bioanalyzer and RNA Pico Kit (Agilent Technologies, Santa Clara, CA). Only samples that yielded clean and undegraded RNA based on the appearance of electropherograms and RNA integrity numbers greater than seven were used. The RNA was reverse transcribed with Taqman Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA).

Microarray Processing and Analysis

Preparation of complementary RNA (cRNA), hybridization to the Affymetrix HG-U133 plus 2.0 GeneChip (Affymetrix, Santa Clara, CA), and scanning of the chip was performed according to the manufacturer's protocol in a core facility at the Harvard Medical School–Partners Healthcare Center for Genetics and Genomics (Cambridge, MA). Data from microarrays were analyzed using

TABLE I.
Patient Demographics for Microarray (n = 30), Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (n = 17), and Immunohistochemistry (n = 10) Techniques.

Assay	n	Males (%)	Mean Age in Years ± SD (Range)	CT Stage Mean ± SD ¹³	Patients with History of Asthma (%)	Prior Sinus Surgeries Mean ± SD
Microarray						
CRS	10	3 (30%)	50.1 ± 13.5 (28–73)	3.4 ± 0.5	1 (10%)	1.7 ± 2.4
ASA	10	3 (30%)	45.5 ± 10.8 (33–64)	3.9 ± 0.3	10 (100%)	3.1 ± 2.1
Control	10	5 (50%)	40.7 ± 19.4 (19–68)	0	0 (0%)	0
Total	30					
PCR						
CRS	5	2 (40%)	55.8 ± 12.5 (36–66)	3.8 ± 0.5	4 (80%)	0.6 ± 0.84
ASA	7	1 (14%)	51.7 ± 13.6 (40–80)	3.6 ± 0.5	7 (100%)	5.4 ± 2.8
Control	5	1 (20%)	47.2 ± 7.7 (38–58)	0	0 (0%)	0
Total	17					
Immunohisto-chemistry						
CRS	4	2 (50%)	47.5 ± 17.4 (34–73)	4 ± 0	2 (50%)	2.25 ± 2.1
ASA	4	0 (0%)	52.3 ± 20.2 (27–75)	3.8 ± 0.5	4 (100%)	3.3 ± 2.6
Control	2	0 (0%)	45.0 ± 2.8 (43–47)	0	0 (0%)	0
Total	10					

ASA = aspirin-sensitive asthma; CRS = chronic rhinosinusitis; CT = computed tomography; SD = standard deviation; n = number.

GenePattern 3.0 platform (Broad Institute, Cambridge, MA).¹⁶ Two unsupervised clustering algorithms—self-organizing maps (SOM)¹⁷ and hierarchical clustering¹⁸—were used to determine which samples clustered without a priori knowledge of which sample came from which phenotypic group. The stability of the identified clusters was assessed using consensus clustering.¹⁹ Consensus matrices were generated assuming two to five clusters, and the best number of clusters determined using visual inspection of the consensus matrices, and the corresponding summary statistics. Class neighbors analysis²⁰ and comparative marker selection²¹ were used to determine which genes best characterize a patient group when there was a priori knowledge of which sample came from what group. Genes were ranked using the *t* test statistic. The false discovery rate (FDR) statistic was used, and set to <10% to adjust for multiple hypothesis testing while estimating probabilities that differences in gene expression represent false positive findings. The robustness of the genes that best characterize a group was tested using K-nearest neighbors (KNN) cross validation.²⁰

Gene set enrichment analysis (GSEA)²² was used to determine whether an a priori-defined set of genes, including cytogenetic bands, metabolic and signaling pathways, and neighborhoods clustered on cancer-related genes, showed statistically significant and concordant differences between the CRS and ASA groups. Correction for multiple hypotheses testing and gene set size was automatically implemented using sample permutations. Gene sets that contained fewer than 25 genes and more than 500 genes were ignored, which is appropriate for datasets with more than 10,000 features.²² We focused on gene sets with an FDR <25% because they are most likely to generate interesting hypotheses and drive further research.

RT-PCR Quantification of Relative mRNA

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to validate expression of five genes determined to be most characteristic of the groups studied. For these genes, 6-FAM linked fluorescent probes and primers were designed and optimized by Applied Biosystems (Foster City, CA). The measurements were carried out on an Applied Biosystems 7700 sequence detector using 96 well plates and conditions as previously described.¹⁵

Immunohistochemistry

Intraoperative specimens were embedded in paraffin and serially sectioned at a thickness of 10 μ m. Immunostaining was done as previously described²³ using primary polyclonal antibodies raised against human Zn- α 2-glycoprotein (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, sc-11238; 1:500 dilution), human hepatocyte growth factor receptor, Met (Santa Cruz Biotechnology, Inc, SC-10; 1:750 dilution), human neurabin 2 (Abcam, Inc, Cambridge, MA, ab18561; 1:500 dilution), human periostin (BioVendor Laboratory Medicine, Inc, Modrice, Czech Republic, RD-181045050; 1:40,000 dilution), and monoclonal antibodies against human prolactin-induced protein (Signet Laboratories, Cambridge, MA, 611-01; 1:1,600 dilution). Control slides, which did not stain, were processed in parallel, but not exposed to the primary antibody.

Informed consent was obtained from all subjects according to the institutional review board study protocol approval by the Human Studies Committee of the Massachusetts Eye and Ear Infirmary.

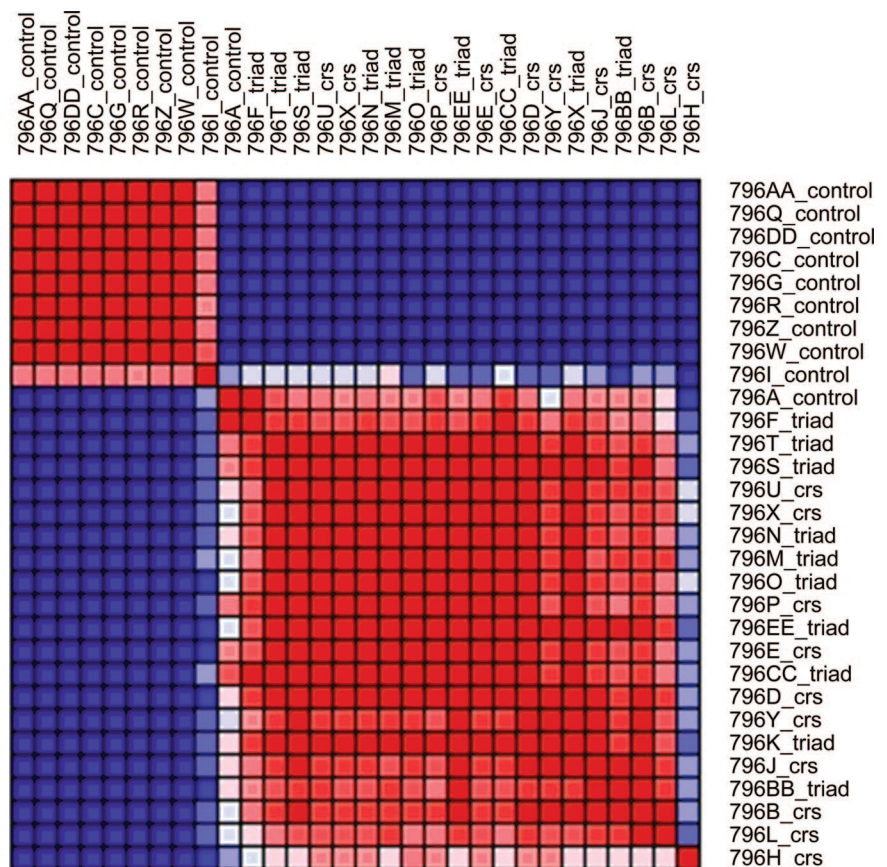


Fig. 1. Heat map of samples that share similar patterns of gene expression. The bright red color indicates samples that always clustered together, whereas the dark blue color indicates samples that never clustered together. Consensus hierarchical clustering was performed assuming three clusters. Control samples formed a distinct cluster (upper red block), whereas the patients with chronic rhinosinusitis alone (CRS) and patients with chronic rhinosinusitis and a history of aspirin-sensitive asthma (ASA) samples intermingled (lower red block).

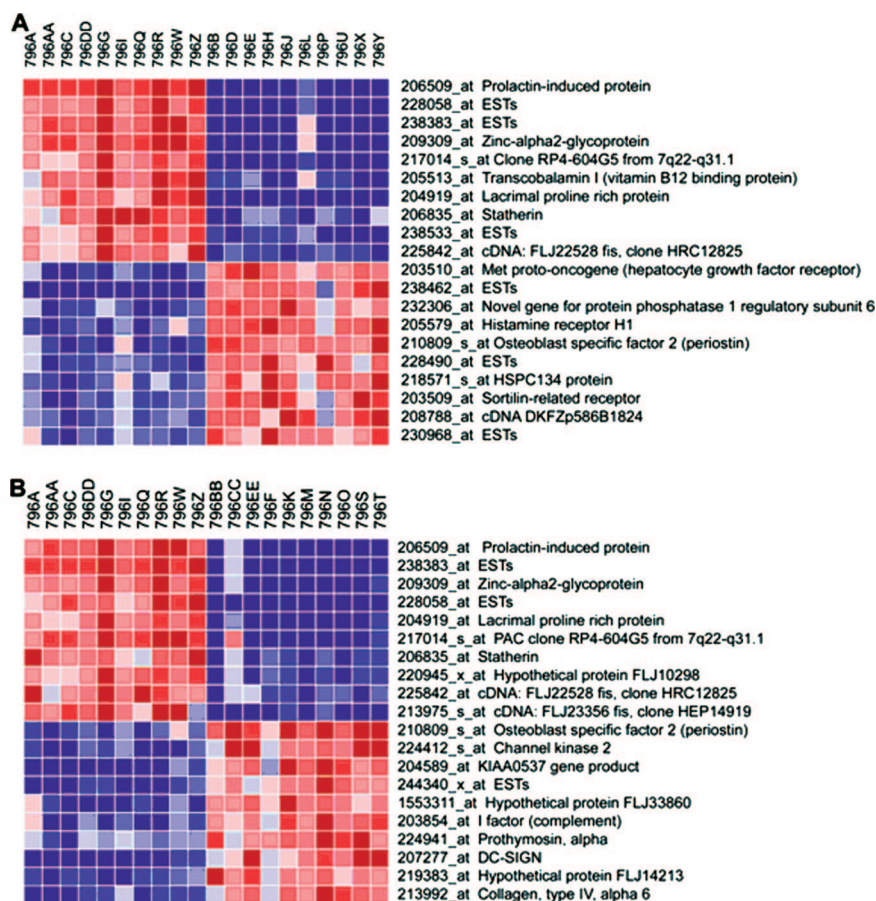


Fig. 2. Heat map of microarray data using class neighbors analysis applied to patients with chronic rhinosinusitis alone (CRS) and control group (A), or patients with chronic rhinosinusitis and a history of aspirin-sensitive asthma (ASA) and control group (B). Red indicates high, and blue indicates low levels of gene expression. The first 10 columns are control samples (A and B), and the last 10 columns are CRS (A) or ASA samples (B). Horizontal labels indicate the Affymetrix (Santa Clara, CA) feature followed by gene name.

RESULTS

Similarities between patterns of gene expression in the samples studied with microarrays are depicted in the square heat map shown in Figure 1. Consensus hierarchical clustering was performed assuming three clusters. Bright red colors indicate samples that always clustered, whereas dark blue colors indicate samples that never clustered. Control samples co-clustered forming the upper red block, whereas CRS and ASA samples intermingled forming the lower red block.

Given how different the CRS and ASA groups were from the control group, we wanted to define the smallest set of genes that best differentiated the groups. The results are summarized in heat maps (Fig. 2) where columns represent different samples, rows represent different genes, and color reflects levels of gene expression with red indicating high and blue indicating low levels of expression. For simplicity, only the 20 most characteristic genes and expressed sequence tags (ESTs) are shown in Figure 2, although 808 genes were expressed at higher levels (that is, upregulated) in controls and 2,724 genes were upregulated in CRS when comparing control and CRS samples; 468 genes were upregulated in controls and 697 genes were upregulated in ASA when comparing control and ASA samples. Figure 2 demonstrates high reproducibility of the measurements with samples from the same group having similar color coding.

The genes that best characterize each phenotypic group are known as predictor genes. These genes do not

necessarily demonstrate the largest difference in expression between two groups, but rather exhibit a small variance within a group in addition to a substantial difference between groups.²⁰ When comparing the CRS group with controls, we found that four genes were sufficient at each cross validation step to correctly predict 19 of 20 samples. Out of 80 possible genes across all 20 steps of cross validation, the following 4 genes were used most commonly (69 of 80 times), and therefore best differentiate the CRS group from controls: prolactin-induced protein (PIP), met proto-oncogene (MET), zinc alpha2-glycoprotein (AZGP1), and a sequence from clone RP4-551D2 on chromosome 20q13.2–13.33 that contains a gene for protein phosphatase 1 regulatory subunit 6 (PPP1R6). When comparing the ASA group with controls, 2 genes were sufficient at each cross validation step to correctly predict all 20 samples. Out of 40 possible genes across all 20 steps of cross validation, the 2 that were used most commonly (30 of 40 times) were periostin (POSTN) and EST Hs.226268. The relative levels of expression of these genes are summarized in Table II.

The microarray data were validated using RT-qPCR. Results are summarized in Table II and in Figure 3 where expression of the five most characteristic genes is plotted for each patient group relative to the control group. Table II indicates that the RT-qPCR data were in overall good agreement with the microarray data, although the magnitude of the divergence between the groups differed somewhat between the two techniques. Protein phosphatase 1

TABLE II.
Fold Increase in Expression of Genes Most Characteristic of the CRS and ASA Groups as Compared With the Control Group and Determined From Microarray and RT-qPCR Analysis.

Gene	CRS vs. Controls (Fold Increase)		ASA vs. Controls (Fold Increase)	
	Microarray	RT-qPCR	Microarray	RT-qPCR
PIP	0.05 (FDR <6%)	0.003+/-0.003 (<i>P</i> = .006)	0.05 (FDR = 9%)	0.002+/-0.001 (<i>P</i> = .001)
MET	3.5 (FDR <6%)	5.4+/-2.7 (<i>P</i> = .01)	3.4 (FDR = 9%)	1.0+/-0.4 (<i>P</i> = .9)
AZGP1	0.04 (FDR <6%)	0.05+/-0.04 (<i>P</i> = .02)	0.07 (FDR = 9%)	0.01+/-0.01 (<i>P</i> = .002)
PPP1R9B	31.5* (FDR <6%)	2.9+/-1.3 (<i>P</i> = .02)	31.7* (FDR = 9%)	1.2+/-0.2 (<i>P</i> = .5)
POSTN	3.6 (FDR <6%)	22.6+/-20.0 (<i>P</i> = .01)	3.6 (FDR = 9%)	4.5+/-2.8 (<i>P</i> = .02)

*Indicates microarray results for sequence from clone RP4-551D2 on chromosome 20q13.2-13.33 that contains a gene for PPP1R6. For RT-qPCR, data are expressed as mean +/- standard error of the mean.

RT-qPCR = real-time quantitative reverse transcription polymerase chain reaction; ASA = aspirin-sensitive asthma; CRS = chronic rhinosinusitis; FDR = false detection rate; PIP = prolactin-induced protein; MET = met proto-oncogene; AZGP1 = zinc alpha2-glycoprotein; PPP1R9B = protein phosphatase 1 regulatory subunit 9B; POSTN = periostin.

regulatory subunit 9B (PPP1R9B) was studied in place of PPP1R6 because the two genes are related, PPP1R9B has been more extensively characterized in the literature, commercially available primers for PPP1R9B exist, and PPP1R6 is only one of the genes in the sequence that also contains a novel cadherin-like protein VR20 gene and the 5' end of the SYCP2 gene for synaptonemal complex protein 2.

The genes most characteristic of CRS (PIP, MET, AZGP1, and PPP1R9B) and ASA (periostin) were all confirmed by RT-qPCR to be expressed at significantly different levels (*P* < .05) compared to controls. Furthermore, three of these genes were found to be expressed at significantly different levels between the CRS and ASA group when assessed with RT-qPCR: periostin (*P* = .01), MET (*P* = .01), and PPP1R9B (*P* = .02). However, when comparing the two polyp groups based on microarray data, no single

gene had a significant difference in expression between the CRS and ASA, even if allowing a false detection rate of 50%. This finding is consistent with the results in Figure 1 where CRS and ASA samples do not segregate into distinct groups. Therefore, GSEA was used to test for sets of related genes that might be systematically altered between the two groups. Three gene sets within distinct chromosomal bands were found to be different between the ASA and CRS groups: 6p22 (FDR = 18%; 39 genes in the set), 22q13 (FDR = 20%; 65 genes in the set) and 1q23 (FDR = 24%; 44 genes in the set). These chromosomal bands do not include genes studied in Figure 3, suggesting that there are additional genes that differentiate CRS and ASA groups.

Immunohistochemistry was used to verify microarray data at the protein level and to determine cellular localization of the gene products of interest. Typical results are summarized in Figure 4, and they are generally consistent with RT-qPCR data. In particular, expression of periostin was high in the basolateral surfaces of the glandular and respiratory epithelium of all tissues studied, but did not stain the apical mucosal boarder (Fig. 4, A-C). The CRS and ASA tissue (Fig. 4, B and C) stained substantially more than the control tissue when the same staining conditions were used. Expression of MET was seen in the glandular cytoplasm and respiratory epithelium of the CRS (Fig. 4E) and control tissues (Fig. 4D), and less in the ASA group (Fig. 4F). PIP showed more expression at the epithelial surface in the control tissue (Fig. 4G) than the CRS (Fig. 4H) or ASA (Fig. 4I) tissue, and consistently stained glandular cytoplasm in all groups. The ASA group, in particular, typically had minimal or no staining of the respiratory epithelium. Expression of AZGP1 was higher in the glandular cytoplasm than the respiratory epithelium of all groups (Fig. 4, J-L). Expression of PPP1R9B was similar in all groups, with staining of the glandular and mucosal epithelium (Fig. 4, M-O).

DISCUSSION

Although several reports have described the use of microarray technology to examine sinonasal tissues,⁵⁻¹² this study is the first to screen the entire human genome

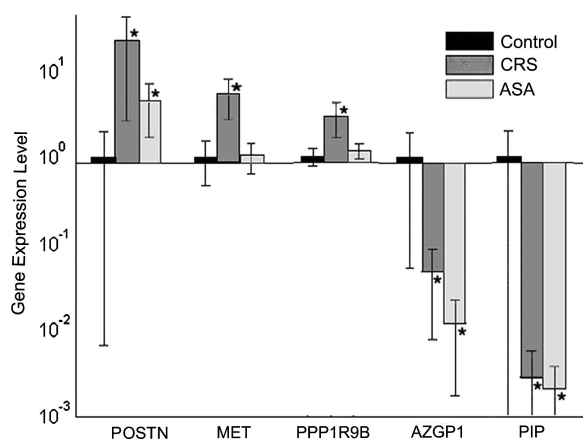


Fig. 3. Normalized levels of expression of select genes in sinonasal tissue expressed relative to controls. Significant differences in the patients with chronic rhinosinusitis alone (CRS) and chronic rhinosinusitis and a history of aspirin-sensitive asthma (ASA) groups relative to control are indicated by an asterisk. For simplicity, the expression of all five genes was set to one in control tissues, although the relative expression of these genes in decreasing abundance was prolactin-induced protein (PIP):periostin (POSTN):zinc-alpha2-glycoprotein (AZGP1):hepatocyte growth factor receptor (MET): neurabin 2 (PPP1R9B) = 97:46:37:1.3:1. Error bars indicate the standard error of the mean (SEM).

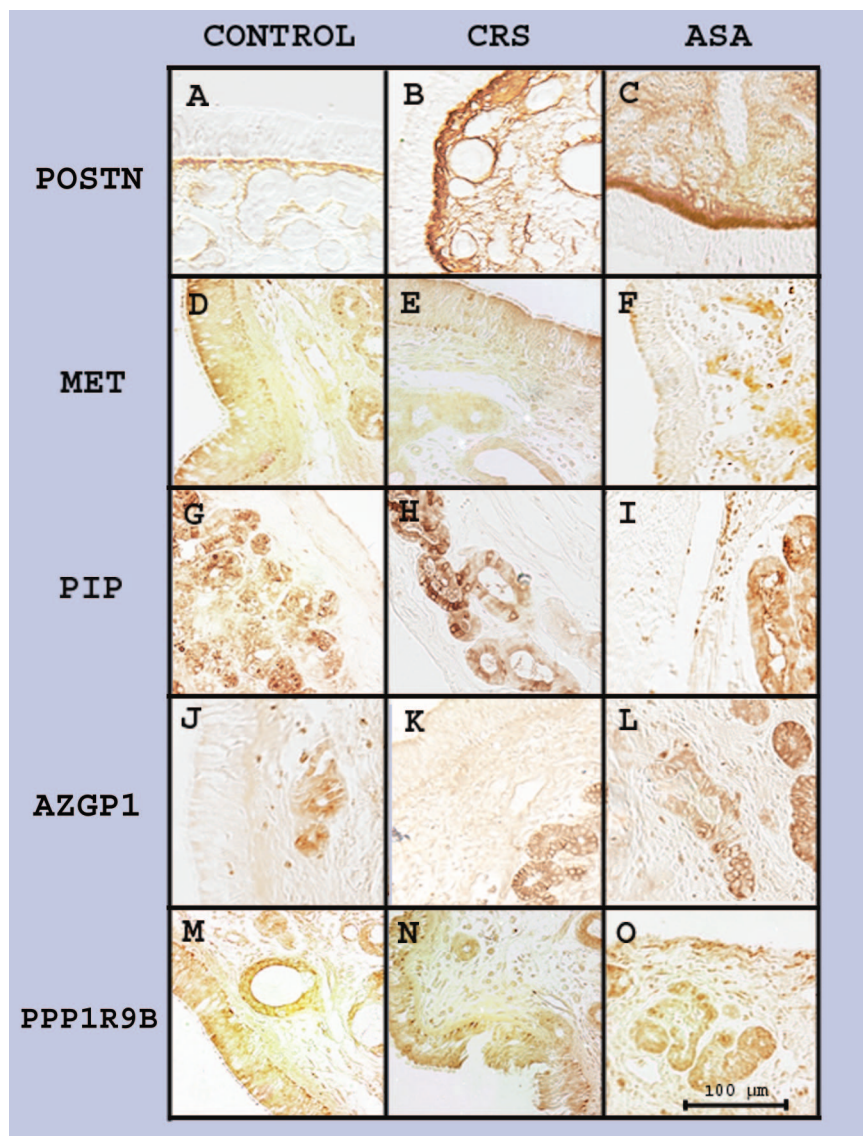


Fig. 4. Sinonasal polyps and control sinonasal mucosa immunostained with antibodies against periostin (POSTN, A–C), hepatocyte growth factor receptor (MET, D–F), prolactin-induced protein (PIP, G–I), zinc-alpha2-glycoprotein (AZGP1, J–L), and neurabin 2 (PPP1R9B, M–O). Scale bar = 100 μ m.

for alterations in gene expression in nasal polyps from patients with sinusitis and asthma. In an attempt to elucidate the genes with greatest impact, the current study screened patients for distinct phenotypes (CRS and ASA), performed bioinformatic analyses on highly reproducible microarray data, and validated select genes with two complementary techniques (RT-qPCR and immunohistochemistry) applied to different patient sets. Five genes were identified for their strong association with these disease entities—POSTN, MET, AZGP1, PIP, and PPP1R9B.

Periostin (POSTN), which has not been previously described in sinonasal tissue, was found to be abundantly expressed in normal mucosa and markedly elevated in polyps from both CRS and ASA patients, suggesting that it plays a role in the normal physiology and pathophysiology of sinonasal mucosa. Periostin is known to be a potent regulator of fibrosis and collagen deposition.²⁴ Its overexpression may be a primary contributor to pathogenesis of sinonasal polyposis by analogy to myocardial tissue where early activation of periostin and resultant fibrosis

is thought to be a primary contributor to cardiac dysfunction, not an advanced secondary phenomenon.²⁵

A second gene found to be overexpressed in CRS polyps is MET (met proto-oncogene), which encodes a receptor tyrosine kinase that plays an important role in various cellular functions, including increased cell growth, reduced apoptosis, altered cytoskeletal function, increased metastasis, and angiogenesis.²⁶ These results confirm the findings of Rho et al.²⁷ who reported an overexpression of c-Met and its ligand, hepatocyte growth factor (HGF), in nasal polyps from patients with sinusitis without asthma or aspirin sensitivity.

Although periostin and MET were found to be overexpressed in nasal polyps, the expression of PIP, a protein secreted by various apocrine glands, was found to be markedly reduced in both CRS and ASA samples compared to controls. This secretory marker of apocrine differentiation in breast carcinoma²⁸ has also been implicated in host defense against infections and tumors.²⁹ The current study results stand in contrast with those of Liu et

al. who reported overexpression of PIP in microarray analysis of nasal polyps from 10 patients, 2 of whom had ASA.⁶ Three additional genes identified by Liu et al. as being upregulated in polyps—statherin, lactoferrin and DMBP1—were found to be downregulated in both CRS and ASA polyps in the current study, and did not emerge as key predictors of CRS or ASA. Moreover, uteroglobin was identified as being downregulated in polyps studied by Liu et al.,⁶ whereas we found no statistically significant difference in expression of this gene in either CRS or ASA polyps compared to controls. A possible explanation of these discrepancies is that all of the subjects studied by Liu et al. received intranasal steroids for 1 month or more prior to surgery, which may have influenced expression levels. Furthermore, two of the four control samples in their study were obtained from the ethmoid sinus in patients who underwent surgery for drainage of maxillary mucocoeles. It is possible that the presence of active maxillary sinus disease in these patients could have affected gene expression in the adjacent ethmoid regions.

Another characteristic gene found to be underexpressed in nasal polyps is zinc- α -2-glycoprotein (AZGP1), a member of a distinct, heterogeneous lineage of major histocompatibility complex class I genes. These genes are implicated in a variety of diverse and important physiologic functions, including antiinfectious and tumor immunity.²⁹ The mechanism of the role of AZGP1 in the immune system is thought to be through the binding and presentation of a lipidic entity to T cells.³⁰ AZGP1 inhibits cell-cycle dependant proliferation, possibly by downregulating cdc2 cyclin dependant kinase,³¹ whose increased expression has been directly linked to increased proliferation and decreased differentiation of advanced tumors. High levels of AZGP1 expression in the normal nasal mucosa and markedly decreased expression in nasal polyps in the present study is consistent with the idea that underexpression of AZGP1 contributes to cellular proliferation characteristic of polyps.

The final marker gene identified in this study was PPP1R9B, a ubiquitously expressed gene that plays a role in cell growth³² and molecular scaffolding³³ by binding the catalytic subunit of protein phosphatase 1. Although this marker was found at a relatively low baseline level in normal mucosa compared to the other four identified marker genes, a significant increase in expression in CRS polyps was observed. It is possible that other regulatory subunits of the protein phosphatase 1 catalytic unit also contribute to the pathophysiology of sinonasal polyposis.

Other microarray studies of smaller patient populations have reported a variety of genes with altered expression in sinonasal polyps. Fritz et al.⁵ reported that mamaglobin, a protein of unknown function found in breast cancer cell lines, was overexpressed in the polyps of three patients with allergic rhinitis compared to controls. Although altered expression of mamaglobin did not emerge as a key predictor of ASA or CRS polyps in our study, we did find a statistically significant increase in expression of mamaglobin 1 in CRS, but not ASA patients, compared to controls. We found no statistically significant changes in mamaglobin 2 in CRS or ASA polyps compared to controls. Benson et al.⁷ observed the

increased expression of uteroglobin, a presumed antiinflammatory gene, in polyp tissue in four patients who underwent a 6-week course of topical steroid therapy whereas we found no alteration in this gene in the CRS or ASA polyps compared to controls. In a microarray study of immune-associated genes, Wang et al.⁸ found increased expression of IL-17 and its receptor in polyp tissue from four patients with chronic rhinosinusitis compared to controls. In our study, there was no statistically significant difference in expression of IL-17 in CRS or ASA polyps compared to controls. Figueiredo et al.¹⁰ studied 96 inflammatory genes in a pooled microarray analysis in patients with non-allergic nasal polyps and found alterations in TGF-B1 and IL-5 compared to controls. The current study found no alterations in these two genes. In a study of polyp tissue from three patients with allergic fungal sinusitis and four with eosinophilic mucin rhinosinusitis, Orlandi et al.¹¹ found four genes—cathepsin B, sialyltransferase 1, GM2 ganglioside activator protein, and S100 calcium binding protein β —that were differentially expressed compared to human referenced RNA controls. Expression of these four genes was not altered in our study. When studying nonallergic chronic rhinosinusitis without polyposis in 14 patients, Anand et al.⁹ found four genes associated with inflammatory pathways—TNF- α , IL-6, IL-12A, and IL-13—that were consistently overexpressed in the diseased tissue compared to controls. Our study found increased expression of IL13 in CRS but not ASA polyps, and no change in the other three genes.

The inconsistent results reported in these previous studies reflect heterogeneity of the studied populations and inherent difficulties in performing microarray analyses. The challenge to obtain meaningful results from the large volume of data generated by a relatively small patient population demands rigorous bioinformatic analyses and validation. In our study, only patients with a demonstrated severe phenotype for either of two sinusitis subsets—CRS and ASA—were enrolled. High throughput technology was used that allowed for expression survey of the entire human genome. Validation of the microarray results was performed on an independent set of patients using two complementary techniques to confirm altered expression of the identified genes and localize their protein products in sinonasal tissues. Application of these methods led to the identification of several genes that likely play a role in the pathogenesis of sinonasal polyposis and are therefore natural targets for novel therapeutic interventions.

Based on the role periostin plays in cell growth, proliferation, motility, and migration, a monoclonal antibody directed against periostin has been proposed as an adjunct to chemotherapy for colorectal cancer.³⁴ The results of our study suggest that topically or systemically applied agents against periostin, including antiperiostin monoclonal antibody, are worthy of investigation as novel agents for treatment of patients with sinonasal polyposis. This therapeutic scenario is particularly interesting given that polyps often respond partially and transiently to steroid treatment and that periostin plays a role in subepithelial fibrosis, seen in early stages of bronchial asthma³⁵ that resists steroid treatment.

Many strategies for interfering with the HGF/Met pathway are currently being investigated²⁶ because c-Met is overexpressed and mutated in a variety of malignancies. The results of our study suggest that only Met-dependent polyps-associated with CRS, but not ASA, would be expected to respond to inhibition of this pathway.

Pharmacologic treatments aimed at increasing AZGP1 production in polyps may be therapeutic, similar to the role of AZGP1 in reducing tumor cell proliferation when added to the culture medium as a protein, or transfected as cDNA.³¹ It is known that dexamethasone, a steroid used to treat nasal polyps, stimulates AZGP1 protein production in other tissues.³⁶ The use of AZGP1 knockout mice³⁰ may prove useful in determining whether AZGP1 deficiency leads to sinonasal polyposis and in exploring novel therapeutic strategies.

The potential application of these therapies to the treatment of patients with asthma, as well as sinusitis, is consistent with the unified airway theory.⁴ Common genetic and environmental factors are thought to have similar effects on both the upper and lower respiratory tracts. As many as one-third of patients diagnosed with sinusitis also present with symptoms of asthma.³⁷ In this study, the ASA cohort manifest symptoms of both disease entities, as well as a history of aspirin allergy. Microarray technology has been used to identify alteration in gene expression in patients with asthma, although not in patients with ASA specifically.^{38,39}

In this study, the GSEA allowed us to identify chromosomal bands on 6p22, 22q13, and 1q23 that contain gene sets which distinguish the CRS and ASA phenotypes. This association suggests that regional alterations with these bands, including chromosomal deletions or amplifications, dosage compensation, and epigenetic silencing, may contribute to the difference between these phenotypes. Given that GSEA optimization is sensitive to initialization, future studies with more samples may be able to increase the accuracy of GSEA. Nonetheless, it is interesting that two of the chromosomal bands that we identified have been associated with asthma. Chromosome 6p has been identified as a susceptibility gene for asthma and allergy,⁴⁰ whereas chromosome 22 is associated with susceptibility for asthma and atopy.⁴¹ Chromosome 1p, but not 1q, has been associated with the development of asthma in patients with environmental exposure to tobacco smoke.⁴² The fact that none of the five genes identified in our study localized to the three chromosomal bands suggests that those genes likely play more direct roles in the pathogenesis of sinonasal polyposis rather than asthma.

CONCLUSION

Sinonasal disease is a problem of major clinical and societal impact for which curative therapeutic modalities are often lacking, and molecular pathogenesis remains elusive. The use of high throughput microarray technology validated by RT-qPCR and immunohistochemistry has led to the identification of five genes (POSTN, MET, PIP, AZGP1, and PPP1R9B) that likely play a role in pathogenesis of sinonasal polyps associated with CRS and ASA. These genes may serve as novel therapeutic targets for

medical management of selected patients with chronic sinusitis and asthma.

BIBLIOGRAPHY

1. Rosenfeld RM, Andes D, Bhattacharyya N, et al. Clinical practice guideline: adult sinusitis. *Otolaryngol Head Neck Surg* 2007;137(3 suppl):S1–S31.
2. Gliklich RE, Metson R. Economic implications of chronic sinusitis. *Otolaryngol Head Neck Surg* 1998;118:344–349.
3. Bateman ND, Fahy C, Woolford TJ. Nasal polyps: still more questions than answers. *J Laryngol Otol* 2003;117:1–9.
4. Krouse JH, Veling MC, Ryan MW, et al. Executive summary: asthma and the unified airway. *Otolaryngol Head Neck Surg* 2007;136:699–706.
5. Fritz SB, Terrell JE, Conner ER, Kukowska-Latallo JF, Baker JR. Nasal mucosal gene expression in patients with allergic rhinitis with and without nasal polyps. *J Allergy Clin Immunol* 2003;112:1057–1063.
6. Liu Z, Kim J, Sypek JP, Wang IM, Horton H, Oppenheim FG, Bochner BS. Gene expression profiles in human nasal polyp tissues studied by means of DNA microarray. *J Allergy Clin Immunol* 2004;114:783–790.
7. Benson M, Carlsson L, Adner M, et al. Gene profiling reveals increased expression of uteroglobin and other anti-inflammatory genes in glucocorticoid-treated nasal polyps. *J Allergy Clin Immunol* 2004;113:1137–1143.
8. Wang X, Dong Z, Zhu DD, Guan B. Expression profile of immune-associated genes in nasal polyps. *Ann Otol Rhinol Laryngol* 2006;115:450–456.
9. Anand VK, Kacker A, Orjuela AF, Huang C, Manarey C, Xiang J. Inflammatory pathway gene expression in chronic rhinosinusitis. *Am J Rhinol* 2006;20:471–476.
10. Figueiredo CR, Santos RP, Silva ID, Weckx LL. Microarray cDNA to identify inflammatory genes in nasal polyposis. *Am J Rhinol* 2007;21:231–235.
11. Orlandi RR, Thibeault SL, Ferguson BJ. Microarray analysis of allergic fungal sinusitis and eosinophilic mucin rhinosinusitis. *Otolaryngol Head Neck Surg* 2007;136:707–713.
12. Bolger WE, Joshi AS, Spear S, Nelson M, Govindaraj K. Gene expression analysis in sinonasal polyposis before and after oral corticosteroids: a preliminary investigation. *Otolaryngol Head Neck Surg* 2007;137:27–33.
13. Gliklich RE, Metson R. A comparison of sinus computed tomography (CT) staging systems for outcomes research. *Am J Rhinol* 1994;8:291–297.
14. Reno C, Marchuk L, Sciore P, Frank CB, Hart DA. Rapid isolation of total RNA from small samples of hypodermal, dense connective tissues. *BioTechniques* 1997;22:1082–1086.
15. Stankovic KM, Kristiansen AG, Bizaki A, Lister M, Adams JC, McKenna MJ. Studies of otic capsule morphology and gene expression in the Mov13 mouse—an animal model of type I osteogenesis imperfecta. *Audiol Neurotol* 2007;12:334–343.
16. GenePattern 3.0 [computer program.] Cambridge, MA: Broad Institute. Available at: <http://www.broad.mit.edu/genepattern/>. Accessed June 20, 2007.
17. Tamayo P, Slonim D, Mesirov J, Zhu Q, Kitarow S, Dmitrovsky E, Lander ES, Golub TR. Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. *Proc Natl Acad Sci U S A* 1999;96:2907–2912.
18. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 1998;95:14863–14868.
19. Monti S, Tamayo P, Mesirov J, Golub T. Consensus clustering: a resampling based method for class discovery and visualization of gene expression microarray data. *Machine Learning* 2003;52:91–118.
20. Golub TR, Slonim DK, Tamayo P, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* 1999;286:531–537.

21. Gould J, Getz G, Monti S, Reich M, Mesirov JP. Comparative gene marker selection suite. *Bioinformatics* 2006;22:1924–1925.
22. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005;102:15545–15550.
23. Adams JC. Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. *J Histochem Cytochem* 1992;40:1457–1463.
24. Norris RA, Damon B, Mironov V, et al. Periostin regulates collagen fibrillogenesis and the biomechanical properties of connective tissues. *J Cell Biochem* 2007;101:695–711.
25. Kim JB, Porreca GJ, Song L, et al. Polony multiplex analysis of gene expression (PMAGE) in mouse hypertrophic cardiomyopathy. *Science* 2007;316:1481–1484.
26. Sattler M, Salgia R. c-Met and hepatocyte growth factor: potential as novel targets in cancer therapy. *Curr Oncol Rep* 2007;9:102–108.
27. Rho HS, Lee SH, Lee HM, et al. Overexpression of hepatocyte growth factor and its receptor c-Met in nasal polyps. *Arch Otolaryngol Head Neck Surg* 2006;132:985–989.
28. Haagenen DE Jr, Dilley WG, Mazoujian G, Wells SA Jr. Review of GCDFP-15. An apocrine marker protein. *Ann N Y Acad Sci* 1990;586:161–173.
29. Bahram S, Bresnahan M, Geraghty DE, Spies T. A second lineage of mammalian major histocompatibility complex class I genes. *Proc Natl Acad Sci U S A* 1994;91:6259–6263.
30. Rolli V, Radosavljevic M, Astier V, et al. Lipolysis is altered in MHC class I zinc-alpha(2)-glycoprotein deficient mice. *FEBS Lett* 2007;581:394–400.
31. He N, Brysk H, Tying SK, Ohkubo I, Brysk MM. Zinc-alpha(2)-glycoprotein hinders cell proliferation and reduces cdc2 expression. *J Cell Biochem* 2001;81:162–169.
32. Sarrouilhe D, di Tommaso A, Metaye T, Ladeveze V. Spinophilin: from partners to functions. *Biochimie* 2006;88:1099–1113.
33. Satoh A, Nakanishi H, Obaishi H, et al. Neurabin-II/spinophilin. An actin filament-binding protein with one pdz domain localized at cadherin-based cell–cell adhesion sites. *J Biol Chem* 1998;243:3470–3475.
34. Tai IT, Dai M, Chen LB. Periostin induction in tumor cell line explants and inhibition of in vitro cell growth by anti-periostin antibodies. *Carcinogenesis* 2005;26:908–915.
35. Takayama G, Arima K, Kanaji T, et al. Periostin: a novel component of subepithelial fibrosis of bronchial asthma downstream of IL-4 and IL-13 signals. *J Allergy Clin Immunol* 2006;118:98–104.
36. Bing C, Bao Y, Jenkins J, Sanders P, Manieri M, Cinti S, Tisdale MJ, Trayhurn P. Zinc-alpha2-glycoprotein, a lipid mobilizing factor, is expressed in adipocytes and is up-regulated in mice with cancer cachexia. *Proc Natl Acad Sci U S A* 2004;101:2500–2505.
37. Larsen K. The clinical relationship of nasal polyps to asthma. *Allergy Asthma Proc* 1996;17:243–249.
38. Guajardo JR, Schleifer KW, Daines MO, et al. Altered gene expression profiles in nasal respiratory epithelium reflect stable versus acute childhood asthma. *J Allergy Clin Immunol* 2005;115:243–251.
39. Yuyama N, Davies DE, Akaiwa M. Analysis of novel disease-related genes in bronchial asthma. *Cytokine* 2002;19:287–296.
40. Ober C. HLA-G: an asthma gene on chromosome 6p. *Immunol Allergy Clin North Am* 2005;25:669–679.
41. Koppelman GH, Stine OC, Xu J, et al. Genome-wide search for atopy susceptibility genes in Dutch families with asthma. In *Genetics of Asthma and Atopy 2001*. [PhD dissertation by GH Koppelman], Groningen, Netherlands: University of Groningen; 2001.
42. Colilla S, Nicolae D, Pluzhnikov A, et al. Evidence for gene environment interactions in a linkage study of asthma and smoking exposure. *J Allergy Clin Immunol* 2003;111:840–846.