

Expression of protease-activated receptors in allergic fungal rhinosinusitis

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Background: The etiology of the intense inflammatory response showed by patients with allergic fungal rhinosinusitis (AFRS) remains a mystery. Potential sources of this inflammation may include fungal proteases. Protease-activated receptors (PARs) are components of the innate immune response that are modulated by proteolytic activity and are involved in potentiating T helper 2 (Th2) responses. The objective of the study was to determine whether there is differential expression of PARs in patients with AFRS compared to controls.

Methods: The study was designed as a comparison of gene expression profiles in patients with AFRS vs diseased and nondiseased controls. Twenty-five patients were enrolled. Patients with AFRS (n = 15) were compared to nondiseased controls (n = 5) undergoing minimally invasive pituitary surgery (MIPS) and patients with chronic rhinosinusitis with nasal polyps (CRSwNP, n = 5) undergoing functional endoscopic sinus surgery (FESS). Ethmoid mucosa RNA was hybridized to 4x44K microarray chips. Four gene probes (PAR1, PAR2, PAR3, and PAR4) were used to assess for differential expression. A linear-mixed model was used to ac-

count for some patients having multiple samples. Significance level was determined at $p < 0.05$.

Results: Of the 4 probes, only PAR3 showed statistically significant differential expression between AFRS and nondiseased control samples ($p = 0.03$) as well as a 2.21-fold change. No additional statistical difference in PAR expression among the comparison groups was noted.

Conclusion: PARs have been shown to enhance production of inflammatory cytokines and potentiate Th2 responses. In this initial report, patients with AFRS have a significantly increased expression of PAR3 compared to nondiseased controls. © 2014 ARS-AAOA, LLC.

Key Words:

allergic; fungal rhinosinusitis; protease; receptors; polyps

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Chronic eosinophilic rhinosinusitis (CERS) describes a refractory phenotype of chronic rhinosinusitis (CRS) with an intense eosinophilic inflammatory response and nasal polyposis. Subtypes of CERS are generally very difficult to control with both medical and surgical management.

Allergic fungal rhinosinusitis (AFRS) is a recalcitrant subtype of CERS that was first described in the late 1970s and early 1980s and is thought to be a similar immuno-

logic and clinical process to allergic bronchopulmonary aspergillosis.¹⁻⁴ Bent and Kuhn developed 5 diagnostic criteria for AFRS, including: type I hypersensitivity; nasal polyposis; computed tomography findings of heterogeneity/bony expansion of the sinuses; eosinophilic mucus; and fungal elements on tissue removed during surgery without evidence of invasion.⁵ Some reports claim that as many as 5% to 10% of those with CRS have AFRS.⁶⁻⁸ The incidence of AFRS varies greatly with geographic region and is strongly concentrated within the southeastern portion of the United States.⁹ Afflicted patients are typically young (mean age at diagnosis is 21.9 years), atopic, and immunocompetent patients who report long-term chronic sinusitis despite prolonged medical therapy and multiple surgeries.⁸

The etiology of this intense inflammatory response showed by patients with AFRS remains a mystery. The most common etiologic factors discussed in AFRS are immune responses to microorganisms including planktonic forms of bacteria and fungi as well as immunoglobulin E (IgE)-mediated allergy.¹⁰ However, this explanation fails

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to describe why only some patients develop recalcitrant inflammation although bacterial and fungal antigen exposure is nearly universal. One explanation of this exaggerated inflammatory process involves an essential component of AFRS: hypersensitivity to environmental fungal allergens. Recent data suggest that fungal proteases (an essential part of fungal physiology and development) are present in most airborne particles and can activate and enhance the innate immune response, resulting in IgE-induced inflammation as well as the production of IgE antibodies to proteins that otherwise would not typically elicit a T helper 2 (Th2)-type response.^{11,12}

Protease-activated receptors (PARs) are a family of proteolytically activated 7-transmembrane G protein-coupled receptors that are widely expressed in airway epithelium, mast cells, eosinophils, neutrophils, monocytes/macrophages, lymphocytes, smooth muscle, endothelium, fibroblasts, and neurons.^{13–17} There are 4 types of PARs (PAR1, PAR2, PAR3, and PAR4) that seem to play an integral role in the immune system's interface with environmental fungal proteases.¹⁶ The proteolytic cleavage of amino acids at the extracellular N-terminus portion of the PAR exposes a tethered ligand domain that binds to another site on the same molecule, thereby activating the receptor.^{11,12,16} This activation is irreversible and leads to G-signaling cascades that increase intracellular phospholipase C, which results in increased intracellular Ca^{++} levels and eventually secretion, degranulation, and smooth muscle contraction.¹¹ PARs ultimately lead to edema, promote angiogenesis, fibrosis, and increased IgE production, as well as eosinophil and neutrophil infiltration.^{12,16} In addition, PAR activation leads to morphologic changes, secretion of proinflammatory cytokines, and development of nasal polyps.^{11,16,18}

PARs were initially recognized in their role for platelet activation and wound healing.¹⁶ However, the focus of recent investigations has targeted their role in the innate immune response and specifically allergic inflammation. To date, there are data that links PAR activation to an allergic inflammatory response.^{11,14,19–24} In a murine model, overexpression of PAR2 increases susceptibility to allergic inflammation.¹⁹ In human polyp epithelial cells, Shin et al.²³ noted upregulation of PAR2 and PAR3 messenger RNA (mRNA) after stimulation with *Alternaria*, *Aspergillus*, and *Cladosporium* allergens. Other authors reported fungal proteases activity in house dust, showing the importance of these enzymes that seem to synergistically stimulate T-cells and dendritic cell signaling.²⁰

DNA microarray use has revolutionized genetic analysis by making it possible to define patterns of gene expression within a particular biologic disease.²⁵ Although research efforts to date have focused on defining the etiologies of CRS and nasal polyps, there is a paucity of literature on the genetic pathogenesis of AFRS. Moreover, results from previous studies using microarray technology in sinonasal inflammatory disease have primarily focused on examining the entire genome for expression differences and are limited

by small sample size, variable inclusion criteria, and lack of adequate controls.^{25–33}

The lack of successful treatment options to adequately control the intense inflammatory response of AFRS makes it an intriguing target for translational research to identify targets of immune dysregulation in the treatment of AFRS. We hypothesize that PAR expression is upregulated in patients with AFRS compared to patients with and without CRS.

Patients and methods

Inclusion and exclusion criteria

This was a University of North Carolina–Chapel Hill Institutional Review Board (IRB)-approved (IRB #09-1538) study. Patients between the ages of 7 and 80 years were eligible for inclusion and recruited through the clinical practices within the Department of Otolaryngology–Head and Neck Surgery. Subjects underwent a thorough history and physical examination to characterize the salient features of their disease presentation for further analysis. AFRS patients were diagnosed based on the criteria outlined by Bent and Kuhn.⁵ All AFRS patients met all 5 of the Bent and Kuhn criteria to be included in the study.⁵ Subjects with inflammatory sinonasal disease were stratified after surgery into either AFRS or chronic rhinosinusitis with nasal polyps (CRSwNP) groups. Patients with AFRS or CRSwNP attempted and, subsequently, failed trials of maximal medical management (including intranasal and/or systemic corticosteroids, antihistamines, and antibiotics for greater than 3 weeks) and had persistence of either radiographic or endoscopic disease. Endoscopic surgical management was offered for the treatment of refractory disease. Patients signed consent forms for the study during their preoperative visit. Patients were excluded from consideration if they had a confirmed diagnosis of an organic disease process, which could account for their disease presentation or confound the interpretation of the genomic data. Specifically, patients with primary ciliary dyskinesia/Kartagener's syndrome, cystic fibrosis, immunodeficiency, human immunodeficiency virus (HIV), organ transplantation, or autoimmune disease were excluded. A control cohort was recruited from those undergoing resection for pituitary tumors via an endonasal, endoscopic transsphenoidal approach. These nondiseased, control patients had no evidence of radiographic or endoscopic inflammatory disease.

A total of 25 subjects (AFRS $n = 15$, normal controls $n = 5$, and $n = 5$ CRSwNP) were recruited and had samples collected (Table 1).

Specimen collection, RNA extraction, hybridization

Intraoperatively, mucosal samples (not fungal material or allergic mucin) were taken from the ethmoid sinuses. Each specimen was placed in sterile saline, bathed in RNAlater® (Qiagen, Inc., Valencia, CA, USA), for 24 hours at 4°C, snap frozen in liquid nitrogen, and stored at the Tissue Procurement Facility at the Lineberger Comprehensive

TABLE 1. Patient cohort demographic data

Patients, n	25
AFRS	15
CRSwNP	5
Controls	5
Gender ratio, males:females	1.3:1
AFRS, males (% male)	10 (67)
CRSwNP, males (% male)	3 (60)
Controls, males (% male)	3 (60)
Race/ethnicity, n (%)	
AFRS	
African American	13 (86)
Caucasian	1 (7)
Hispanic	1 (7)
CRSwNP	
African American	3 (60)
Caucasian	2 (40)
Hispanic	0 (0)
Controls	
African American	1 (20)
Caucasian	4 (80)
Hispanic	0 (0)
Age, years, mean $\pm$ SD	
AFRS	20.2 $\pm$ 6.7
CRSwNP	42.6 $\pm$ 16.8
Controls	57.8 $\pm$ 15.2
Mean total IgE, kU/L	
AFRS	1276

AFRS = allergic fungal rhinosinusitis; Controls = patients undergoing MIPS; CRSwNP = chronic rhinosinusitis with nasal polyps; IgE = immunoglobulin E; MIPS = minimally invasive pituitary surgery; SD = standard deviation.

Cancer Center (University of North Carolina–Chapel Hill) in preparation for mRNA extraction. At the time of mRNA extraction, the tissue was disrupted and homogenized in guanidine-isothiocyanate-containing lysis buffer using PRO200 homogenizer (Intermountain Scientific Co., Kaysville, UT, USA). The RNeasy Extraction Mini Kit (Qiagen, Inc., Valencia, CA, USA) was used for mRNA isolation. The mRNA was quantified by ultraviolet spectrophotometry at an absorbance of 260 nm and its integrity was confirmed by agarose gel electrophoresis. Only tissue samples that yielded RNA integrity numbers greater than 7 were included in the microarray hybridization. The Stratagene Universal human reference mRNA (Agilent Technologies, Inc., Wilmington, DE, USA), which

comprises mRNA from 10 different human cell lines, was used as a control. If there were RNA isolates that did not meet the required quantity for hybridization, these samples underwent amplification in order to provide a sufficient quantity of mRNA for further processing. Hybridization to the Agilent (Agilent Technologies, Inc., Wilmington, DE, USA) Two-Color 4 $\times$ 44K Whole Genome Microarray chip was performed according to protocol. For each sample, a minimum of 1 μ g of mRNA and 1 μ g of reference RNA was used. The sample was prepared for hybridization by generating fluorescent complementary RNA (cRNA) from the extracted isolate. The Agilent Low Input Quick Amp Labeling Kit used a T7RNA polymerase to amplify label the product with a cyanine 3-labeled or cyanine 5-labeled cytidine triphosphate. With the use of this kit, there was at least a 100-fold amplification of the mRNA to cRNA. A flipped-dye paradigm was used for sample validation. Once the cRNA was measured and quantified, the sample was hybridized. The microarray chip then underwent a series of washes and was prepared for scanning on the Axon 4000B Scanner. Agilent feature extraction was then performed in order to use information garnered from the probe features of the microarray data set to measure the degree of differential expression between the samples. Background data correction was performed by subtracting the local background for each channel. LOESS and quartile normalization was applied to each of the 50 slides (25 subjects $\times$ 2 sinus pairs) to reduce interslide variability and ensure that intensities were comparable across arrays.³⁴ For each gene on each slide, the gene expression value was computed as the log (Cy3/Cy5) ratio. Following quality control and filtration of unexpressed genes, the difference in gene expression values was computed for each gene.

Statistical analysis

A linear mixed model was used for the statistical analysis to account for some patients having repeated samples. A log₁₀ transformation was used to achieve normalized variance. Benjamini-Hochberg multiple comparison correction was applied within comparisons and a total of 12 tests were performed.³⁵ To adjust for multiple comparisons, the false discovery rate (FDR)^{35,36} was controlled at the 0.10 level. Genes above this threshold were considered differentially expressed. The microarray results were not used to narrow down the entire list of genes expressed. The analysis of the 4 PAR genes (PAR1, PAR2, PAR3, and PAR4) was decided a priori. The PAR genes were only tested for differential expression between patient groups of interests. A fold change of ≥ 1.5 with a $p \leq 0.05$ was considered significant. (A threshold of fold change of ≥ 1.5 may identify a greater number of biologically important genes because overall few genes have differential expression that exceed this threshold. Thus, the significant difference observed at $p \leq 0.05$ and fold change > 2 may result in missing important differences in gene expression.^{37,38})

Statistical comparisons of demographic factors (means, standard deviations, confidence intervals, and *t* tests) were

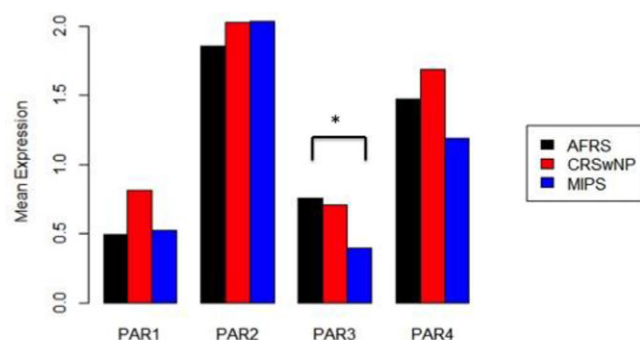


FIGURE 1. Direct overall expression of PAR genes in each comparison group. AFRS (black), control (MIPS) subjects (blue), and CRSwNP (red). PAR3 was the only gene with statistically significant differential expression (*) among the comparison groups. AFRS = allergic fungal rhinosinusitis; CRSwNP = chronic rhinosinusitis with nasal polyps; MIPS = minimally invasive pituitary surgery; PAR = protease-activated receptor.

calculated using Microsoft Excel for Mac 2011 software (Microsoft Corporation; version 14.3.5). Values of $p < 0.05$ were determined a priori to be statistically significant.

Results

A total of 25 patients were enrolled in the study. Fifteen patients were diagnosed with AFRS by meeting all 5 Bent-Kuhn criteria (Table 1). Five patients with CRSwNP and 5 patients undergoing minimally invasive pituitary surgery (MIPS) were also recruited. There was a predominance of males in all subject groups, whereas the AFRS group was comprised of mostly African Americans (86%). The AFRS had a statistically significant younger population with a mean age of 20.2 ± 6.7 years compared to CRSwNP (42.6 ± 16.8 years, $p = 0.04$) and MIPS controls (57.8 ± 15.2 years, $p = 0.004$). Of note, the mean total IgE for AFRS patients was 1276 kU/L.

Microarray analysis

The overall expression of PAR distributed among the groups is shown in Figure 1. Of the 4 PAR genes, PAR3 expression was noted to have the highest fold

change (2.21, $p = 0.03$) when comparing those with AFRS to the control cohort (MIPS) (Table 2, Figs. 1 and 2). Expression of PAR1, PAR2, and PAR4 were not statistically higher in AFRS subjects compared to controls (Fig. 2). When comparing patients with CRSwNP to those with AFRS, none of the genes met fold-change criteria (>1.5 fold-change) (Table 1). Of note, PAR1 expression did approach p value significance prior to Benjamini-Hochberg multiple comparison correction ($p = 0.07$), but did not meet the fold-change criterion. Furthermore, patients with CRSwNP showed no statistically significant upregulation of any of the PAR genes compared to the controls (MIPS) and only PAR3 was noted to meet the fold-change criterion of ≥ 1.5 (Fig. 2).

Discussion

Previous attempts to apply microarray technology to sinonasal disease have found some success in describing the underlying inflammatory mechanisms of CRS. Wang et al.³³ showed differential expression of 87 genes between nasal polyp tissue and inferior turbinates in patients with CRS with nasal polyposis. The majority of these genes had immunologic activity, including cytokines and chemokines and their receptors, adhesion molecules, and immune signal transduction molecules. In another comparative analysis between the genetic profiles of patients with CERS, Orlandi et al.³¹ showed differential expression of 4 genes that mediated lysosomal activity and played roles in various neoplastic and inflammatory disease states. Other studies similarly applied microarray technology to examine the pathogenic mechanisms at work in patients with CRS as well as AFRS. These studies found numerous genes resulting in differential expression, but no definitive insight on the pathogenesis of CRS has been elucidated.^{29,30,32}

Although AFRS represents a refractory phenotype of CERS notable for an intense eosinophilic inflammatory response, nasal polyps, hypersensitivity, and fungal elements on operative specimen, few etiologic hypotheses exist. Potential contributors to the exaggerated inflammatory process are environmental fungal allergens. Fungal proteases are a critical component of fungal physiology

TABLE 2. Comparison of PAR expression*

	Comparisons					
	AFRS vs MIPS		AFRS vs CRSwNP		CRSwNP vs MIPS	
	<i>p</i>	Fold change	<i>p</i>	Fold change	<i>p</i>	Fold change
PAR1	0.81	0.86	0.26	0.62	0.67	1.39
PAR2	0.81	1.09	0.82	0.91	0.72	1.18
PAR3	0.03	2.21	0.59	1.32	0.67	1.66
PAR4	0.58	1.26	0.84	0.96	0.67	1.30

*These data show a statistically significant higher expression of PAR3 in patients with AFRS compared to controls (patients undergoing MIPS), while no statistical significance is seen in comparisons of AFRS vs CRSwNP or CRSwNP vs MIPS. Reported p values represent Benjamini-Hochberg-corrected values.

AFRS = allergic fungal rhinosinusitis; CRSwNP = chronic rhinosinusitis with nasal polyps; MIPS = minimally invasive pituitary surgery; PAR = protease-activated receptor.

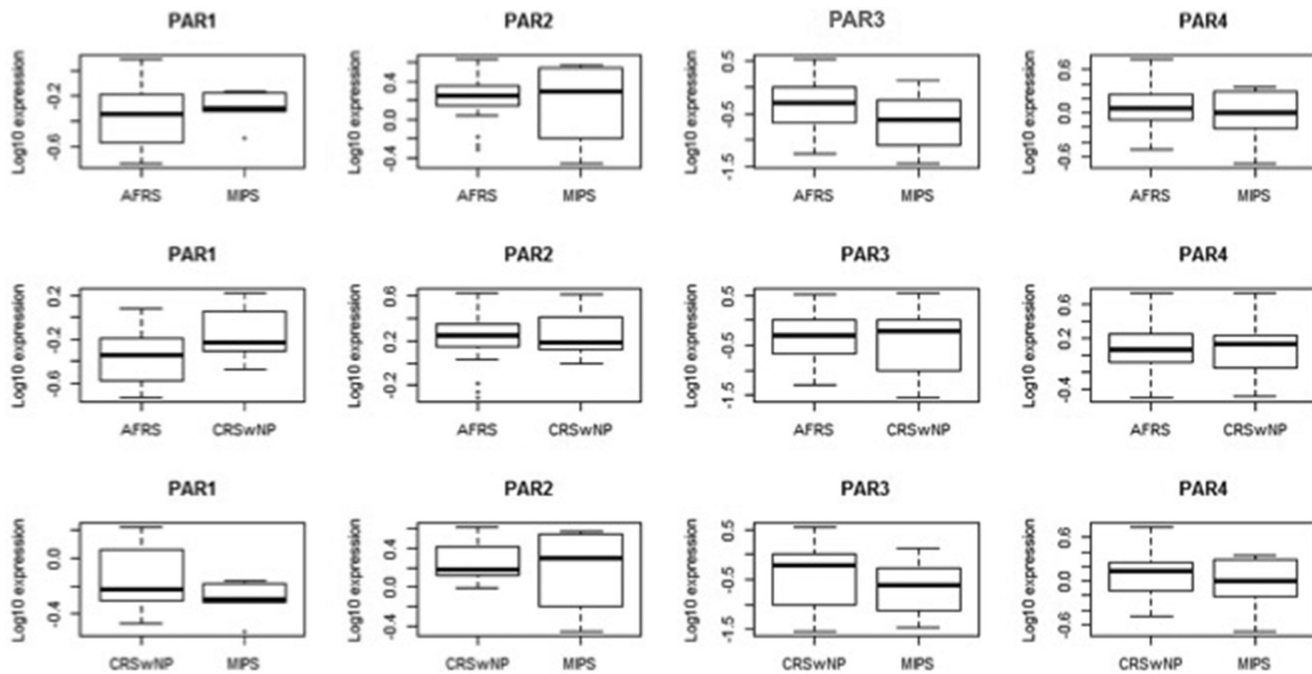


FIGURE 2. Box plots showing direct comparisons of PAR expression in patients with AFRS vs control (MIPS) subjects, AFRS vs CRSwNP, and CRSwNP vs control (MIPS). PAR3 expression was significantly higher compared to other PAR genes ($p = 0.03$) in the AFRS vs control (MIPS). No other statistically significant differential expression was noted among any of the comparison groups among the PAR genes. The values on the y-axes are converted to $\log_{10}$ in order to lie on a normal scale. AFRS = allergic fungal rhinosinusitis; CRSwNP = chronic rhinosinusitis with nasal polyps; MIPS = minimally invasive pituitary surgery; PAR = protease-activated receptor.

and development. These proteases have been found in most airborne particles and shown to stimulate and heighten the innate immune response resulting in IgE-induced inflammation.^{11,12}

The sinonasal epithelium acts as a protective barrier against harmful insults of pathogenic microorganisms. This protective barrier is composed of physical barriers (mucous layer, tight junctions, and mucociliary clearance) and mechanical barriers (surface receptors capable of identify microorganisms and/or soluble proteins).²³ When stimulated, the respiratory epithelial cells are capable of participating in the inflammatory response through the secretion of cytokines and other mediators.^{23,39,40} Airborne fungi and their proteases may have a significant role in this inflammatory response.

It is thought that fungal proteases act much like cysteine protease and serine-like proteases from dust mites and result in epithelial permeability by eroding tight junctions between epithelial cells, thereby providing allergens access to the cells of the innate immune response.^{12,41} Tai et al.⁴² showed the breakdown of occludin, a tight junction protein, by the serine protease of *Penicillium chrysogenum*. In addition, proteases from *Aspergillus fumigatus* have resulted in damage of the integrity of the basement membrane integrity by disrupting the actin cytoskeleton and destroying cell attachment.^{12,43}

PARs represent a component of the innate immune system's interface with these fungal proteases. PARs have been

found in numerous cells, including airway epithelium, mast cells, eosinophils, neutrophils, monocytes-macrophages, lymphocytes, smooth muscle, endothelium, fibroblasts, and neurons.^{13–17} Moreover, fungi have been shown to induce expression of PARs in sinonasal epithelial cells and result in the production of Regulated on activation, normal T cell expressed and secreted (RANTES), eotaxin, interleukin 8 (IL-8), and granulocyte macrophage colony stimulating factor (GM-CSF).²³

To date, there are no data exploring the role of PAR expression in AFRS. In the present study, only PAR3 gene expression in those with AFRS showed differential expression compared to nondiseased controls (MIPS). While previous studies have linked PAR2 expression to allergic-type inflammation,⁴⁴ there is evidence that PAR3 is similarly involved with the innate inflammatory response. In 1 study, sinonasal epithelial cells that did not express PAR mRNA prior to stimulation with fungi were noted to express PAR2 and PAR3 after fungal stimulation.²³ In addition, there is some thought that mPAR3 may also act as a cofactor for other PARs, thereby acting as an ancillary molecule to present a ligand to another receptor.⁴⁵

Investigations into fungal proteases and their interaction with the innate and adaptive immune system may provide interesting insight into the intense inflammatory response demonstrated by patients with AFRS. While PARs likely play a critical role in this process, the small sample size and lack of validation data are limiting factors in this

Conclusion

PARs represent a component of the innate immune system's interface with environmental fungal proteases. PARs have been found in cells throughout the upper airway and have been shown to enhance production of inflammatory cytokines and potentiate Th2 responses. In this initial report, patients with AFRS have a significant increase in PAR3 expression as compared to nondiseased controls. 8

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