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Elevation of S100 calcium binding protein A9 in sputum of neutrophilic inflammation in severe uncontrolled asthma

Tae-Hyeong Lee, PhD candidate^{*}; An-Soo Jang, MD, PhD^{*}; Jong-Sook Park, MD, PhD^{*}; Tae-Hoon Kim, PhD^{*}; Young Suk Choi, MS^{*}; Hye-rim Shin, MS^{*}; Sung-Woo Park, MD, PhD^{*}; Soo-Taek Uh, MD, PhD[†]; Jae-Sung Choi, MD, PhD[‡]; Young Hoon Kim, MD, PhD[‡]; YongBae Kim, PhD[§]; Sungryul Kim, PhD[§]; Il Yup Chung, PhD^{||}; Sung Hwan Jeong, MD, PhD[¶]; and Choon-Sik Park, MD, PhD^{*}

^{*} Genome Research Center for Allergy and Respiratory Disease, Soonchunhyang University Bucheon Hospital, Bucheon, Korea

[†] Division of Allergy and Respiratory Medicine, Soonchunhyang University Seoul Hospital, Seoul, Korea

[‡] Division of Allergy and Respiratory Disease, Soonchunhyang University Chunan Hospital, Chunan, Korea

[§] Soonchunhyang Environmental Health Center for Asbestos-Related Disease, College of Medicine, Soonchunhyang University Cheonan Hospital, Cheonan, Korea

^{||} Seoul College of Science and Technology, Hanyang University, Ansan, Korea

[¶] Department of Internal Medicine, Gachon University Gil Medical Center, Incheon, Korea

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ABSTRACT

Background: Neutrophilic airway inflammation is frequently observed in severe uncontrolled asthma (UA) and controlled asthma (CA). However, there is no sputum biomarker to differentiate the 2 conditions.

Objective: To identify biomarkers of severe uncontrolled asthma with neutrophilic airway inflammation.

Methods: Sputum with a neutrophil content larger than 70% was pooled from 5 patients with severe UA and from 10 patients with CA. Two-dimensional electrophoresis was adopted for differential display proteomics, and candidate proteins were identified using matrix-assisted laser desorption/ionization–time of flight mass spectrometric analysis. S100 calcium binding protein A9 (S100A9) was identified by western blot and its level was measured in sputum from asthmatics with varying disease severity, patients with chronic obstructive lung disease, and normal controls using enzyme-linked immunosorbent assay.

Results: Fourteen protein spots exhibited differences in relative intensity between patients with severe UA and those with CA. Matrix-assisted laser desorption/ionization–time of flight/time of flight of these spots showed an increase in human neutrophil peptide-2, S100A9, β -amylase, neutrophil gelatinase-associated lipocalin, 4-aminobutyrate transaminase, and cystatin SA in patients with UA compared with patients with CA. There was a decrease in the plunc precursor, complement C3 component, immunoglobulin heavy-chain variable region, glial fibrillary acidic protein isoform-1, IgM κ IIIb SON, MLL-AF4 der(11) fusion protein, cytokeratin-8, and recombinant IgG4 heavy chain. S100A9 was detected at a higher level in western blots of neutrophilic sputum from patients with severe UA vs CA. S100A9 levels were significantly increased, as measured by enzyme-linked immunosorbent assay, in neutrophilic UA compared with CA, eosinophilic UA and CA, and chronic obstructive lung disease.

Conclusion: S100A9 in sputum may be a biomarker of neutrophilic inflammation in severe UA.

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Reprints: Choon-Sik Park, MD, PhD, Division of Allergy and Respiratory Medicine, Department of Internal Medicine, Soonchunhyang University Bucheon Hospital, 1174, Jung-Dong, Wonmi-Ku, Bucheon, Gyeonggi-Do, 420-020, South Korea; E-mail: mdcspark@daum.net.

Tae-Hyeong Lee and An-Soo Jang contributed equally as first authors.

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Introduction

Asthma is a heterogeneous disease with subgroups defined by etiology, pathology, severity, physiologic parameters, and response to treatment.¹ Severe uncontrolled asthma (UA) is a specific phenotype of asthma and constitutes about 5% of total asthma.^{2,3} This subgroup has difficult characteristics in terms of clinical approach and treatment as manifested by the high medication requirements (eg, Global Initiative for Asthma [GINA] step 4 or 5 to control persistent symptoms) and exacerbations or continuing airflow obstruction.² However, these goals are frequently not achieved despite high-intensity treatment.³ Severe asthma is composed of subgroups with potentially different types of airway inflammation.

Airway inflammation can be measured directly and invasively by bronchoscopic biopsies⁴ and bronchoalveolar lavages.⁵ It can be measured indirectly and noninvasively by sputum⁶ and exhaled nitric oxide measurement.⁷ Asthmatics are divided into subtypes based on 2 inflammatory patterns, eosinophilic and neutrophilic, that possess distinct physiologic and clinical characteristics.^{8–10} Pathologic studies have found that neutrophils are present in larger quantities in the airways of severe asthmatics compared with the airways of patients with mild asthma or normal control subjects.^{8,11} In addition to their increased numbers, neutrophils are functionally activated in the airways of severe asthmatics. The airway neutrophilia is accompanied by increased levels of matrix metalloproteinase-9,¹² interleukin (IL)-8,¹³ and IL-6.¹⁴ In addition, oncostatin M is increased in severe asthmatics with incompletely reversible airflow obstruction.¹⁴ However, to date, few biomarkers of severe UA have been defined in fluids lining the airway compared with controlled asthma (CA).

The authors' previous study reported that patients with severe UA, despite long-term and high-dose inhaled corticosteroids, exhibited neutrophil-dominant airway inflammation.⁹ However, the difference in neutrophil fraction was marginal between subjects with severe UA and those with severe CA.¹⁵ These findings prompted the authors to search for biomarkers in the neutrophil-dominant sputum to reflect severe UA.

High-throughput technologies are the preferred method to screen candidate proteins related to the activated neutrophils in the airways of asthmatics, but few screens have been tried. Recently, Hastie et al¹⁶ used a protein chip to profile neutrophilia-associated proteins in the sputum of severe asthmatics and found increases in brain-derived neurotrophic factor, IL-1 β , and macrophage inflammatory protein-3 α /CCL20. Advances in 2-dimensional gel electrophoresis (2DE), mass spectrometry, protein arrays, and bioinformatics may allow the discovery of protein profiles and provide new knowledge of the molecular basis of asthma.^{17–20} Airway-lining fluid proteomics can be used to identify biomarkers of asthma,²¹ chronic obstructive pulmonary disease (COPD),²² and other lung diseases.^{23–26} Therefore, the elucidation of protein expression differences in airway-lining fluids of the severe UA subgroup may broaden the scope of the mechanism behind airway neutrophilia. The aim of this study was to identify airway neutrophilia-related proteins in the sputum of severe asthmatics and to validate one of the differentially expressed proteins, S100 calcium binding protein A9 (S100A9).

Methods

Subjects

Subjects were recruited as described in the authors' previous study.⁹ The diagnosis of asthma was based on the GINA guidelines.³ All subjects had a clinical diagnosis of asthma that was supported by at least 1 of the following criteria: (1) variability in maximum diurnal peak expiratory flow rate greater than 20% over the course of 14 days, (2) an increase in forced expiratory volume in 1 second (FEV₁) greater than 15% after inhalation of albuterol 400 μ g, or (3) a 20% decrease in FEV₁ in response to a provocative concentration of inhaled methacholine (PC20) of less than 10 mg/mL. Spirometry was performed before and after inhalation of a bronchodilator. Basal and post-bronchodilator FEV₁ and forced vital capacity (FVC) were measured at regular intervals of 1 or 2 months. All patients underwent a standardized assessment, which included analyses of induced sputum specimens, complete blood cell count with differential counts, total IgE, chest radiography, and allergy skin prick tests at the initial visit. Atopy was defined as a wheal reaction of at least 3 mm in diameter.

Study Design

Asthmatics who never smoked and those who quit smoking (<5 pack-years), with a regular follow-up of at least 2 years, were

selected for the study. Study subjects had taken daily treatment of long-acting β 2-agonists, theophylline, and/or leukotriene antagonists in addition to controller medication of inhaled or systemic corticosteroids, as recommended by the GINA guidelines.³ Asthma control was defined according to the GINA guidelines³ as CA, UA, or partially controlled asthma (PC). The diagnosis of COPD was based on Global Initiative for Chronic Obstructive Lung Disease guidelines.²⁷

All subjects were Korean and gave informed written consent to donate their sputum to a biobank in Soonchunhyang University Hospital. All human sputum samples from asthmatics (n = 76), patients with COPD (n = 21), and normal controls (n = 8) in the present study were obtained from the biobank after the protocol was approved by the local ethics committee of Soonchunhyang University Hospital (institutional review board number schbc-biobank-2011-009).

Sputum Preparation

Sputum was induced using isotonic saline that contained a short-acting bronchodilator, and samples were processed within 2 hours of collection, as described previously.^{28,29} Briefly, all portions with visibly greater solidity were carefully selected and placed in a preweighed Eppendorf tube. The samples were treated by adding 8 volumes of 0.05% dithiothreitol (DTT) (Sputolysin; Calbiochem Corp, San Diego, California) in Dulbecco phosphate buffered saline. One volume of protease inhibitor (methylenediamine tetra-acetic acid 0.1 mol/L and phenyl methyl sulfonyl fluoride 2 mg/mL) was added to 100 volumes of homogenized sputum, and the total cell count was determined using a hemocytometer. Sputum cells were collected by cytocentrifugation and 500 cells were examined after staining with Diff-Quick (American Scientific Products, Chicago, Illinois). Sputum that contained more than 10% squamous epithelial cells was not analyzed. The remainder of the homogenized sputum sample was centrifuged at 1,000g for 5 minutes, and the supernatant was collected and stored at -70°C for subsequent proteomic analyses. Of the total 125 subjects, 20 subjects (16%) had more than 10% squamous cells in their sputum and were excluded from the study. Based on the eosinophil and neutrophil proportions in the sputum, subjects were categorized into inflammatory subtypes as described previously^{9,30}: eosinophilic asthmatics (sputum eosinophils >3.0%) or neutrophilic asthmatics (sputum neutrophils >70%). The volume and protein concentration of each sputum sample were measured for studies using 2DE, western blots, and enzyme-linked immunosorbent assay (ELISA). Only 4 sputum samples were required for 2DE, western blots, and ELISA of neutrophilic inflammation of UA. One sample was adequate for 2DE and ELISA. Because the other 5 samples had low protein concentration, only ELISA was applied. For patients with neutrophilic CA, 10 samples had an adequate protein concentration only for 2DE and ELISA, and 4 samples were adequate for an additional western blot study.

2DE of Sputum, Intra-Gel Digestion, and Mass Spectrometric Analyses

A pooled sputum sample (1 mg) was prepared from 5 subjects with neutrophilic-type UA (sputum 200 μ g per subject), and a 1-mg sputum sample was pooled from 10 subjects with neutrophilic CA. The pooled sputum was diluted 4-fold with equilibration buffer and filtered through a 0.22- μ m microcentrifuge filter tube (Agilent Technologies, Santa Clara, California). The flow-through fractions were concentrated by centrifugation in Amicon Ultra-4 tubes (5-kDa molecular weight cutoff; Millipore, Billerica, Massachusetts). The pellet was air dried for a few minutes and dissolved in sample buffer (urea 7 mol/L, thiourea 2 mol/L, 4% [w/v] 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.5% [v/v] ampholyte, DTT 100 mmol/mL, Tris-HCl 40 mmol/L [pH 7.5], and 0.002% bromophenol blue) for 2DE. Protein concentration was

quantified using the 2D-quant kit protein assay.³¹ Immobiline Dry Strips (Amersham Biosciences, Piscataway, New Jersey) were used for isoelectric focusing, which was carried out with 1 mg of the extracted protein on MultiPhorII (GE Healthcare, Milwaukee, Wisconsin). After isoelectric focusing separation, the proteins were separated in the second dimension by sodium dodecyl sulfate polyacrylamide gel electrophoresis. For image analysis, the gels were visualized with Coomassie Brilliant Blue G-250 (Sigma, St. Louis, Missouri), according to the manufacturer's instructions. The 2D gels were scanned with an ImageScanner (Amersham Biosciences, Piscataway, New Jersey) in transmission mode. Spot detection and matching were performed using ImageMaster 2D 5.0 (Amersham Biosciences). Digitized images were analyzed using the ImageMaster program to calculate the 2D spot intensity by integrating the optical density over the spot area and were normalized, as described previously.^{18,19}

Differentially expressed protein spots were excised from the gels, cut into smaller pieces, and digested with trypsin (Promega, Madison, Wisconsin).³² For matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometric (MS) analyses, the tryptic peptides were concentrated on POROS R2 columns (Applied Biosystems, Foster City, California). After successive column washings with 40% methanol, 100% acetonitrile, and ammonium bicarbonate 50 mmol/L, the samples were applied to the R2 column and eluted in cyano-4-hydroxycinnamic acid 2 mL before MALDI-TOF analyses. The protein spectra were obtained using a Voyager DE PRO MALDI-TOF spectrometer (Applied Biosystems, Foster City, California). The protein database search was performed with the MS Fit program (<http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm>) using mono-isotopic peaks. A mass tolerance within 50 ppm was allowed for the first analysis; the system was subsequently recalibrated at 20 ppm using the protein lists obtained from the initial analyses. The spectra also were calibrated internally using trypsin autolysis products. Peptide matching and protein searches against the Swiss-Prot and National Center for Biotechnology Information databases were performed using the Mascot search program (<http://www.matrixscience.com/>) and Pro Found program (<http://prowl.rockefeller.edu/>).

S100A9 Expression

Western blot for S100A9 was performed on sputum supernatants from subjects with UA and CA. Protein (30 µg) was loaded and electrophoresed with a discontinuous system consisting of a 15% polyacrylamide gel. The proteins were transferred to nitrocellulose membrane at 120 V for 1 hour. The membrane was blocked for 1 hour at room temperature in 5% skim milk in 0.1% Tween-20 in Tris-buffered saline and incubated overnight at 4°C with a 1:300 dilution of a rabbit polyclonal S100A9 antibody (Abcam, Inc, Cambridge, United Kingdom). The membrane was incubated for 1 hour at room temperature with a horseradish peroxidase–conjugated anti-rabbit IgG (1:5,000 dilution; Thermo, Inc, Fremont, California). The target protein was detected by an enhanced chemiluminescence solution (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) on x-ray film.

Quantitative Measurement of S100A9 Using ELISA

The protein concentration in each sample was determined using a bicinchoninic acid kit (Pierce Biotechnology, Rockford, Illinois). S100A9 concentrations in sputum were measured by quantitative sandwich ELISA kits according to the manufacturer's protocol (Cusabio Boitech Co, Ltd, Wuhan, China). The detection limit for S100A9 was 1.17 ng/mL. The levels were normalized to the total protein concentration of the samples. Interassay and intra-assay variations were less than 15%.

Statistical Analyses

The data were double-entered into a statistical software package (SPSS 14.0; SPSS, Inc, Chicago, Illinois). Data are expressed as median (interquartile range). Group differences were compared using analysis of variance. Group differences between patients with UA and those with CA were compared using a 2-sample *t* test, Mann-Whitney test, or Pearson χ^2 test for normally distributed, skewed, or categorical data, respectively. *P* values less than .05 were considered statistically significant.

Results

Demographic Characteristics

The demographic characteristics of the study population are listed in Table 1. All subjects were adults older than 20 years. Although there were no age differences among subjects with CA, PC, and UA of the eosinophilic or neutrophilic subtype, subjects with COPD tended to be older. Women were dominant in the CA groups, whereas men were dominant in the COPD group (*P* < .05). The physiologic parameters and sputum cell analyses are presented in Table 1. Subjects with PC and UA of the neutrophilic and eosinophilic subtypes had significantly lower FEV₁ and FEV₁/FVC compared with the CA group (*P* < .05), whereas the initial FEV₁ and initial FEV₁/FVC were comparable between groups. FEV₁, FEV₁/FVC, and body mass index were significantly lower in the COPD group than in the UA neutrophilic or eosinophilic subtypes (*P* < .05). Total cell counts tended to be elevated in neutrophilic subtypes compared with the eosinophilic subtypes of each group (*P* > .05). All asthmatics were taking high doses of regular inhaled corticosteroid (fluticasone propionate >500 µg or equivalent dose) plus long-acting β_2 -agonists when sputum was collected. The daily total dosage of inhaled corticosteroids was higher in the UA group than in the PC or CA group (*P* < .05 or *P* < .001, respectively).

Sputum Protein Identification

The differential expression of proteins in neutrophilic sputum between the UA and CA groups was evaluated using 2DE (Fig 1). After Coomassie blue staining, 532 protein spots in the UA group and 490 in the CA group were detected in 1 mg of sputum protein. All identified spots were localized at an isoelectric point of 4 to 10, with a molecular mass range of 10 to 150 kDa. Of these, 13 proteins showed significant differences in relative intensity between the 2 groups. The relative intensities (percentages) of the spots are listed in Table 2 and the locations of the identified protein spots are shown in a master image (Fig 1). Six protein spots had a greater than 3-fold increase in relative intensity in the sputum of the UA vs CA group. These spots were excised from the gels, incubated with trypsin for in-gel digestion, and identified by MALDI-TOF/TOF. They included spot 1 (human neutrophil peptide-2 [HNP-2]), spot 2 (S100A9), spot 3 (neutrophil gelatinase-associated lipocalin [NGAL]), spot 4 (4-aminobutyrate transaminase), spot 5 (cystatin SA), and spot 6 (protein Plunc precursor). In contrast, spot 9 (glial fibrillary acidic protein isoform-1), spot 11 (MLL-AF4 der[11] fusion protein), and other immune-related proteins, including spot 7 (complement component C3), spot 8 (immunoglobulin heavy-chain variable region), spot 10 (IgM κ IIIb SON), and spot 13 (recombinant IgG4 heavy chain) were greater than 4-fold lower in sputum of patients with UA compared with those with CA (Fig 1, Table 2). These proteins are associated with inflammation, anti-inflammation, enzymatic activity, immunity, signaling, cytoskeleton, and cysteine protease inhibition, respectively (Table 2).

S100A9 Protein in Sputum

Among the protein spots identified as neutrophil-derived proteins, including HNP-2, S100A9, NGAL, and protein Plunc

Table 1

Physiologic parameters and sputum analysis

Variable	UA		PC		CA		COPD	NC
	Neutrophilic	Eosinophilic	Neutrophilic	Eosinophilic	Neutrophilic	Eosinophilic		
Subjects (n)	8	12	15	20	10	11	21	8
Age (y)	61 (45–79)	64 (48–83)	62 (31–75)	54 (29–78)	54 (23–78)	56 (33–74)	71 (52–88)	58 (52–75)
Duration of asthma (y)	19.8 (2–40) ^c	5.4 (2–10)	10.1 (1–50)	8.9 (1–30)	5.4 (1–15)	1.2 (1–3) ^d		
Men/women	3/5	4/8	5/10	6/14	1/9	3/8	20/1 ^a	2/6
Smoking status, NS/ES/CS	7/1/0	9/3/0	10/5/0	11/9/0	10/0/0	10/1/0	2,000/12/9	7/1/0
Atopy (%)	25	33.3	53.3	40	50	54.5	19	0
BMI (kg/m ²)	23.5 (19.6–29.6)	23.4 (18.8–28.7)	23.3 (18.9–27.2)	23.5 (17.7–25.3)	23.3 (16.4–30.5)	23 (18.6–26.8)	21.1 ^b (16.5–27.6)	25.9 (18.1–35.6)
Use of inhaled steroid (fluticasone, µg/d)	1,187.5 (500–2,000)	1,666.7 (1,000–2,000)	800 ^{cd} (500–1,000)	725 ^{cd} (500–1,000)	500 ^b –500	863.6 (500–1,000)	666.6 (500–1,000)	
Sputum total cell count/mL × 10 ⁵	17.8	5.8	22	7.3	28.8	6.1	12.4	8.2
Eosinophils (%)	0.3 (0–2.29)	36.3 (3.25–67.5) ^f	0.7 (0–2.9)	51.9 (27.8–97.5) ^f	0.2 (0–1)	71.1 (58.5–93.7)	0.5 (0–3.5)	0.3 (0.3–2.3)
Neutrophils (%)	91.7 (80.66–100) ^a	49 (8–67.3) ^f	90.5 (76.3–100)	37.5 (5.9–69.8) ^{af}	88.6 (77.5–99.5)	19.7 (4.3–28.3)	94.1 (75–100)	51.9 (30.3–57.3)
Macrophages (%)	8.7 (0–29.34)	7.0 (0.5–23.5)	3.6 (0–18.3)	8.0 (0.2–21.3) ^a	1.4 (0.5–4.5)	7.7 (1.3–25.7)	4.0 (0–13.3)	45.0 (34–54.7)
Lymphocytes (%)	2.0 (0–11)	2.1 (0–11)	1.0 (0–5.4)	0.8 (0–3.2)	0.1 (0–0.5)	1.2 (0–5.5)	1.3 (0–7.8)	0.9 (0–2.3)
Columnar epithelial cells (%)	0.1 (0–1)	3.2 (0–4.5)	0.4 (0–3.9)	1.2 (0–5.5)	0 (0)	0.3 (0–1.5)	0.4 (0–2.6)	2.1 (0–8.29)
FVC (%), predicted	67.2 (31–103) ^a	73.7 (39–106) ^f	74.3 (62–104)	83.0 (61–116) ^{af}	92.2 (73–110)	90.5 (75–116)	55.3 (16–79)	88.6 (47–109)
FEV ₁ (%), predicted	57.3 (31–78) ^a	62.9 (32–79) ^{ef}	71.9 (65–88)	73.3 (65–89) ^{af}	97.5 (80–122)	95.2 (83–122)	40.4 (16–59) ^b	107.9 (85–126)
FEV ₁ /FVC (%)	65.5 (46–87) ^a	61.5 (38–81) ^f	72.1 (47–95)	61.6 (47–69) ^{af}	80.2 (67–93)	79.2 (68–92)	49.9 (38–61) ^b	88.0 (80–100)

Abbreviations: BMI, body mass index; CA, controlled asthma; CS, current smoker; ES, ex-smoker; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; NC, normal control; NS, nonsmoker; PC, partially controlled asthma; UA, uncontrolled asthma.

Data are mean (standard error of the mean) unless indicated otherwise.

^a*P* < .01 compared with controlled neutrophilic asthma.

^b*P* < .01 compared with uncontrolled eosinophilic and neutrophilic asthma.

^c*P* < .05 compared with uncontrolled eosinophilic asthma.

^d*P* < .05 compared with uncontrolled neutrophilic asthma.

^e*P* < .01 compared with partially controlled eosinophilic asthma.

^f*P* < .01 compared with controlled eosinophilic asthma.

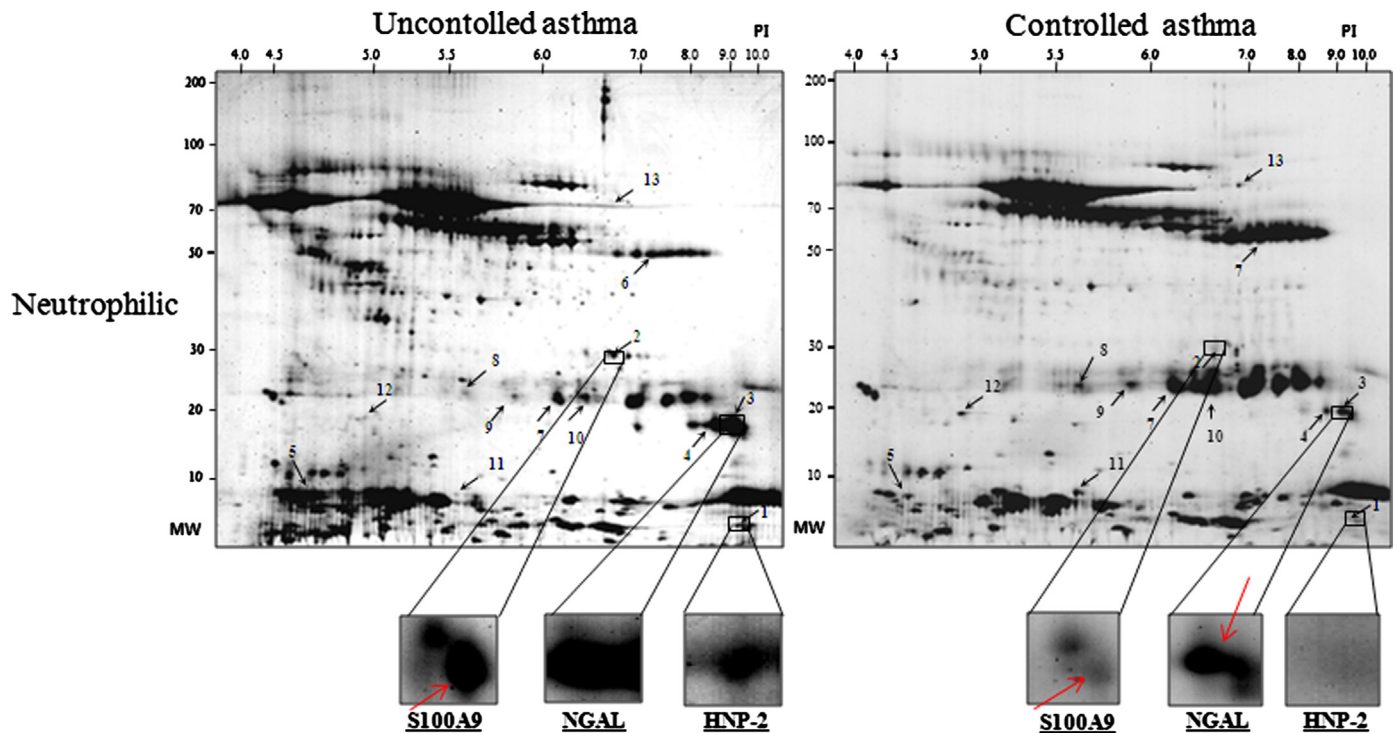


Figure 1. Photographs of 2-dimensional electrophoresis protein separation of pooled sputum ($n = 5$) obtained from uncontrolled asthmatics and that ($n = 10$) from controlled asthmatics with neutrophilic inflammation. Protein spots identified by matrix-assisted laser desorption/ionization–time of flight/time of flight (arrows) are identified by their spot numbers. Enlargements of spots 1, 2, and 4 from each group are presented as insets. HNP-2, human neutrophil peptide-2; NGAL, neutrophil gelatinase-associated lipocalin; S100A9, S100 calcium binding protein A9.

precursor, S100A9 was validated. Western blotting was performed using sputum obtained from patients with UA ($n = 4$) and those with CA ($n = 4$) and neutrophilic inflammation who were enrolled in the proteomics study. Demographic parameters of these patients are listed in eTable 1. The intensity of the S100A9 (13 kDa) band was greater (Fig 2A) in sputum from the UA group. The densities were normalized to that of albumin; S100A9 was significantly increased in sputum from patients with UA vs those with CA and neutrophilic inflammation (2.53 [2.37–3.08] vs 0.23 [0.11–0.47], $P = .029$; Fig 2B).

S100A9 protein levels were measured by ELISA and normalized to the total protein concentration. The levels were significantly higher in sputum from patients with neutrophilic UA than from

those with eosinophilic UA (3.02 ng/ μ g [2.24–5.72] vs 1.12 ng/ μ g [0.50–1.77], $P = .009$; Fig 3) and from the CA and PC subgroups ($P < .05$; Fig 3) and patients with COPD ($P = .001$; Fig 3). The S100A9 levels in all asthma subphenotypes were significantly higher compared with normal controls ($P < .05$; Fig 3). S100A9 levels of the 76 asthmatics were significantly higher than those of normal controls ($P = .005$) and comparable to those of patients with COPD ($P = .127$; Fig 3). The levels of patients with COPD were significantly higher than those of normal controls ($P = .024$).

The S100A9 levels were not correlated with FEV₁ (percentage predicted; $n = 76$, $r = 0.004$, $P = .55$) or with the percentages of neutrophils ($n = 33$, $r = 0.083$, $P = .38$) and eosinophils ($n = 43$, $r = 0.02$, $P = .20$) in the sputum of asthmatics.

Table 2

List of differentially expressed proteins identified by matrix-assisted laser desorption/ionization–time of flight/time of flight

Number	Protein name	Accession number	Determined sequence	MW (kDa)/PI	Relative intensity		Fold change	Function
					UA	CA		
1	human neutrophil peptide-2	75765819	R.YXTCIYQGR.L	34.28/8.67	0.175	> 0.028	6.23	anti-inflammatory
2	S100 calcium binding protein A9	4506773	R.NIETINTFHQYSVK.L	13.234/5.71	0.124	> 0.02	6.2	inflammatory
3	neutrophil gelatinase-associated lipocalin	300181	K.WYVVLGAGNAILR.E	20.536/8.87	1.831	> 0.32	5.718	inflammatory
4	4-aminobutyrate transaminase	84702675	K.GLIFLTAGRK.A	46.748/8.63	0.04	> 0.007	5.399	enzyme activity
5	cystatin SA	359513	R.EQIVGGVNYFFDIEVGR.T	13.843/4.85	0.597	> 0.111	5.383	cysteine protease inhibitor
6	Plunc	7706119	K.VTDPQLLELGLVQSPDGHR.L	26.696/5.41	0.086	> 0.021	3.97	inflammatory
7	complement component C3	179665	K.VQLSNDFFEYIMAIEQTIK.S	18.704/6.02	0.016	< 0.123	0.13	immunity
8	immunoglobulin heavy chain variable region	194719561	_EVQLVESGGGLVQPGGVLRL	12.965/6.45	0.005	< 0.03	0.167	immunity
9	glial fibrillary acidic protein isoform 1	4503979	K.LALDIEIATYR.K	49.850/5.42	0.009	< 0.044	0.204	inflammatory
10	IgM κ IIIb SON	224377	R.TVAAPSVFIFPPSDEQLK	13.756/8.65	0.076	< 0.352	0.215	immunity
11	MLL-AF4 der(11) fusion protein	347377	K.HFESSSKVAQAPSPCIAR.S	24.986/10.07	0.026	< 0.12	0.216	signaling
12	cytokeratin 8	30313	K.LSELEAALQRA	30.840/4.91	0.009	< 0.041	0.219	cytoskeleton
13	recombinant IgG4 heavy chain	9857759	K.GFYPSDIAVEWESNGQPENNYK.T	42.895/5.72	0.112	< 0.475	0.235	immunity

Abbreviations: CA, controlled neutrophilic asthma; MW, molecular weight; PI, isoelectric point; UA, severe uncontrolled neutrophilic asthma.

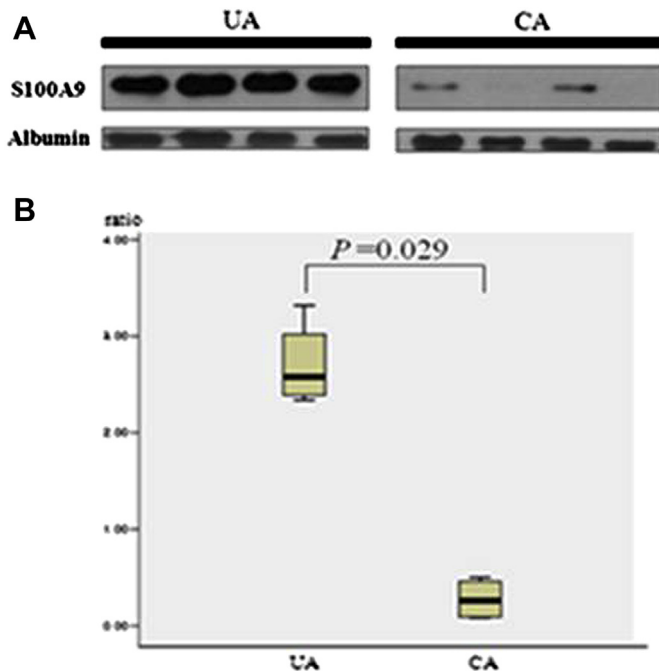


Figure 2. Western blot analysis of S100 calcium binding protein A9 (S100A9) in sputum from patients with severe uncontrolled asthma (UA; $n = 4$) and controlled asthma (CA; $n = 4$) with neutrophilic inflammation. (A) Bands of S100A9 (molecular weight 13 kDa) and albumin are well visualized in each sample of the 2 groups. (B) The intensity of S100A9 is normalized with that of albumin and expressed as a ratio with the median (interquartile range).

Discussion

In the present study, the authors aimed to detect protein biomarker(s) of neutrophilic inflammation in severe UA using proteomic analyses of sputum. Use of the proteomic approach has generated sputum protein profiles.^{23–25} Differential expression of

13 proteins was found in the neutrophilic sputum of subjects with UA compared with those with CA, suggesting that these proteins may be potential biomarkers of severe UA. Among them, the authors determined whether S100A9 was specific to clinically UA with neutrophilic-type sputum. Western blotting was performed using sputum obtained from patients with UA ($n = 4$) and those with CA ($n = 4$) and neutrophilic inflammation. The intensity of the S100A9 (13 kDa) band was greater (Fig 2A) in sputum from patients with UA. HNP-2, S100A9, NGAL, and protein Plunc precursor were clearly observed in western blots of sputum from patients with UA (data not shown). Among them, S100A9 was validated by measuring its levels in sputum by ELISA in a case–control study, because this protein is functionally related to the activation of neutrophils³³ and an ELISA kit was available. S100A9 levels were significantly higher in neutrophilic UA than in neutrophilic CA. These data suggest that the S100A9 may be synthesized in the airway at higher levels in the former than in the latter. Because the fraction of neutrophils was similar in the 2 groups, S100A9 elevation may represent a biomarker of severe UA.

S100A9 forms a complex with S100A8 and belongs to the large Ca^{2+} -binding S100 protein family. It is one of the most abundant proteins in neutrophils, macrophages, and epithelial cells during chronic inflammation.³³ In the present study, the authors did not determine the source of S100A9 in severe UA. S100A9 forms homodimers and a heterodimer, called calprotectin (S100A8/A9). An increase in blood calprotectin concentration has been reported in patients with Crohn disease, colorectal carcinoma, cystic fibrosis, multiple sclerosis, human immunodeficiency virus infection, and those who have undergone major surgery.³⁴ A more marked increase in calprotectin concentration has been noted in body fluids from local inflammatory sites, such as the synovial fluid of patients with rheumatoid arthritis compared with blood.³⁵ However, quantitative measurement of S100A9 in the sputum of severe asthmatics has not been performed to date. Although S100A8/A9 has been suspected as a mediator of the neutrophilic type of severe asthma,³³ this hypothesis has not been evaluated. In the present study, S100A9 protein levels were significantly higher in neutrophilic UA than in eosinophilic UA, suggesting that S100A9 may be specific to the neutrophilic airway inflammation of severe UA. In addition, S100A9 levels were compared in 3 subgroups of asthma with a similar neutrophilic fraction in the sputum. The COPD group was included as a prototype of neutrophilic airway inflammation. The S100A9 level was markedly increased in neutrophilic UA compared with the neutrophilic PC and CA groups or the COPD group. This result indicates that an elevated S100A9 level is specifically limited to neutrophilic inflammation in subjects with UA, which is a steroid-resistant asthma subtype.

The proposed function of calprotectin is the regulation of inflammation, including macrophage and neutrophil chemotaxis, activation of neutrophil functions, and inhibition of lymphocyte immunoglobulin synthesis.^{35–39} This suggests that it may affect inflammatory processes and immune reactions. Recently, S100A8 and S100A9 have been identified as important endogenous activators of Toll-like receptor 4 and advanced glycation end products, which are expressed by lung and airway mesenchymal cells.³⁶ They are associated with the innate immunity that programs local airway inflammation and fibroproliferative pulmonary diseases. Altered expression of this protein has been observed in bronchoalveolar lavage fluids of patients with idiopathic pulmonary fibrosis.³⁶ Considering that elevated S100A9 levels are limited to patients with UA, the fibroproliferative function of S100A8/A9 might lead to airway remodeling, which is related to the resistance of airways to inhaled steroid treatment in severe UA.

Gray et al.²³ reported a high level of S100A8, but not S100A9, in the sputum of asthmatics, although patients with cystic fibrosis and those with bronchiectasis exhibit increased levels of these 2 proteins.

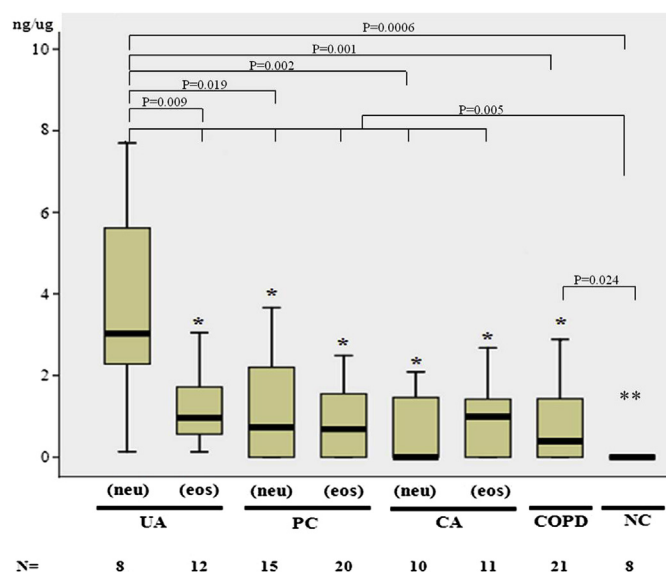


Figure 3. Comparison of S100 calcium binding protein A9 (S100A9) levels in sputum between the neutrophilic (neu) and eosinophilic (eos) subtypes of severe uncontrolled asthma (UA), partially controlled asthma (PC), controlled asthma (CA), chronic obstructive pulmonary disease (COPD), and normal control (NC) using enzyme-linked immunosorbent assay. Data are presented as median (interquartile range). * $P < .05$ vs normal controls; ** $P = .005$, total asthmatics vs normal controls.

The differences between the present results and their results may be due to the different clinical status of the subjects, including age, asthma severity, and extent of neutrophilic inflammation in sputum. The neutrophil fraction of asthmatics in their study²³ was lower than the neutrophilic subtype of UA in the present study (50.6% vs >70%). Neutrophils and monocytes are rich in S100A8/A9, although activated epithelial and endothelial cells also express these proteins.^{37,38}

In the sputum analysis process, DTT was added to solubilize mucus. Because DTT can disturb the measurement of S100A9 by ELISA, the authors checked the effect of DTT. The recovery rate of S100A9 in DTT-treated samples was 70%, suggesting that the presence of DTT is not critical in the interpretation of the values measured. As a limitation of the present study, the authors could not localize the S100A9 protein in airways because bronchoscopy biopsy was not feasible in the subjects with severe UA.

In addition to S100A9, HNP-2 and NGAL levels were increased in the sputum of patients with neutrophilic UA compared with those with eosinophilic UA. Lipocalin-2, also known as NGAL, is a protein associated with neutrophil gelatinase.³⁹ Lipocalin-2 is believed to bind small lipophilic substances, such as bacteria-derived lipopolysaccharide and formyl peptides, and may function as a modulator of inflammation. The binding of lipocalin-2 to bacterial siderophores is pivotal in the innate immune response to bacterial infection.⁴⁰ In the present study, the increased NGAL and HNP-2 levels in neutrophilic sputum suggest that enhanced innate immunity may play a role in the pathogenesis and resistance to steroid treatment of severe UA. These questions will be addressed in future studies.

Wu et al⁴¹ reported a very interesting result regarding the variable effects of inhaled treatment with glucocorticoids in patients with CA and those with UA. The percentages of eosinophils and neutrophils and the levels of IL-17 and stromal cell-derived factor-1 decreased significantly in all patients after glucocorticosteroid therapy for 4 weeks, whereas the percentage of sputum neutrophils and the levels of IL-17 and stromal cell-derived factor-1 increased significantly in patients with UA compared with the CA and PC groups. These data indicate that the percentage of neutrophils might be more elevated in sputum of patients with UA in the present study by an elevation of IL-17 and stromal cell-derived factor-1 levels.

In conclusion, 13 differentially expressed proteins in sputum related to the neutrophilic inflammation associated with severe UA were identified. Of these, use of S100A9 as a biomarker was investigated in a case–control study. The elevation of S100A9 was limited to the neutrophilic inflammation group with severe UA. Based on these results, sputum S100A9 level may represent a biomarker of severe neutrophilic UA. Further studies are needed to understand fully the role of S100A9 in severe UA.

Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2013.06.028>.

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eTable 1

Demographic parameters of subjects by western blot

Variable	Neutrophilic	
	UA	CA
Subjects (n)	4	4
Age (y)	65.9 (45–79)	58.5 (48–73)
Duration of asthma (y)	17.8 (2–40)	6.4 (2–10)
Male/female	4/0	1/3
Smoking status, NS/ES/CS	3/1/0	1/3/0
Atopy (%)	25	50
BMI (kg/m ²)	23.1 (19.6–25.6)	22.4 (18.8–26.5)
Inhaled steroid used (fluticasone, μ g/d)	1,625 (500–2,000)	500
Sputum total cell count/mL $\times 10^5$	26.5	26.4
Eosinophils (%)	0.6 (0–2.9)	0.3 (0–1.5)
Neutrophils (%)	91.1 (85–100)	88.5 (77.5–99.5)
Macrophage (%)	6.8 (0–29.3)	1.4 (0.5–4.5)
Lymphocytes (%)	1.8 (0–11)	0.1 (0–0.5)
Columnar epithelial cells (%)	0.1 (0–1)	3.2 (0–4.5)
FVC (%), predicted	57.3 (22–103)	84.4 (51–110)
FEV ₁ (%), predicted	52.4 (25–78)	86.9 (73–122)
FEV ₁ /FVC (%)	68.3 (46–95)	77.4 (67–93)

Abbreviations: BMI, body mass index; CA, controlled neutrophilic asthma; CS, current smoker; ES, ex-smoker; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; NC, normal control; NS, nonsmoker; UA, uncontrolled severe neutrophilic asthma.