Pathophysiological relevance of sputum MUC5AC and MUC5B levels in patients with mild asthma

AUTHOR(S):

Tajiri, Tomoko; Matsumoto, Hisako; Jinnai, Makiko; Kanemitsu, Yoshihiro; Nagasaki, Tadao; Iwata, Toshiyuki; Inoue, Hideki; ... Oguma, Tsuyoshi; Ito, Isao; Niimi, Akio

CITATION:

Tajiri, Tomoko ...[et al]. Pathophysiological relevance of sputum MUC5AC and MUC5B levels in patients with mild asthma. Allergology International 2022, 71(2): 193-199

ISSUE DATE:

2022-04

URL:

http://hdl.handle.net/2433/284953

Allergology International 71 (2022) 193–199

Contents lists available at ScienceDirect

Allergology International

journal homepage: http://www.elsevier.com/locate/alit

Original Article

Pathophysiological relevance of sputum MUC5AC and MUC5B levels in patients with mild asthma

Tomoko Tajiri [a, b, *], Hisako Matsumoto [a, c], Makiko Jinnai [a], Yoshihiro Kanemitsu [a, b], Tadao Nagasaki [a], Toshiyuki Iwata [a], Hideki Inoue [a], Hitoshi Nakaji [a], Tsuyoshi Oguma [a], Isao Ito [a], Akio Niimi [a, b]

[a] Department of Respiratory Medicine, Kyoto University, Kyoto, Japan
[b] Department of Respiratory Medicine, Allergy and Clinical Immunology, Nagoya City University School of Medical Sciences, Aichi, Japan
[c] Department of Respiratory Medicine and Allergology, Kindai University Faculty of Medicine, Osaka, Japan

ARTICLE INFO

Article history:
Received 9 May 2021
Received in revised form
28 August 2021
Accepted 7 September 2021
Available online 13 October 2021

Keywords:
Airway hyperresponsiveness
Airway hypersecretion
Asthma
MUC5AC
MUC5B

Abbreviations:
CA_{NO}, alveolar nitric oxide; Dmin, cumulative dose of inhaled methacholine at the inflection point at which respiratory resistance begins to increase; FeNO50, fractional exhaled nitric oxide at expiratory flow of 50 mL/s; FVC, forced vital capacity; IOS, impulse oscillometry; R_{rs}, respiratory resistance; SRrs, slope of the methacholine–respiratory resistance dose–response curve

ABSTRACT

Background: Airway mucus hypersecretion is an important pathophysiological feature of asthma. MUC5AC and MUC5B are the major secreted polymeric mucins in airways, and their compositions affect mucus properties. Despite the increasing appreciation of MUC5AC and MUC5B compositions in asthmatic airways, their pathophysiological relevance remains to be fully understood in humans.
Methods: In this cross-sectional study, we prospectively enrolled newly referred steroid-untreated patients with mild asthma and healthy controls. We compared induced sputum MUC5AC and MUC5B levels between patients and controls. Subsequently, we assessed the correlation between MUC5AC and MUC5B levels and clinical indices in patients. Sputum MUC5AC and MUC5B levels were measured using enzyme-linked immunosorbent assays.
Results: Sputum MUC5AC and MUC5B levels were significantly higher in patients ($n = 87$) than in controls ($n = 22$) ($p = 0.0002$ and $p = 0.006$, respectively). The ratio of sputum MUC5AC to MUC5B tended to be higher in patients than in controls ($p = 0.07$). Sputum MUC5AC levels significantly and positively correlated with fractional exhaled nitric oxide at expiratory flow of 50 mL/s (Spearman's rho = 0.29, $p = 0.006$), sputum eosinophil proportion (rho = 0.34, $p = 0.0013$), and airway sensitivity (rho = 0.39, $p = 0.0005$). By contrast, sputum MUC5B levels significantly and positively correlated with airway sensitivity (rho = 0.35, $p = 0.002$) and negatively correlated with airway reactivity (rho = −0.33, $p = 0.004$).
Conclusions: Sputum MUC5AC is increased by protein levels and involved in airway type 2/eosinophilic inflammation and airway hyperresponsiveness in steroid-untreated patients with mild asthma.

Introduction

Airway mucus normally plays a vital role to protect the epithelium against inhaled particles or pathogens through mucociliary clearance. However, pathologic mucus hypersecretion contributes to the pathophysiology of respiratory diseases, such as cystic fibrosis,[1] asthma,[2,3] and COPD.[4,5] Mucus hypersecretion is linked to infectious exacerbations, pulmonary function decline, disease severity, and increased mortality associated with these respiratory diseases.

Mucus consists of 97% water and 3% solids, including mucin, non-mucin proteins, salts, lipids, and cellular debris.[6] Mucin, a large glycoprotein, accounts for less than 30% of the solids.[6] Mucin concentrations are critical for effective mucus transport, and the abnormal increase in mucin concentrations affects mucus viscoelasticity and impairs mucociliary clearance.[7] In chronic inflammatory airway diseases, such as cystic fibrosis, asthma, and COPD, airway mucin levels are higher than in controls.[8] We previously demonstrated that total sputum mucin levels were significantly higher in 49 patients with asthma than in 11 healthy controls.[9]

* Corresponding author. Department of Respiratory Medicine, Allergy and Clinical Immunology, Nagoya City University School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan.
E-mail address: tomokot@med.nagoya-cu.ac.jp (T. Tajiri).
Peer review under responsibility of Japanese Society of Allergology.

https://doi.org/10.1016/j.alit.2021.09.003

To date, 20 human mucin genes have been identified, which are classified into two groups: secreted polymeric mucins and membrane-associated mucins.[7,10,11] Among them, the major mucins produced in airways are secreted polymeric mucins MUC5AC and MUC5B.[12] MUC5AC is produced in epithelial surface goblet cells,[13] while MUC5B is mainly produced in mucous cells of submucosal glands.[12,13] The composition of MUC5AC and MUC5B varies with the state of health. In a previous study of adult patients with asthma on inhaled or oral corticosteroids, MUC5AC was the predominant mucin in sputum from patients, whereas MUC5B was the predominant mucin from healthy subjects.[14] Another study of children with asthma on treatment showed that sputum MUC5AC levels were significantly higher in patients with acute asthma than in controls, and the ratio of MUC5B to MUC5AC was significantly lower in acute asthma than in controls.[15] Few studies have examined sputum MUC5AC and MUC5B levels in steroid-untreated patients with asthma.

Some studies have assessed the pathophysiological involvement of MUC5AC and MUC5B by using transgenic mouse models or induced sputum from human subjects. *Muc5ac* was the most highly expressed mucin gene in allergic murine airways.[16] The ratio of sputum MUC5AC to MUC5B protein was significantly higher in asthmatic patients with sputum eosinophilia (>2%) than in those without.[14] Additionally, *Muc5b* was the earliest and most abundantly expressed mucin gene throughout lung development in fetal mice[17] and ensured normal mucociliary clearance.[18] Despite an increasing appreciation of MUC5AC and MUC5B compositions, their pathophysiological relevance remains to be fully understood in human subjects.

We previously showed that total sputum mucin levels in patients with asthma were positively correlated with sputum eosinophil count and negatively correlated with respiratory resistance and airway sensitivity in a small subset of patients with asthma.[9] To further investigate the roles of individual mucins, we herein compared sputum MUC5AC and MUC5B protein levels in steroid-untreated patients with mild asthma and healthy controls. Subsequently, we analyzed the correlations between sputum MUC5AC and MUC5B levels and clinical and pathophysiological indices in these patients.

Methods

Study design and patients

This was a cross-sectional study. This study was approved by the ethics committee of Kyoto University (C436), and was registered in the UMIN Clinical Trials Registry (Registry ID UMIN000004068). Written informed consent was obtained from all participants.

We prospectively enrolled newly referred patients with asthma at the respiratory clinic of Kyoto University Hospital from July 2010 to February 2013. Healthy controls were recruited from among hospital personnel. The diagnosis of asthma was based on identifying both variable respiratory symptoms, such as wheezing, cough, dyspnea, and chest tightness, and variable expiratory airflow limitation, as demonstrated clinically or by airway hyperresponsiveness, according to American Thoracic Society criteria.[19] Patient were considered atopic when they were positive for one or more serum allergen-specific IgE antibodies against house dust, Japanese cedar pollen, mixed Gramineae pollen, mixed weed pollen, mixed mold, cat dander, dog dander, and *Trichophyton rubrum*. Exclusion criteria were as follows: 1) failure of sputum induction, 2) history of upper respiratory tract infection in the preceding 8 weeks, 3) taking inhaled or oral corticosteroids, leukotriene receptor antagonists, antibiotics, anti tussives, and mucolytics within the previous 4 weeks, 4) taking β_2-agonists, anticholinergics, and theophylline within the previous 48 h, and 5) smoking history of >5 pack-years or within the previous 6 months. Healthy controls also had successful sputum induction and had neither history of upper respiratory tract infection in the preceding 8 weeks nor history of smoking >5 pack-years or within the previous 6 months.

Study protocol

Patients with asthma underwent the following examinations: asthma control questionnaire, fractional exhaled nitric oxide (FeNO) levels, prebronchodilator pulmonary function [impulse oscillometry (IOS) and spirometry], and sputum induction, in this order on the first day, because FeNO levels were affected by spirometry and other respiratory maneuvers.[20] Methacholine airway responsiveness test and capsaicin cough challenge were performed, in this order on a separate day within one week, because neither methacholine nor β2 agonists influence cough sensitivity to capsaicin.[21] It took about 40 min for patients with asthma to complete the whole examinations on the first day. Healthy controls underwent pre-bronchodilator pulmonary function and sputum induction, in this order.

Sputum MUC5AC[22] and MUC5B[23] levels were measured using enzyme-linked immunosorbent assays (ELISAs) as previously described,[22,23] and levels were compared between patients with asthma and controls. Correlations between sputum MUC5AC and MUC5B levels and clinical and pathophysiological indices and sputum cell differentials were investigated in patients with asthma.

Pulmonary function tests

FeNO levels were determined using a chemiluminescence analyzer (Sievers Model 280NOA; Sievers, Boulder, CO, USA). FeNO levels were determined at three expiratory flow rates of 50 (FeNO50), 100, and 200 mL/s. Alveolar NO (CA_{NO}) was calculated using the following equation: CA_{NO} = slope − intercept/740, after plotting NO output (i.e., FeNO level × expiratory flow).[24]

We subsequently measured respiratory impedance using Jaeger Master-Screen IOS™ (Erich Jaeger GmbH, Hoechberg, Germany) according to standard recommendations.[25] We measured respiratory resistance at 5 Hz (R5) and 20 Hz (R20), the difference between R5 and R20 (R5 − R20), reactance at 5 Hz, and the integrated area of low-frequency reactance, as proxies of total and large airway resistance and ventilation heterogeneity or small airway disease.[26,27]

After IOS measurements, spirometry was performed according to the recommendation.[28] Prebronchodilator forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1) were evaluated using Chestac-8800 (Chest, Tokyo, Japan).

We determined airway responsiveness by measuring respiratory resistance (Rrs; $cmH_2O/L/s$) (Astograph™; Chest) under continuous methacholine inhalation.[29–31] Briefly, twofold increasing concentrations of methacholine chloride diluted in physiologic saline in 10 dose steps (49–25,000 μg/mL) were prepared. They were inhaled during tidal breathing from nebulizers with an output of 0.15 mL/min. After recording the baseline Rrs during inhalation of physiologic saline for 1 min, methacholine was sequentially inhaled, beginning with the lowest concentration, at 1-min intervals. Dmin, the cumulative dose of inhaled methacholine at the inflection point at which Rrs begins to increase, was used as the index of airway sensitivity. This variable was measured in terms of a unit defined as 1-min inhalation of methacholine (1 mg/

mL). Lower Dmin indicated increased airway sensitivity. Methacholine inhalation was continued until Rrs reached twice the baseline value. The slope of the methacholine respiratory resistance dose–response curve (SRrs) was used as the measure of airway reactivity.[31] Higher SRrs indicated increased airway reactivity. Airway sensitivity and airway reactivity are two major components of airway hyperresponsiveness.[32,33]

After methacholine inhalation challenge, cough sensitivity was tested by continuous inhalation of capsaicin solution using Astograph™ as described previously.[34,35] Ten doubling concentrations of capsaicin solution (0.61–312 μmol/L) were inhaled until ≥5 coughs were induced. Each concentration of capsaicin was inhaled for 15 s during tidal breathing every 60 s. Capsaicin concentrations causing ≥2 and ≥ 5 coughs are referred to as C2 and C5, respectively.

Sputum was induced and processed as described previously.[36] Briefly, after premedication with salbutamol (200 μg), subjects inhaled a hypertonic (3%) saline solution for 15 min from an ultrasonic nebulizer. All adequate sputum plugs were separated from saliva and weighed. The expectorated sputum plugs were treated with 0.1% dithiothreitol (Sputasol, Oxoid Ltd., Hampshire, UK) at two times the weight (mg) of the sputum samples, and then treated with the same volume of Dulbecco's phosphate-buffered saline (PBS). After centrifugation, supernatants were collected and stored at −80 °C until analyses. The cell pellet was resuspended in PBS solution. The total cell count, excluding squamous cells, was determined using a standard hemocytometer and expressed as cells × 10^5/g wet weight sputum. Cells were then centrifuged and stained by the May–-Grünwald–Giemsa method to determine cell differential counts. Induction was considered successful if subjects expectorated sputum containing more than 200 non-squamous cells.[37]

MUC5AC and MUC5B protein levels in supernatants were measured separately with ELISA.[22,23] Briefly, standards (porcine gastric mucin) (Sigma–Aldrich, Saint Louis, MO, USA) and samples were prepared with PBS-Tween 20 at multiple dilutions, and 50 μL of each standard or sample were incubated with 50 μL of bicarbonate-carbonate buffer (Sigma–Aldrich) at 37 °C in a 96-well plate until dry. After blocking with 2% BSA-PBS-Tween 20, the plate was incubated with 50 μL of mouse monoclonal MUC5AC antibody (1:40 dilution; clone 45M1; Thermo Fisher Scientific Anatomical Pathology, Fremont, CA, USA) or rabbit polyclonal MUC5B antibody (1:30 dilution; clone H-300; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 h. Primarily incubation was followed by incubation for 1 h with 100 μL of HRP-conjugated horse anti-mouse IgG (1:1000 dilution; Vector Laboratories, Burlingame, CA, USA) for MUC5AC or HRP-conjugated polyclonal swine anti-rabbit IgG (1:4000 dilution; Dako, Glostrup, Denmark) for MUC5B at room temperature. The color reaction was developed with 3,3′,5,5′-tetramethylbenzene peroxidase solution (BD Biosciences, San Diego, CA, USA), and stopped with 2 N H_2SO_4. The absorbance was measured at 450 nm MUC5AC and MUC5B content in each supernatant was estimated in comparison with a standard mucin (porcine gastric mucin)[23] and expressed as micrograms of standard reactivity per milliliter of diluted resolubilized supernatant. The limit of detection for the mucin ELISA was 1 μg/mL.

Statistical analysis

Results are presented as means ± standard deviation values or medians (ranges). Two groups were compared using the χ^2 test or Wilcoxon rank-sum test as appropriate. Spearman's correlation was used to determine correlations between variables. All statistical analyses were conducted using JMP Pro 14 (SAS Institute, Tokyo, Japan). Values of $p < 0.05$ were considered statistically significant.

Results

Subject characteristics

Subject characteristics in each group are shown in Table 1. The controls were younger than patients with asthma. Sputum eosinophil proportion was higher in patients with asthma than in controls (Table 1). The number of 10 patients could not perform airway sensitivity test because of low FEV_1 (n = 1), epilepsy during treatment (n = 1), uncontrolled hypertension (n = 1), and asthma attack (n = 7).

Sputum MUC5AC and MUC5B levels

Sputum MUC5AC levels were significantly increased in patients with asthma (n = 87) than in controls (n = 22) (65.1 ± 122.0 μg/mL vs. 16.2 ± 16.9 μg/mL, respectively, p = 0.0002) (Fig. 1). Sputum MUC5B levels were also significantly higher in patients with asthma than in controls (357.9 ± 367.6 μg/mL vs. 170.7 ± 150.8 μg/mL, respectively, p = 0.006) (Fig. 2).

The ratio of sputum MUC5AC to MUC5B tended to be higher in patients with asthma than in controls (0.22 ± 0.37 vs. 0.12 ± 0.08, respectively, p = 0.07) (Fig. 3). This tendency remained even after the highest outlier among patients with asthma was excluded from the analysis (p = 0.08).

Table 1
Characteristics of subjects.

	Control	Asthma	p-value*
Number	22	87	–
Age, year	39 ± 11	52 ± 19	0.01
Male/female	12/10	30/57	0.08
Disease duration, year	–	3.5 ± 6.3	–
Total ACT score	–	16.8 ± 4.3	–
Serum IgE, IU/mL	NA	96 (0.34–5490)	–
Atopic/nonatopic†	NA	75/12	–
FeNO50, ppb	NA	33 ± 33	–
CA_{NO}, ppb	NA	4.5 ± 4.2	–
FVC, % predicted	115.8 ± 13.8	111.4 ± 21.8	0.24
FEV_1, % predicted	107.6 ± 21.7	102.8 ± 14.1	0.27
Sputum eosinophil, %	0.6 ± 1.5	5.5 ± 12.7	<0.0001
Sputum neutrophil, %	72.0 ± 24.1	69.7 ± 20.8	0.36
Dmin‡, units	NA	9.6 ± 13.0	–
SRrs‡, cm H_2O/L/s/min	NA	5.8 ± 22.0	–
C2, μg/mL	NA	4.3 ± 2.3	–
C5, μg/mL	NA	5.2 ± 2.3	–
R5, kPa/L/s	NA	0.39 ± 0.17	–
R20, kPa/L/s	NA	0.32 ± 0.11	–
R5 − R20, kPa/L/s	NA	0.07 ± 0.09	–
X5, kPa/L/s	NA	−0.14 ± 0.11	–
AX, kPa/L	NA	0.76 ± 1.12	–

Values are given mean ± standard deviation, median (range), or number.
ACT, asthma control test; IU, international units; FeNO50, fractional exhaled nitric oxide at expiratory flow of 50 mL/s; CA_{NO}, alveolar nitric oxide; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; Dmin, cumulative dose of inhaled methacholine at the inflection point where respiratory resistance (Rrs) begins to increase; SRrs, slope of the methacholine–Rrs dose–response curve; C2, capsaicin concentrations causing ≥2 coughs; C5, capsaicin concentrations causing ≥5 coughs; R5, respiratory resistance at 5 Hz; R20, respiratory resistance at 20 Hz; R5 − R20, difference between R5 and R20; X5, reactance at 5 Hz; AX, integrated area of low-frequency reactance; NA, not assessed.
* χ^2 test or Wilcoxon rank-sum test.
† Patients were considered atopic when they were positive for one or more serum allergen-specific IgE antibodies against house dust, Japanese cedar pollen, mixed Gramineae pollen, mixed weed pollen, mixed mold, cat dander, dog dander, and *Trichophyton rubrum*.
‡ Examined in 77 patients with asthma.

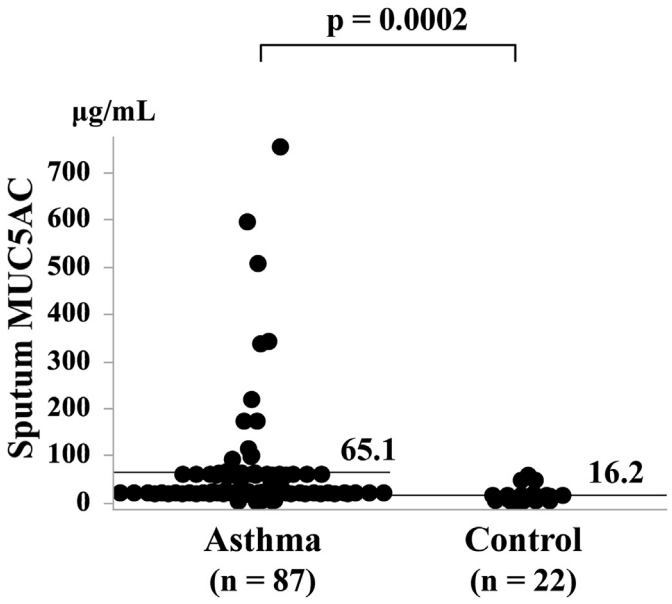

Fig. 1. Sputum MUC5AC levels in patients with asthma and controls. MUC5AC levels are expressed as micrograms of standard reactivity per milliliter of diluted resolubilized supernatants. MUC5AC levels were significantly increased in patients with asthma than those in controls (p = 0.0002, Wilcoxon rank-sum test). Bars represent means.

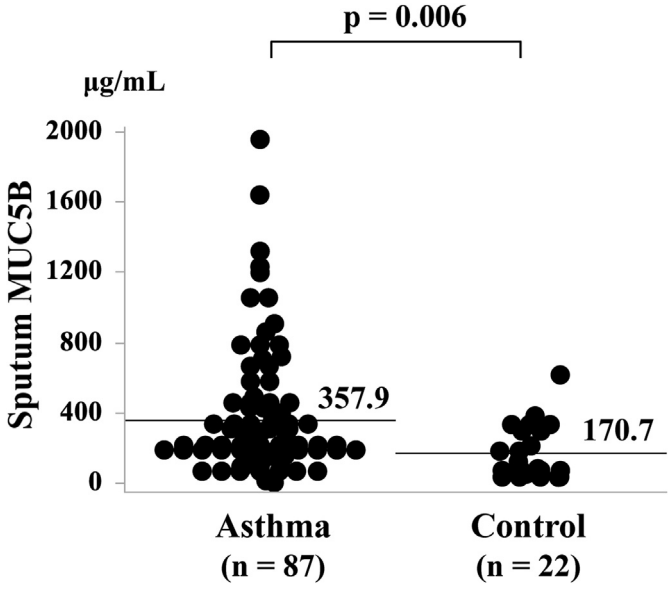

Fig. 2. Sputum MUC5B levels in patients with asthma and controls. MUC5B levels are expressed as micrograms of standard reactivity per milliliter of diluted resolubilized supernatants. MUC5B levels were significantly increased in patients with asthma than those in controls (p = 0.006, Wilcoxon rank-sum test). Bars represent means.

Sputum MUC5AC and clinical indices

Correlations between sputum MUC5AC levels and clinical and pathophysiological indices in patients with asthma are presented in Table 2. No correlation was found for age, disease duration, spirometry results, capsaicin cough sensitivity, and IOS indices. However, sputum MUC5AC levels were significantly and positively correlated with FeNO50 (Spearman's rho = 0.29, p = 0.006) and sputum eosinophil proportion (rho = 0.34, p = 0.0013). When using FeNO50 ≥ 27 ppb as a measure of airway type 2 inflammation,[38]

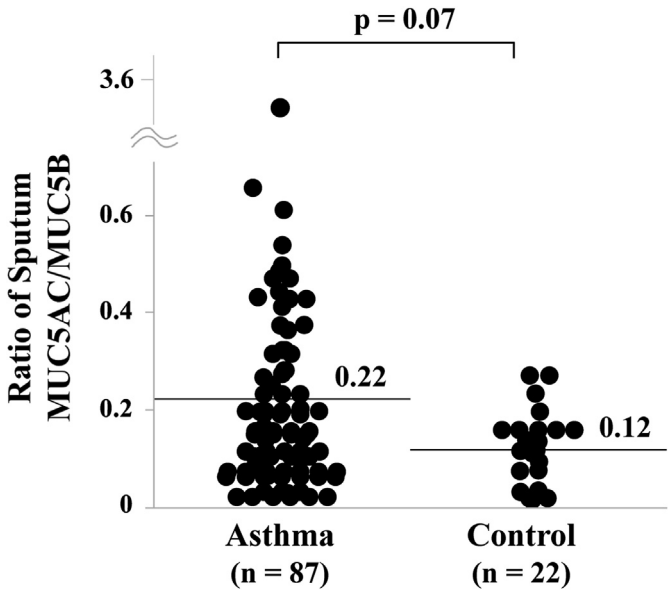

Fig. 3. Ratio of sputum MUC5AC to MUC5B in patients with asthma and that in controls. The ratio of sputum MUC5AC to MUC5B tended to be significantly increased in patients with asthma than that in controls (p = 0.07, Wilcoxon rank-sum test). Bars represent means.

sputum MUC5AC levels were significantly higher in patients with type 2 asthma (n = 37) than in those without (n = 50) (69.7 ± 147.5 μg/mL vs. 58.1 ± 66.4 μg/mL, respectively, p = 0.027), and the ratio of sputum MUC5AC to MUC5B was significantly higher in patients with type 2 asthma than in those without (0.31 ± 0.56 vs. 0.17 ± 0.16, respectively, p = 0.018). When using sputum eosinophil proportion >2% as a measure of airway type 2 inflammation,[14] consistent results were yielded; sputum MUC5AC levels were significantly higher in patients with type 2 asthma (n = 32) than in those without (n = 55) (89.2 ± 139.0 μg/mL vs. 51.1 ± 109.8 μg/mL, respectively, p = 0.003), and the ratio of sputum MUC5AC to MUC5B was significantly higher in patients with type 2 asthma than in those without (0.23 ± 0.14 vs. 0.21 ± 0.46, respectively, p = 0.006). Sputum MUC5AC levels were significantly and negatively correlated with Dmin (rho = −0.39, p = 0.0005) (Fig. 4), indicating involvement in airway hypersensitivity. Dmin (rho = −0.29, p = 0.01), but not SRrs, was also significantly and negatively correlated with sputum eosinophil proportion.

Sputum MUC5B and clinical indices

Correlations between sputum MUC5B levels and clinical and pathophysiological indices in patients with asthma are presented in Table 2. No correlation was found for age, disease duration, spirometry results, FeNO, sputum cell differentials, capsaicin cough sensitivity, and IOS indices. When using FeNO50 ≥ 27 ppb or sputum eosinophil proportion >2% as a measure of airway type 2 inflammation, there was no difference in sputum MUC5B levels between patients with type 2 asthma and those without (p > 0.10). Sputum MUC5B levels were significantly and negatively, but peculiarly, correlated with both Dmin (rho = −0.35, p = 0.002) and SRrs (rho = −0.33, p = 0.004), indicating involvement in airway hypersensitivity, but inhibitory property on airway reactivity.

Discussion

In this study, we showed that 1) sputum MUC5AC and MUC5B levels were significantly increased in steroid-untreated patients

Table 2
Correlations between sputum MUC5AC or MUC5B levels and clinical and pathophysiological indices in patients with asthma (n = 87).

Characteristics	Sputum MUC5AC vs:		Sputum MUC5B vs:	
	rho value	p value*	rho value	p value
Age, year	0.11	0.30	0.08	0.44
Disease duration, year	−0.05	0.64	0.03	0.76
Total ACT score	0.02	0.86	−0.03	0.75
FVC, % predicted	−0.15	0.17	0.04	0.74
FEV_1, % predicted	−0.14	0.20	0.04	0.69
FeNO50, ppb	0.29	0.006	0.03	0.79
CA_{NO}, ppb	−0.003	0.98	−0.04	0.72
Sputum eosinophil, %	0.34	0.0013	0.09	0.39
Sputum neutrophil, %	0.003	0.98	−0.06	0.61
Sputum macrophage, %	−0.04	0.72	0.16	0.15
Dmin†, units	−0.39	0.0005	−0.35	0.002
SRrs†, cm H_2O/L/s/min	−0.12	0.29	−0.33	0.004
C2, μg/mL	0.08	0.46	0.05	0.65
C5, μg/mL	0.10	0.37	0.03	0.78
R5, kPa/L/s	−0.02	0.86	−0.009	0.93
R20, kPa/L/s	−0.03	0.80	−0.001	0.99
R5 − R20, kPa/L/s	−0.08	0.48	−0.12	0.27
X5, kPa/L/s	0.01	0.92	0.07	0.55
AX, kPa/L	−0.01	0.93	−0.09	0.41

ACT, asthma control test; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; FeNO50, fractional exhaled nitric oxide at expiratory flow of 50 mL/s; CA_{NO}, alveolar nitric oxide; Dmin, cumulative dose of inhaled methacholine at the inflection point where respiratory resistance (Rrs) begins to increase; SRrs, slope of the methacholine–Rrs dose–response curve; C2, capsaicin concentrations causing ≥2 coughs; C5, capsaicin concentrations causing ≥5 coughs; R5, respiratory resistance at 5 Hz; R20, respiratory resistance at 20 Hz; R5 − R20, difference between R5 and R20; X5, reactance at 5 Hz; AX, integrated area of low-frequency reactance.
* Spearman's correlation coefficient.
† Examined in 77 patients.

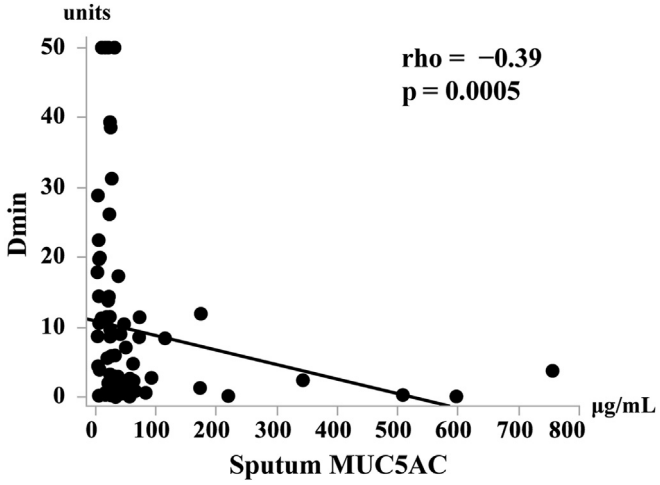

Fig. 4. Correlation between sputum MUC5AC and airway sensitivity. Sputum MUC5AC significantly correlated with Dmin (rho = −0.39, p = 0.0005, Spearman's correlation coefficient). Dmin, cumulative dose of inhaled methacholine at the inflection point where respiratory resistance begins to increase. Bar represents correlation line.

with mild asthma than in controls, with a trend towards the predominance of MUC5AC to MUC5B; and 2) sputum MUC5AC levels were correlated with airway type 2/eosinophilic inflammation and airway sensitivity in patients. We firstly assessed correlations between sputum MUC5AC and MUC5B levels and detailed clinical and pathophysiological indices, including FeNO, pulmonary function, and airway responsiveness in steroid-untreated patients with mild asthma.

Mucins are important components of mucus that contribute to viscoelastic properties of mucous secretion.[8] Among the 20 identified mucin genes to date, 11 mucins are expressed at either mRNA and/or protein levels in human airways.[8] The predominant mucins in human airways are MUC5AC and MUC5B.[12] The proportion of MUC5AC and MUC5B varies with the state of health.[6] A previous study[39] showed that asthmatic sputum (patients' background unknown) contains higher MUC5AC and MUC5B levels than healthy sputum, and there seemed to be a trend towards the predominance of MUC5AC to MUC5B in asthmatic sputum. Another study of adult patients with asthma on inhaled or oral corticosteroids and healthy controls[14] showed that MUC5AC was the predominant mucin in sputum from asthmatic patients, whereas MUC5B was the predominant mucin from healthy controls. The ratio of sputum MUC5B to MUC5AC in patients with asthma was lower than that in healthy controls.[14] Another study of children with asthma on treatment and control subjects[15] demonstrated that sputum MUC5AC levels were significantly higher in patients with acute asthma than in control subjects, whereas MUC5B levels were similar.[15] Furthermore, the ratio of MUC5B to MUC5AC was significantly lower in patients with acute asthma than in control subjects. In agreement with these studies, we also found that sputum MUC5AC levels were significantly higher in steroid-untreated patients with mild asthma than in controls, with a trend towards the predominance of MUC5AC to MUC5B in such patients. Despite the different background of patients among studies, the predominance of sputum MUC5AC to MUC5B seems to be a consistent feature of asthma.

MUC5AC gene expression on airway epithelial cells is stimulated by inflammatory cytokines, especially Th2 cytokines (interleukin (IL)-4 and IL-13)[8] both *in vitro* and *in vivo*.[11] IL-4 and IL-13 also mediate eosinophil differentiation and function[33] and produce NO by stimulating inducible NO synthase in airway epithelial cells.[40] In a previous study, the ratio of sputum MUC5AC to MUC5B was significantly higher in patients with sputum eosinophilia (>2%) than in those without.[14] Consistent with this study, we also showed that sputum MUC5AC levels and the ratio of sputum MUC5AC to MUC5B were significantly higher in patients with high FeNO50 (≥27 ppb) or sputum eosinophilia (>2%) than in those without. FeNO50 and sputum eosinophil proportion are representative markers of airway type 2 inflammation. Therefore, MUC5AC may be involved in the pathophysiology of airway type 2/eosinophilic inflammation, mediated by Th2 cytokines, in patients with asthma. Our previous results[9] of positive association between total mucin levels and sputum eosinophilia may be explained by the predominance of MUC5AC observed among patients with asthma in the present study.

MUC5B gene expression is upregulated by IL-6 and IL-17, but the effect of IL-13 has been inconsistently reported.[8] In the present study, there was no significant correlation between sputum MUC5B levels and airway inflammatory parameters, such as FeNO, sputum eosinophils, neutrophils, and macrophages in patients with asthma. Though MUC5B does not seem to be involved in airway type 2 inflammation, further studies are needed to identify the role(s) of MUC5B in human airway diseases including asthma.

Airway hyperresponsiveness (AHR) is a consistent and defining feature of asthma and provides a quantitative measure of asthma severity. A previous study showed that *Muc5ac* gene is predominantly induced in antigen-challenged murine airways.[16] The present study has shown that sputum MUC5AC levels were significantly and positively correlated with airway sensitivity in patients with asthma. Airway sensitivity was also positively correlated with sputum eosinophil proportions. As for MUC5B, a previous study showed that *Muc5b* gene is induced, albeit not significantly, in antigen-challenged mouse airways.[16] In the present study, sputum MUC5B levels were significantly and positively correlated with airway sensitivity and negatively correlated with airway reactivity in patients with asthma. AHR consists of two

major components: airway sensitivity and airway reactivity.[31–33] Airway sensitivity represents the strength of the stimulus that triggers airway narrowing and is enhanced by epithelial damage or malfunction, neural control, and inflammatory cell number/activity.[32] This component is variable or inducible and occurs after allergen exposure. This component reflects airway inflammation, in particular eosinophilic inflammation.[33] Meanwhile, airway reactivity represents the responsiveness of airways to the stimulus and is determined by airway smooth muscle contractility, viscous and elastic loads, swelling of the airway wall, and intraluminal secretions.[32] This component is persistent and relates to structural airway changes known as airway remodeling.[33] When considering the association between sputum MUC5AC or MUC5B levels and AHR in the present study, MUC5AC and MUC5B may be involved in different components of AHR. MUC5AC may augment airway sensitivity through eosinophilic inflammation, whereas MUC5B may decrease or exert protective effects on airway reactivity or airway narrowing by intraluminal secretions. MUC5AC is secreted from superficial epithelia of proximal airways, whereas MUC5B is secreted from submucosal grands and superficial epithelia of small airways.[41] MUC5AC expression is concentrated in proximal airways, whereas MUC5B expression is predominant in distal airways. The different regional distribution of MUC5AC and MUC5B in the airways may partly explain their different correlations with AHR through airway particles deposition. The positive association between MUC5AC or MUC5B levels and airway sensitivity in the present study was inconsistent to our previous findings, which showed the negative association between sputum mucin levels and airway sensitivity.[9] Sputum mucin composition other than MUC5AC and MUC5B, such as MUC2,[39] might differently affect airway sensitivity. Otherwise, our previous results might have been biased by the small sample size and different background of asthmatics; only 23 patients underwent AHR measurement, some of whom were taking inhaled corticosteroids.

In this study, there was no correlation between sputum MUC5AC or MUC5B levels and capsaicin cough sensitivity. The previous study showed that capsaicin cough sensitivity was not related to eosinophilic inflammation of the airway or airway hyperresponsiveness in patients with allergic asthma.[42] Our results suggest that sputum MUC5AC and MUC5B may not be involved in capsaicin cough reflex mechanism.

Our study has several limitations. First, patients with asthma were significantly older than controls. Although age-related changes in MUC5AC and MUC5B production remain unknown, this might have affected the results. However, there was no correlation between sputum MUC5AC and MUC5B levels and age in both patients with asthma and healthy controls in the present study (data not shown). Second, only patients with mild asthma were enrolled in the present study. The correlation between asthma severity and sputum MUC5AC and MUC5B production remains unknown. Despite the different asthma severity among studies, the predominance of sputum MUC5AC to MUC5B was maintained. Third, we did not measure type 2 cytokines in induced sputum. In order to strengthen the correlation between sputum MUC5AC levels and airway type 2 inflammation, the future study is needed to assess the correlation.

We conclude that sputum MUC5AC is involved in airway type 2/eosinophilic inflammation and AHR in steroid-untreated patients with mild asthma. The effect of inhaled corticosteroid on sputum MUC5AC has not been clarified yet. Future studies are needed to explore the correlation between sputum MUC5AC levels and asthma control or asthma severity, and the effect of inhaled corticosteroid on sputum MUC5AC levels. Because airway hypersecretion is often refractory to corticosteroid treatment,[43] MUC5AC could be a potential therapeutic target in asthmatic patients with airway hypersecretion.

Acknowledgements

The authors are grateful to Ms. Aya Inazumi and Ms. Yuko Maeda for help with induced sputum processing.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

TT had measured methacholine challenge, cough sensitivity, sputum induction and sputum mucin, and had acquired and interpreted the data, written and drafted the manuscript. HM had recruited and managed patients, and advised measurement of sputum mucin. MJ had advised measurement of sputum mucin. YK, TN, and TO had measured pulmonary function. TI and HI had measured exhaled nitric oxide. II had recruited and managed patients. AN had conceived the study, recruited and managed patients, and revised the manuscript.

References

1. Smyrnios NA, Irwin RS, Curley FJ. Chronic cough with a history of excessive sputum production. The spectrum and frequency of causes, key components of the diagnostic evaluation, and outcome of specific therapy. *Chest* 1995;**108**:991–7.
2. Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T. Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* 1992;**101**:916–21.
3. Kuyper LM, Pare PD, Hogg JC, Lambert RK, Ionescu D, Woods M, et al. Characterization of airway plugging in fatal asthma. *Am J Med* 2003;**115**:6–11.
4. James A, Rebecca H, Gavin D, Seif S, Diana K, Jadwiga W. The presence of chronic mucus hypersecretion across adult live in relation to chronic obstructive pulmonary disease developement. *Am J Respir Crit Care Med* 2016;**193**:662–72.
5. Vestbo J, Prescott E, Lange P. Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group. *Am J Respir Crit Care Med* 1996;**153**:1530–5.
6. Fahy JV, Dickey BF. Airway mucus function and dysfunction. *N Engl J Med* 2010;**363**:2233–47.
7. Ridley C, Thornton DJ. Mucins: the frontline defence of the lung. *Biochem Soc Trans* 2018;**46**:1099–106.
8. Thai P, Loukoianov A, Wachi S, Wu R. Regulation of airway mucin gene expression. *Annu Rev Physiol* 2008;**70**:405–29.
9. Jinnai M, Niimi A, Ueda T, Matsuoka H, Takemura M, Yamaguchi M, et al. Induced sputum concentrations of mucin in patients with asthma and chronic cough. *Chest* 2010;**137**:1122–9.
10. Lillehoj EP, Kato K, Lu W, Kim KC. Cellular and molecular biology of airway mucins. *Int Rev Cel Mol Biol* 2013;**303**:139–202.
11. Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev* 2006;**86**:245–78.
12. Thornton DJ, Rousseau K, McGuckin MA. Structure and function of the polymeric mucins in airways mucus. *Annu Rev Physiol* 2008;**70**:459–86.
13. Groneberg DA, Eynott PR, Oates T, Lim S, Wu R, Carlstedt I, et al. Expression of MUC5AC and MUC5B mucins in normal and cystic fibrosis lung. *Respir Med* 2002;**96**:81–6.
14. Lachowicz-Scroggins ME, Yuan S, Kerr SC, Carrington SD, Fahy JV. Abnormalities in MUC5AC and MUC5B protein in airway mucus in asthma. *Am J Respir Crit Care Med* 2016;**194**:1296–9.
15. Welsh KG, Rousseau K, Fisher G, Bonser LR, Vradding P, Brightling CE, et al. MUC5AC and a glycosylated variant of MUC5B alter mucin composition in children with acute asthma. *Chest* 2017;**152**:771–9.
16. Young HW, Williams OW, Chandra D, Bellinghausen LK, Perez G, Suarez A, et al. Central role of Muc5ac expression in mucous metaplasia and its regulation by conserved 5' elements. *Am J Respir Cel Mol Biol* 2007;**37**:273–90.
17. Roy MG, Rahmani M, Hernandez JR, Alexander SN, Ehre C, Ho SB, et al. Mucin production during prenatal and postnatal murine lung development. *Am J Respir Cel Mol Biol* 2011;**44**:755–60.
18. Roy MG, Livraghi-Butrico A, Fletcher AA, McElwee MM, Evans SE, Boerner RM, et al. Muc5b is required for airway defence. *Nature* 2014;**505**:412–6.
19. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *Am Thorac Soc Am J Respir Crit Care Med* 1995;**152**:S77–121.
20. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. *Am J Respir Crit Care Med* 2005;**171**:912–30.
21. Fujimura M, Sakamoto S, Kamio Y, Matsuda T. Effects of methacholine induced bronchoconstriction and procaterol induced bronchodilation on cough receptor sensitivity to inhaled capsaicin and tartaric acid. *Thorax* 1992;**47**:441–5.

22. Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, et al. Epidermal growth factor system regulates mucin production in airways. *Proc Natl Acad Sci U S A* 1999;**96**:3081–6.
23. Viswanathan H, Brownlee IA, Pearson JP, Carrie S. MUC5B secretion is up-regulated in sinusitis compared with controls. *Am J Rhinol* 2006;**20**:554–7.
24. Matsumoto H, Niimi A, Jinnai M, Nakaji H, Takeda T, Oguma T, et al. Association of alveolar nitric oxide levels with pulmonary function and its reversibility in stable asthma. *Respiration* 2011;**81**:311–7.
25. Oostveen E, MacLeod D, Lorino H, Farre R, Hantos Z, Desager K, et al. The forced oscillation technique in clinical practice: methodology, recommendations and future developments. *Eur Respir J* 2003;**22**:1026–41.
26. Yamaguchi M, Niimi A, Ueda T, Takemura M, Matsuoka H, Jinnai M, et al. Effect of inhaled corticosteroids on small airways in asthma: investigation using impulse oscillometry. *Pulm Pharmacol Ther* 2009;**22**:326–32.
27. Shi Y, Aledia AS, Tatavoosian AV, Vijayalakshmi S, Galant SP, George SC. Relating small airways to asthma control by using impulse oscillometry in children. *J Allergy Clin Immunol* 2012;**129**:671–8.
28. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, COates A, et al. Standardisation of spirometry. *Eur Respir J* 2005;**26**:319–38.
29. Nakamura Y, Tamaoki J, Nagase H, Yamaguchi M, Horiguchi T, Hozawa S, et al. Japanese guidelines for adult asthma 2020. *Allergol Int* 2020;**69**:519–48.
30. Takishima T, Hida W, Sasaki H, Suzuki S, Sasaki T. Direct-writing recorder of the dose-response curves of the airway to methacholine. Clinical application. *Chest* 1981;**80**:600–6.
31. Niimi A, Matsumoto H, Takemura M, Ueda T, Chin K, Mishima M. Relationship of airway wall thickness to airway sensitivity and airway reactivity in asthma. *Am J Respir Crit Care Med* 2003;**168**:983–8.
32. Sterk PJ, Bel EH. Bronchial hyperresponsiveness: the need for a distinction between hypersensitivity and excessive airway narrowing. *Eur Respir J* 1989;**2**:267–74.
33. Cockcroft DW, Davis BE. Mechanisms of airway hyperresponsiveness. *J Allergy Clin Immunol* 2006;**118**:551–9. quiz 60-1.
34. Fukumitsu K, Kanemitsu Y, Asano T, Takeda N, Ichikawa H, Yap J, et al. Tiotropium attenuates refractory cough and capsaicin cough reflex sensitivity in patients with asthma. *J Allergy Clin Immunol Pract* 2018;**6**:1613–20.
35. Kanemitsu Y, Fukumitsu K, Kurokawa R, Takeda N, Szuki M, Yap J. Increased capsaicin sensitivity in severe asthmatics associated with worse clinical outcome. *Am J Respir Crit Care Med* 2020;**201**:1168–77.
36. Matsumoto H, Niimi A, Takemura M, Ueda T, Minakuchi M, Tabuena R, et al. Relationship of airway wall thickening to an imbalance between matrix metalloproteinase-9 and its inhibitor in asthma. *Thorax* 2005;**60**:277–81.
37. Matsuoka H, Niimi A, Matsumoto H, Ueda T, Takemura M, Yamaguchi M, et al. Patients' characteristics associated with unsuccessful sputum induction in asthma. *J Allergy Clin Immunol* 2008;**121**:774–6.
38. Schleich F, Brusselle G, Louis R, Vandenplas O, Michils A, Pilete C, et al. Heterogeneity of phenotypes in severe asthmatics. The Belgian severe asthma registry (BSAR). *Respir Med* 2014;**108**:1723–32.
39. Kirkham S, Sheehan JK, Knight D, Richardson PS, Thonrnton DJ. Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B. *Biochem J* 2002;**361**:537–46.
40. Suresh V, Mih JD, George SC. Measurement of IL-13-induced iNOS-derived gas phase nitric oxide in human bronchial epithelial cells. *Am J Respir Cel Mol Biol* 2007;**37**:97–104.
41. Okuda K, Chen G, Subramani DB, Wolf M, Gilmore RC, Kato T, et al. Localization of secretory mucins MUC5AC and MUC5B in normal/healthy human airways. *Am J Respir Crit Care Med* 2019;**199**:715–27.
42. Minoguchi H, Minoguchi K, Tanaka A, Matsuo H, Kihara N, Adachi M. Cough receptor sensitivity to capsaicin does not change after allergen bronchoprovocation in allergic asthma. *Thorax* 2003;**58**:19–22.
43. Teeter JG, Bleecker ER. Relationship between airway obstruction and respiratory symptoms in adult asthmatics. *Chest* 1998;**113**:272–7.