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Original Article

Airway inflammation phenotype prediction in asthma patients using lung sound analysis with fractional exhaled nitric oxide

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LSA, Lung sound analysis; FeNO, fractional exhaled nitric oxide; FEV_{1.0}, forced expiratory volume in one second; FVC, forced vital capacity; V₅₀ and V₂₅, maximal expiratory flow at 50% and 25%; E/I LF, the expiration-to-inspiration sound power ratio in a low-frequency range; E/I MF, the expiration-to-inspiration sound power ratio in a mid-frequency range; PC₂₀, provocative concentration of acetylcholine causing a 20% decrease in FEV_{1.0}

ABSTRACT

Background: We previously reported the results of lung sound analysis in patients with bronchial asthma and demonstrated that the exhalation-to-inhalation sound pressure ratio in the low frequency range between 100 and 200 Hz (E/I LF) was correlated with the presence of airway inflammation and airway obstruction. We classified asthma patients by airway inflammation phenotype using the induced sputum eosinophil and neutrophil ratio and determined whether this phenotype could be predicted using E/I LF and fractional exhaled nitric oxide values.

Methods: Steroid-naïve bronchial asthma patients were classified into four phenotypes, including “Low inflammation” (35 patients), “Eosinophilic type” (58 patients), “Neutrophilic type” (15 patients), and “Mixed type” (15 patients) based on the results of induced sputum examinations. The E/I LF data and FeNO levels were then evaluated for the four phenotype groups; the prediction powers of these two indices were then analyzed for each phenotype.

Results: The median E/I LF value was highest in the “Mixed type” and lowest in the “Low inflammation” group. FeNO differentiated between the “Low inflammation” and “Eosinophilic type” groups, “Low inflammation” and “Neutrophilic type” groups, and “Neutrophilic type” and “Mixed type” ($p < 0.0001$, $p = 0.007$, and $p = 0.04$, respectively). E/I LF differentiated between the “Low inflammation” and “Eosinophilic type” groups ($p = 0.006$). E/I LF could distinguish the “Mixed type” group from the “Low inflammation” and “Eosinophilic type” groups ($p = 0.002$).

Conclusions: A combination of the E/I LF value and FeNO may be useful for the classification of the airway inflammation phenotype in patients with bronchial asthma.

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Introduction

Bronchial asthma is a chronic inflammatory disease that presents as a mixture of various phenotypes.^{1–3} The classification of phenotypes using cluster analysis is expected to help elucidate asthma pathophysiology and impact personalized medicine

according to individual responses to drugs.^{4,5} Currently available methods to evaluate airway inflammation in bronchial asthma include exhaled nitric oxide (FeNO) measurement, bronchofiberscopic biopsy and bronchoalveolar lavage (BAL), differential cell counts and inflammatory cytokine measurement in induced sputum, and inflammatory cytokine measurement in exhaled breath condensate.^{6–10} Differential cell counts of induced sputum can distinguish between eosinophilic and neutrophil inflammation; however, this method requires complex processing and may involve sample collection errors.

In contrast, FeNO measurement and lung sound analysis (LSA) have the advantages of being simple and noninvasive.^{7,11} FeNO is

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currently used as an airway eosinophilic inflammation index. Regarding LSA, we previously reported that the exhalation-to-inhalation sound pressure ratio in the low frequency (LF) range between 100 and 200 Hz (E/I LF) was correlated with airway inflammation.¹¹ E/I LF may indicate the inflammation level by evaluating airway turbulence but does not indicate the inflammation phenotype (neutrophilic or eosinophilic). However, this index may shed light on the physiological characteristics of asthma by assessing the sound pressure ratio of inhalation versus exhalation.

Based on these findings and hypothesis, we classified asthma patients into four phenotype groups (low inflammation, eosinophilic, neutrophilic, and both eosinophilic and neutrophilic) based on the proportion of eosinophils vs. neutrophils of induced sputum. By comparing E/I LF and FeNO values across the four groups, we determined whether E/I LF is useful for distinguishing airway inflammation phenotypes in bronchial asthma.

Methods

Subjects and study design

We examined 190 patients diagnosed with persistent asthma who first visited our hospital from November 2009 to November 2012. An LSA, a blood examination, pulmonary function tests, an FeNO measurement, an acetylcholine (Ach) bronchial provocation test, and an induced sputum analysis were performed. All of the patients included in this study fulfilled the diagnostic criteria of the Global Initiative for Asthma (GINA) Guidelines.¹ All included patients reported a history of asthmatic symptoms, including recurrent cough, wheezing or dyspnea, and exhibited airway hyper-responsiveness, i.e., PC₂₀ for Ach <8000 mcg/mL. Airway reversibility was confirmed in 80% of patients but not in the remaining 20% of patients, for whom bronchial asthma was diagnosed based on medical history and positive PC₂₀ results. Chest X rays and high resolution computed tomography (CT, as needed) identified no indications of chronic obstructive pulmonary disease (COPD) in any patient. All patients demonstrated normal diffusion capacity. COPD was ruled out by an FEV₁/FVC over 70% after bronchodilator inhalation and smoking history. No patients had previously used inhaled or oral corticosteroids. The asthmatic subjects treated with bronchodilators were not excluded. Anti-asthma drugs, including bronchodilators, were discontinued for at least 24 h prior to examination. Of the 190 patients examined, 137 patients were nonsmokers or past smokers who were successful in collecting induced sputum. Among these patients, those with a large number of squamous cells in the sputum and those considered ineligible due to suspected infection were excluded from the analysis. The remaining 123 patients were classified into four phenotype groups as follows: eosinophils <3% with neutrophils <60% (Low inflammation); eosinophils ≥3% with neutrophils <60% (Eosinophilic type); eosinophils <3% with neutrophils ≥60% (Neutrophilic type); and eosinophils ≥3% with neutrophils ≥60% (Mixed type).^{12,13} As a result, 35 patients (28%), 58 patients (47%), 15 patients (12%), and 15 patients (12%) were classified into the “Low inflammation”, “Eosinophilic type”, “Neutrophilic type”, and “Mixed type” groups, respectively.

The ethics committee of Fukuoka National Hospital approved the study protocol (protocol No.: 20-12); all participants received verbal and written information about the study before providing their informed consent.

LSA

LSA was performed according to the procedure described in previous studies.^{11,14} The sound recording was performed in quiet room, but not in a soundproof booth, in the outpatient department.

The patients breathed deeply during the breath sound recording. Lung sounds were recorded using a hand-held microphone for ≥30 s over the left lung base. The recording system consisted of an electro-stethoscope containing a wide-range audio sensor attached to the inside of a diaphragm (Bio-Sound Sensor BSS-01; Kenz Medico, Saitama, Japan), a signal processing system, and a personal computer. The sensor had a band-pass filter range of 40–2500 Hz and a reliable sound collecting ability in the 40–2000 Hz range. The recorded sound was analyzed by fast Fourier analysis using a sound spectrometer (Easy-LSA; Fukuoka, Japan) and was displayed as a spectrograph, with the frequency in Hz on the vertical axis and time on the horizontal axis. The recording system was calibrated with a reference sound pressure (1 kHz; 94 dB [0 dB = 20 μPa]).

We defined a frequency range of 100–200 Hz as LF and determined the inspiration sound power, expiration sound power, and the E/I in the LF range. E/I LF data were converted from logarithmic values (dBSPL) to actual values.

Measuring the fractional exhaled nitric oxide (FeNO) concentration

Following the guidelines published by the American Thoracic Society (ATS), FeNO was measured using the online single-breath method and a fast response (0.02 s) chemiluminescence analyzer (Sievers Nitric Oxide Analyzer NOA 280i, GE Analytical Instruments, Boulder, CO, USA).¹¹ All measurements were obtained using a mouth pressure of 16 cm H₂O, corresponding to an expiratory flow of 50 mL/s. The FeNO concentrations were recorded as the average of 3 FeNO values.

Sputum induction and processing

The participants inhaled 5 mL 3% NaCl solution through an ultrasonic nebulizer to induce sputum collection. The subjects were asked to cough during and after the inhalation and to expectorate into empty containers. Sputum was induced over a 20-min period. The sputum samples were processed within 30 min according to a method described by Metso *et al.*¹⁰ The sputum cells were separated by centrifugation at 2000g for 10 min. The cell suspension was cytocentrifuged (Cytospin 3; Shandon, Astmoor, UK) onto microscope slides at 450 rpm for 6 min. The cytospin products from the sputum were air-dried for 30 min and then stained using the Giemsa staining method (Muto Pure Chemicals, Tokyo, Japan). At least 400 non-squamous cells were counted differentially, including eosinophils, neutrophils, lymphocytes, macrophages, and ciliated epithelial cells. The results are expressed as the percentages of the total non-squamous cell counts.

Other examinations

The measurements of flow–volume curves and bronchial hyperresponsiveness to Ach were performed in accordance with previous papers.^{11,14}

Statistical analysis

We examined potential differences in the background or measured values between four groups (Low inflammation”, “Eosinophilic type”, “Neutrophilic type” and “Mixed type”) using a box plot. The Steel–Dwass test was used to test the significance of differences between the pairs of groups.

The ROC curve and chi square test were performed for each index to clarify the useful threshold to distinguish any two groups. Next, the chi square values were compared between using FeNO only and using FeNO and E/I LF together to determine E/I LF value addition.

Table 1
Patient characteristics.

	Low inflammation	Eosinophilic type	Neutrophilic type	Mixed type
N, Men/women [†]	35, 14 (40)/21 (60)	58, 14 (24)/44 (76)	15, 6 (40)/9 (60)	15, 6 (40)/9 (60)
Age, years [‡]	38 (31–53)	40 (31–50)	38 (33–58)	43 (35–61)
BMI, kg m ⁻² [‡]	22.9 (21.0–25.6)	22.5 (20.2–24.6)	21.6 (19.9, 25.6)	21.6 (19.8–25.1)
Severity, GINA Step 1, 2, 3 [§]	21 (60), 14 (40), 0 (0)	18 (31), 35 (60), 5 (9)	7 (47), 6 (40), 2 (13)	8 (53), 4 (27), 3 (20) [¶]
Allergic rhinitis, no/yes [†]	19 (54)/16 (46)	34 (59)/24 (41)	6 (40)/9 (60)	10 (67)/5 (33)
Sinusitis, no/yes [†]	33 (94)/2 (6)	55 (95)/3 (5)	13 (87)/2 (13)	13 (87)/2 (13)
Hypertension, no/yes [†]	33 (94)/2 (6)	54 (93)/4 (7)	13 (87)/2 (13)	13 (87)/2 (13)
Atopic/non-atopic ^{‡,}	18 (53)/16 (47)	35 (63)/20 (36)	8 (67)/4 (33)	10 (67)/5 (33)
Age of onset, years [‡]	37 (25–50)	35 (20–47)	36 (24–52)	33 (20–58)
Disease duration, years [†]	0.7 (0.08–7.0)	3.0 (0.3–14.5)	5.0 (0.4, 17.0)	6.0 (0.03–21.0)
Disease duration, less than 5 years/more than 5 years [†]	25 (71)/10 (29)	33 (57)/25 (43)	7 (47)/8 (53)	7 (47)/8 (53)
History of childhood asthma, no/yes [†]	26 (74)/9 (26)	40 (69)/18 (31)	12 (80)/3 (20)	11 (73)/4 (27)
Smoking, never/past/current [†]	24 (69)/11 (31)/0	40 (69)/18 (31)/0	8 (53)/7 (47)/0	13 (87)/2 (13)/0
Smoked amount, pack-years [‡]	0 (0–4)	0 (0–4)	0 (0–27)	0 (0–0)

[†] The numbers (percentages) are shown for the categorical variables.[‡] Data are shown as the median (25–75%).[§] GINA (Global Initiative for Asthma) step refers to the Step.[¶] $p < 0.05$ vs. Low inflammation.^{||} Atopic status was defined if the patient had 1) atopic disease complication, 2) atopic family history, 3) high level of serum IgE, or 4) positive specific IgE-RAST to any antigen.

These statistical analyses were conducted by using JMP Pro version 11 (SAS Institute Inc., Cary, North Carolina, USA) and R statistical software 3.1.2.

Results

Patient characteristics

The patients in the “Low inflammation”, “Eosinophilic type”, “Neutrophilic type” and “Mixed type” groups were not significantly different in terms of gender ratio, age, BMI, prevalence of allergic rhinitis and sinusitis, blood pressure status, history of childhood asthma, proportion of past smokers, or cumulative pack-years. None of the patients in these four groups were current smokers (Table 1). The “Mixed type” group had a significantly higher proportion of patients with a greater severity of GINA Step 3 than the “Low inflammation” group ($P < 0.05$).

Comparison of respiratory function, PC₂₀, IgE and blood eosinophils (%) among the four groups

Patients in the “Eosinophilic type” and “Mixed type” groups had lower FEV_{1.0}/FVC, FEV_{1.0}%predicted, V₅₀%predicted, and V₂₅%predicted and higher blood eosinophils, FeNO, and E/I LF than the patients in the “Low inflammation” group (Table 2). The “Mixed type” group also had lower V₅₀%predicted and higher blood eosinophils, FeNO, and E/I

LF than the patients in the “Low inflammation” group. The “Neutrophilic type” group had higher FeNO than the patients in the “Low inflammation” group. The “Eosinophilic type” group had higher FeNO than the “Neutrophilic type” group. The “Mixed type” group had higher E/I LF than the “Eosinophilic type” group.

Prediction thresholds to distinguish between groups using FeNO or E/I LF

The prediction thresholds of FeNO and E/I LF to distinguish between the groups are shown in Table 3. FeNO differentiated between the “Low inflammation” and “Eosinophilic type” groups, “Low inflammation” and “Neutrophilic type” groups, and “Neutrophilic type” and “Mixed type” groups ($p < 0.0001$, $p = 0.007$, and $p = 0.04$, respectively). However, FeNO could not distinguish the “Mixed type” group from the combination of the “Low inflammation” and “Eosinophilic type” groups. E/I LF differentiated between the “Low inflammation” and “Eosinophilic type” groups ($p = 0.006$). E/I LF could distinguish between the “Mixed type” group and the combination of the “Low inflammation” and “Eosinophilic type” groups ($p = 0.002$).

Discussion

In a recent classification of airway inflammation phenotypes in patients with bronchial asthma, attention was placed not only on

Table 2
Comparison of respiratory function, PC₂₀, IgE and blood eosinophils among the four groups.

	Low inflammation N = 35	Eosinophilic type N = 58	Neutrophilic type N = 15	Mixed type N = 15
FEV _{1.0} /FVC, %	81.6 (73.1–85.1)	74.9 (67.5–82.7)*	76.7 (72.2–84.1)	73.8 (67.8–78.8)
FEV _{1.0} %predicted	96.2 (87.8–105.3)	86.7 (74.2–94.3)***	96.5 (85.7–105.1)	85.9 (67–96.9)
V ₅₀ %predicted	78.6 (63.8–101.1)	56.7 (37.6–74.3)***	68.8 (58.1–93.2)	56.4 (37.7–72.6)**
V ₂₅ %predicted	56.9 (44.7–73.7)	36.6 (26.6–58.8)**	51.7 (38.5–72.0)	37.7 (33.7–59.2)
%DLCO/VA, %	99.6 (89.9–119.6)	106.1 (95.1–121.5)	88.0 (85.5–114.6)	98.6 (89.5, 140.8)
logPC ₂₀	3.1 (2.8–3.5)	2.5 (2.3–2.9)	2.9 (2.5–3.5)	2.7 (2.4–3.0)
logIgE [†]	1.9 (1.5–2.5)	2.3 (1.8–2.7)	2.3 (1.6–2.6)	2.4 (2.0, 2.9)
Blood eosinophils, %	2.7 (1.6–5.1)	8.0 (5.1–11.5)***	2.7 (2.1–5.0)	6.3 (3.8–8.0)*
FeNO, ppb	17.3 (11.8–30.7)	91.1 (56.6–151.3)***‡	31.1 (23.1–117.8)*	79.6 (33.3–195.6)***§
E/I LF	0.47 (0.32–0.55)	0.56 (0.44–0.69)*	0.62 (0.28–0.89)	0.72 (0.62–0.86)***§

Data are shown as the median (25–75%). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Low inflammation group.[†] IgE was expressed as a logarithmic conversion because of its wide range.[‡] $p < 0.05$ between the Eosinophilic type and Neutrophilic type.[§] $p < 0.05$ between the Eosinophilic type and Mixed type.

Table 3
Prediction thresholds to distinguish between the groups using FeNO or E/I LF.

Distinguished groups	Threshold	Odds ratio (95% CI)	chi-square	p value
FeNO, ppb				
Low inflammation vs Eosinophilic type	40	37.5 (11.2–125.2)	50.6	<0.0001
Low inflammation vs Neutrophilic type	28	5.8 (1.6–21.5)	7.4	0.007
Eosinophilic type vs Mixed type	45	0.46 (0.1–1.7)	1.3	0.25
Neutrophilic type vs Mixed type	31	5.7 (0.9–34.5)	4.1	0.04
Low inflammation/Eosinophilic type vs Mixed type	53.5	1.2 (0.4–3.5)	0.08	0.78
E/I LF				
Low inflammation vs Eosinophilic type	0.50	3.4 (1.4–8.1)	7.7	0.006
Low inflammation vs Neutrophilic type	0.49	2.5 (0.7–8.8)	2.2	0.14
Eosinophilic type vs Mixed type	0.64	4.1 (1.2–13.7)	5.6	0.02
Neutrophilic type vs Mixed type	0.71	1.7 (0.4–7.3)	0.54	0.46
Low inflammation/Eosinophilic type vs Mixed type	0.64	5.8 (1.8, 18.5)	9.24	0.002

eosinophilic but also on non-eosinophilic inflammation as well as a mixed type of both eosinophilic and neutrophilic inflammation. In a study by Moore *et al.* using cluster analysis from the Severe Asthma Research Program (SARP), patients with the highest severity of airway narrowing exhibited a higher proportion of neutrophils in sputum samples.² The authors classified the patient population into four clusters, including “Eosinophil predominant” and “Neutrophil predominant” types, and described the characteristics of patients in each group.³ McGrath *et al.* reported that patients with the non-eosinophilic type of asthma, based on induced sputum testing, were resistant to steroids.⁴ Mohamed *et al.* also demonstrated that patients with neutrophilic inflammation were less responsive to inhaled steroid therapy.⁵ The results in our present study indicate that patients with a mixed type of eosinophilic and neutrophilic inflammation tend to present with more severe cases of asthma. The eosinophilic type and mixed type tended to have lower lung function and higher blood eosinophils as well as FeNO, as in previous reports.⁶ The high FeNO of the “Neutrophilic type” may suggest that this group also has eosinophilic inflammation. This speculation was supported in that the FeNO was higher in the “Mixed type” group than the “Neutrophilic type” group. FeNO could not identify the additional neutrophilic inflammation observed in the “Eosinophilic type” and/or “Low inflammation” patient groups.

The strength of lung sounds heard on auscultation varies by airflow volume and velocity; a larger airflow produces a stronger sound.^{15–19} Airflow is sensitive to deep breathing, necessitating data correction. In bronchial asthma patients with a narrowed airway due to inflammation, the inspiratory sound is weaker than in healthy control subjects because the inspiratory airflow is not affected by disturbance. However, the expiratory sound is similar or slightly stronger than that in healthy control patients as a result of two mechanisms: 1) the acceleration of sound power due to a larger airflow and 2) an increase in sound power due to a higher airflow velocity as a result of airway narrowing.^{11,14} Wang *et al.* reported that bronchial asthma patients exhibited a significant reduction in vibration energy at the peak of inhalation and exhalation compared to healthy volunteers and patients with congestive heart failure.²⁰ Studies have also reported variations in respiratory airflow and power at the time of airway narrowing in the methacholine challenge test.^{21,22} We have reported a correlation of the E/I LF with airway inflammation and airway narrowing in patients with bronchial asthma of the eosinophilic type.¹¹ The E/I LF is an indicator of eosinophilic inflammation, but the function of the E/I LF as an indicator of neutrophilic inflammation is unclear, because the smokers were excluded in these previous studies. In the present study, we compared the four phenotypes of “Low inflammation”, “Eosinophilic type”, “Neutrophilic type” and “Mixed type” and found a significantly higher E/I LF in the “Mixed type” group compared with the “Eosinophilic type” and “Low inflammation” groups. Therefore, it appears that the E/I LF increases when airway

inflammation worsens as a result of the activation of neutrophil migration in the presence of eosinophils. As in the “Mixed type” phenotype, the E/I LF may also be elevated in the “Eosinophilic type” if the airway obstruction is severe. Our results suggested the possibility of differentiating the “Mixed type” patients from the “Low inflammation” plus “Eosinophilic type” patients using E/I LF data from LSA.

At this time, differential cell counting in samples from the induced sputum test is commonly used as an indicator of airway inflammation. This method requires cumbersome sputum processing. In contrast, E/I LF measurement has the advantage of being noninvasive, simple, and time-saving. When utilizing the E/I LF in bronchial asthma diagnosis, precautions should be taken to have the patient breathe deeply, and the examination should be performed in an environment with a minimum noise level whenever possible.^{18,23} Smoking is one of the most important factors that contributes to neutrophil inflammation in patients with asthma. We excluded current smokers from the present analysis to eliminate the presence of neutrophilic inflammation associated with smoking. Inclusion of smokers is likely to increase the proportion of patients in the “Neutrophilic type” group, but we decided to exclude smokers in the present analysis because this subpopulation exhibits larger inter-individual variations in lung function and LSA without any discernible trend. In the study, the “Neutrophilic type” did not include a substantially higher ratio of past smokers. Patients who are currently smoking and those exhibiting complications with alveolar abnormalities, such as emphysema, asthma and COPD overlap, should be included in future studies.

In conclusion, the E/I LF has the potential to serve as an indicator of not only eosinophilic but also mixed type inflammation involving both eosinophils and neutrophils, while FeNO is an indicator of eosinophilic inflammation. Therefore, the combination of FeNO and E/I LF can be used in place of induced sputum differential cell counts to distinguish airway inflammation phenotypes in patients with bronchial asthma.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

TS designed the study, participated in the data analysis and wrote the manuscript. YO assisted in the data analysis. YN assisted in the lung sound analysis.

HN performed the lung sound analysis. RK assisted in the design of the study and performed the clinical examinations. TI coordinated the patient recruitment process.

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