hemorrhage in the second cycle, and therefore received docetaxel at a dose of 60 mg/m² from the third cycle. In Cycle 4, he developed interstitial lung disease (ILD), and showed an increase of AST, ALT and creatinine before death.

Ten (50%) of the 20 patients at dose level 2 needed dose reduction throughout the treatment period because of various toxicities. Among these patients and the patients at dose level 1, who received docetaxel at 70 mg/m², only 3 (19%) of 16 patients needed a dose reduction to 60 mg/m².

A total of nine patients were withdrawn from the study due to treatment-related toxicities. Two patients discontinued because of a dose delay due to non-recovery of the neutrophil count and worsening PS, respectively. Three patients refused the study treatment due to maximum Grade 2 fatigue. Two patients developed ILD: one died as described above and the other recovered. The remaining two patients were withdrawn from the study by the investigators because of Grade 2 but refractory limb edema and subungual abscess, respectively.

For the 20 patients who received a dose of 75 mg/m², only 62% of the cycles (58 of 94 cycles) could be delivered at the initial dose in comparison with 89% (33 of 37 cycles) in the 6 patients who received a dose of 70 mg/m².

Efficacy

There were four partial responses (one patient at dose level 1 and three patients at dose level 2) and no complete responses, yielding an overall response rate of 15.4%. One patient at dose level 2 was not evaluable for response because of ILD in Cycle 2. The median number of treatment cycles for the patients overall was 4 (range, 2–22 cycles). All 26 patients were assessable for PFS and OS. After a median follow-up period of 12.5 months (range, 3.2–36.0 months), 12 patients were still alive. The median PFS of the patients overall was 4.0 months (95% CI 1.4–6.6 months; Fig. 1A), and that for patients at dose level 1 and dose level 2 was 3.3 and 4.0 months, respectively. The median OS for all patients was 14.6 months (95% CI 2.9–26.2 months; Fig. 1B).

Post-discontinuation Chemotherapy

Except for 3 patients who did not show disease progression, 20 (87%) of the 23 patients received third- or fourth-line chemotherapy. Among the seven patients who had EGFR mutations, three had already received EGFR-TKI as the first- or second-line chemotherapy before entering the study and the rest received EGFR-TKIs after failure of the study treatment. Among patients with wild-type EGFR or an unknown EGFR mutation status, nine received erlotinib after failure of the study treatment.

DISCUSSION

This is, to our knowledge, the only study to have re-evaluated the feasibility of docetaxel at doses of up to 75 mg/m² in

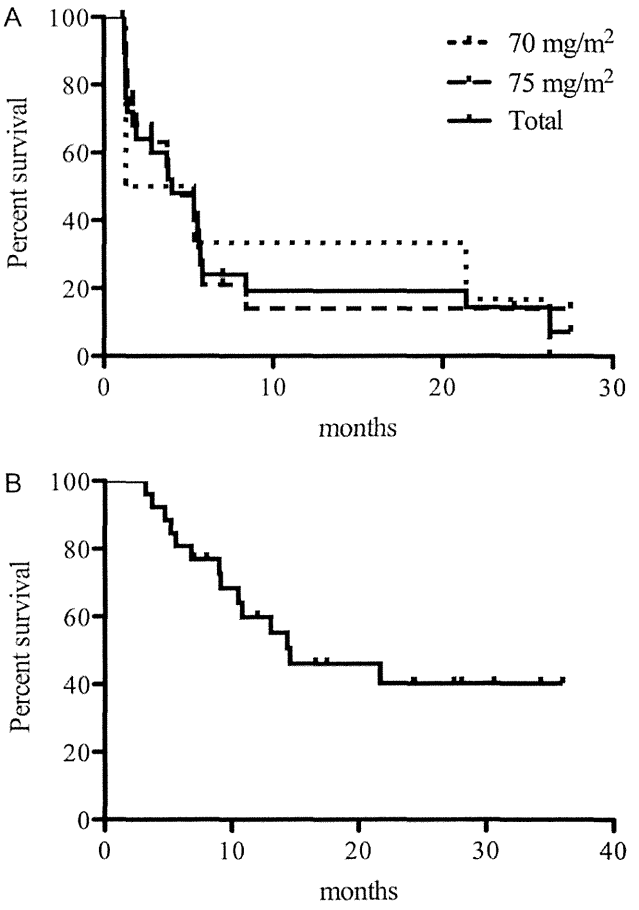

Figure 1. (A) Progression-free survival (PFS) of all patients. PFS relative to docetaxel dose (70 and 75 mg/m²) is also shown. (B) Overall survival of all patients.

Japanese patients with previously treated NSCLC. Even at 70 mg/m², no previous report has documented the safety and efficacy of docetaxel prospectively, and for this reason we decided to start the present study at 70 mg/m².

As DLT was observed in 1 patient at dose level 1 and in 1 of the first 6 patients at dose level 2, we were able to accrue 20 patients at dose level 2, as originally planned. At dose level 2, we observed DLT in 6 patients (30%) in the first cycle, including 5 patients with FN. These accounted for less than one-third of the patients at dose level 2. Eight out of the 20 patients (40%) finally developed FN at some point during the treatment period. In addition, because of DLT, another two patients at this dose level required dose reduction to 70 mg/m² after the second cycle or later. As a result, only 62% of cycles could be delivered at the initial dose, the rate being very similar to that reported by Shepherd et al. (2) for a dose of 100 mg/m². However, unlike that study, which recorded a 10% early death rate, no early deaths due to toxicity occurred in our study. On the other hand, among 6 patients at dose level 1 and 10 patients at dose level 2 who required dose reduction and received docetaxel at 70 mg/m² thereafter, DLT was observed in only 3 of the 16 patients (19%), including 2 with FN during the treatment period. Therefore, docetaxel at an

Table 5. Prospective studies of docetaxel for previously treated NSCLC in Japan or other countries

First author	Phase	Dose (mg/m^2)	No. of patients	Grade 3/4 neutropenia (%)	FN (%)	RR (%)
Mukohara (18)	II	60	22	72.7	18.0	18.2
Takeda (19)	III	60	64	85.9	25.0	6.8
Segawa (20)	RPII	60	29	89.7	13.8	20.7
Maruyama (24)	III	60	239	73.6	7.1	12.8
Shepherd (2)	III	75	55	67.3	1.8	5.5
Hanna (3)	III	75	288	40.2	12.7	8.8
Kim (5)	III	75	715	58.2	10.1	7.6
Present study	—	70/75	6/20	100/85.0	33.3/40.0	16.7/15.0

NSCLC, non-small cell lung cancer; RR, response rate; RPII, randomized phase II.

initial dose of 75 mg/m^2 was manageable with dose reduction or appropriate supportive care, although the toxicity seemed to be more severe in these Japanese patients than in previously reported cohorts from other countries.

Severe neutropenia (Grade 3 or 4) was observed in nearly 90% of the patients in this study, being approximately equivalent to the rate documented previously in Japan for a dose of 60 mg/m^2 (18–20). However, as shown in Table 5, the incidence of FN in our patients at a dose of 75 mg/m^2 was clearly higher than that reported previously. FN is a critical toxicity and a potentially life-threatening oncologic emergency. According to the American Society of Clinical Oncology Clinical Practice Guidelines published in 2006, primary prophylactic CSF is recommended when the risk of FN is ~20% or higher (21). However, this does not mean that prophylactic CSF should be used for all patients receiving such a high-risk regimen. The primary consideration is the aim of the treatment, i.e. curative, prolongation of life or palliative. If treatment is not curative, a change to an alternative regimen or dose modification should be considered, rather than using prophylactic CSF. Unfortunately, because chemotherapy is unable to achieve complete cure for advanced or recurrent NSCLC at this time, the role of chemotherapy for these patients is prolongation of life, palliation or symptom control. On the basis of this rationale, we did not use prophylactic CSF for our patients.

In this study we found a considerable difference in the incidence of FN between the two dose levels, even though the difference in dose was only 5 mg/m^2. Although the reason for this is unclear, it has been reported that docetaxel exposure (i.e. docetaxel clearance) is a significant predictor of Grade 4 neutropenia and FN (22). Furthermore, Yamamoto et al. (23) have reported that when docetaxel was administered on the basis of body surface area, the interpatient variability in the area under the concentration–time curve (AUC) became large in Japanese patients. In the present study, there was no significant difference in the incidence of Grade 4 neutropenia (ANC < 500) between the two dose levels. However, 10 of 20 patients (50%) in the 75 mg/m^2 group developed an ANC nadir of <200, whereas only 1 patient in the 70 mg/m^2 group did so (data not shown). Because all patients who developed FN had an ANC nadir of <200, which significantly increased the likelihood of FN, more patients with a high AUC in the 75 mg/m^2 group developed a lower ANC nadir than those in the 70 mg/m^2 group, and this may have affected the incidence of FN. However, non-hematological toxicities other than FN were mild, and similar in severity to those reported previously in both Japan and Western countries (1,2,18–20,24). Therefore, some genetic or other distinctive factors may be associated with FN, and further research is needed.

This study may have had some potential drawbacks in that the number of patients was small and we did not stipulate a specific statistical design, because our main purpose was to re-evaluate the feasibility of the global standard dose of docetaxel on a clinical basis. Moreover, we did not perform PK/pharmacodynamic or pharmacogenomic examinations. Therefore, it is unknown whether ethnic differences could have fully explained the difference in sensitivity to FN at a dose of 75 mg/m^2. The possibility that the incidence of FN in our study could have been attributable to chance also cannot be ruled out.

In summary, considering the toxicities comprehensively, docetaxel monotherapy at an initial dose of 75 mg/m^2 according to the protocol definition is considered to be feasible for Japanese patients with previously treated NSCLC. However, the administration of docetaxel at 75 mg/m^2 may increase the incidence of FN in Japanese patients. Therefore, it is highly recommended that feasibility should be confirmed beforehand when Japanese patients with NSCLC participate in global studies using docetaxel at 75 mg/m^2 in combination with other agents.

Conflict of interest statement

None declared.

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The progression of comorbidity in IL-18 transgenic chronic obstructive pulmonary disease mice model

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ABSTRACT

Patients with severe COPD are known to have comorbidities such as emaciation, cor pulmonale and right heart failure, muscle weakness, hyperlipemia, diabetes mellitus, osteoporosis, muscle atrophy, arterial sclerosis, hypertension, and depression. Therefore, treatment for COPD needs to focus on these comorbidities as well as the lungs. We previously reported a new mouse model of COPD utilizing the human surfactant protein C promoter SP-C to drive the expression of mature mouse IL-18 cDNA; constitutive IL-18 overproduction in the lungs of transgenic (Tg) mice induces severe emphysematous change, dilatation of the right ventricle, and mild pulmonary hypertension with aging. In the present study, we evaluated the progression of comorbidity in our COPD model. In female Tg mice, significant weight loss was observed at 16 weeks and beyond, when compared with control wild-type (WT) mice. This weight loss was suppressed in IL-13-deficient (knockout; KO) Tg mice. Muscle weight and bone mineral density were significantly decreased in aged Tg mice relative to control WT and IL-13 KO Tg mice. The aged Tg mice also showed impaired glucose tolerance. IL-18 and IL-13 may play important roles in the pathogenesis of comorbidity in COPD patients.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is an important pulmonary inflammatory disease whose prevalence and associated mortality rates have been increasing [1,2]. COPD often coexists with other diseases (comorbidities) that may have a significant impact on prognosis. Comorbidities include cardiovascular disease (CVD) (such as ischemic heart disease, heart failure, hypertension, and pulmonary hypertension), osteoporosis, diabetes, infections, and lung cancer, and are common at any severity of COPD, so that differential diagnosis can often be difficult (see review [3]). It is thought that several common genetic or constitutional factors may predispose individuals with COPD to both pulmonary and systemic inflammation [4].

The proinflammatory cytokines IL-1, IL-18, IL-33, IL-36, IL-37, and IL-38 belong to the IL-1 family [5]. IL-18 is well known to play an important role in Th1 polarization, and can also act as a co-factor for Th2 cell development and IgE production [6–9]. IL-18 has been reported to take part in the differentiation of Th17 cells by amplifying IL-17 production by polarized Th17 cells in synergy with IL-23 [10]. IL-18 plays important roles in the pathogenesis of inflammatory diseases such as atopic dermatitis [11], rheumatoid arthritis (RA), adult-onset Still's disease, Sjögren's syndrome, and inflammatory bowel diseases including Crohn's disease [see review [6]]. IL-18 is also involved in the development of lung diseases including lung injury [12,13] and idiopathic pulmonary fibrosis (IPF) [14]. It has been shown that IL-18 and its receptor are involved in the pathogenesis of COPD [15–17]. Previously, we established a new animal model of COPD in which constitutive overproduction of mature IL-18 protein in the lungs of transgenic (Tg) mice resulted in severe emphysema accompanied by pulmonary inflammation. IL-13 gene deletion resulted in suppression of emphysema and inflammation in the IL-18 Tg mice [18]. In the present study, we evaluated comorbidity (in terms of body and muscle weight, bone mineral density, and glucose tolerance) and the roles of IL-13 in our COPD mouse model.

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2. Materials and methods

2.1. Lung-specific IL-18-transgenic (Tg) mice

We used female IL-18 Tg mice with a C57BL/6N (B6) background in which mature mouse IL-18 was overproduced in the lungs under the control of the human surfactant protein (SP) C promoter [18]. We established B6 background IL-13 deficient (knockout; KO) IL-18 Tg (IL-13KO/IL-18 Tg) mice by backcrossing IL-18 Tg mouse line A with B6 IL-13 KO mice, as reported previously [19]. Age-matched female B6 wild-type (WT) mice, purchased from Charles River Japan (Yokohama, Japan), were used as controls. All procedures were approved by the Committee on the Ethics of Animal Experiments, Kurume University (Approval No. H22-079-084). Animal care was provided in accordance with the procedures outlined in the "Principle of laboratory animal care" (National Institutes of Health Publication No. 86-23, revised 1985).

2.2. Histological examinations

For the histological analysis, mice were sacrificed with an intraperitoneal injection of sodium pentobarbital (2.5–5 mg per mouse). After gross examination, the extracted tissues were placed in 10% buffered formalin and further fixed for at least 24 h. Sections (4 μm thick) were cut from paraffin-embedded tissues, placed on poly-L-lysine-coated slides, and then incubated overnight at 55–60 °C. Deparaffinized sections were stained with hematoxylin and eosin (HE), as reported previously [12,20,21].

2.3. Measurement of bone mineral density

Mice aged 24 weeks were sacrificed, and the right thighbone of each was extirpated and cut into 20 slices 1 mm thick. Bone mineral density was analyzed by the DEXA (dual-energy X-ray absorptiometry) method using a DCS-600EX-IIIR instrument (Aloka Corporation, Tokyo, Japan). The weight and surface area of each slice was measured, and the bone density (weight/surface area) calculated [22].

2.4. Glucose tolerance test

Glucose tolerance tests were performed as reported previously [23]. Briefly, a dose of glucose (1 g/kg) was administered by intraperitoneal (i.p.) injection, and the blood glucose level was measured at 0, 30, 60 and 120 min after the injection.

2.5. Statistical analyses

Results are expressed as means ± standard error of the mean (SEM). ANOVA was used to compare differences between groups. The SAS 9.1.3 software package, Japanese edition (SAS Institute, Cary, NC, USA), was used for statistical analysis. $P < 0.05$ was considered to represent statistical significance.

3. Results

3.1. Aging-related body weight loss in IL-18 Tg mice

We examined the body weight of female WT, IL-13 KO/IL-18 Tg, and IL-18 Tg mice every week from 7 to 24 weeks after birth ($n = 4$–5 in each group). Representative results are shown in Fig. 1. In WT, IL-13 KO/IL-18 Tg, and IL-18 Tg mice, body weight increased until 15 weeks of age. There was no significant difference in body weight among the groups until that time. From 16 to 24 weeks, body weight decreased significantly in IL-13 KO/IL-18 Tg and IL-18 Tg mice, when compared to WT mice. Interestingly, from 22 to 24 weeks, IL-18 Tg mice were significantly lighter than IL-13KO/IL-18 Tg mice. These results showed that weight loss was suppressed in IL-13KO/IL-18 Tg mice.

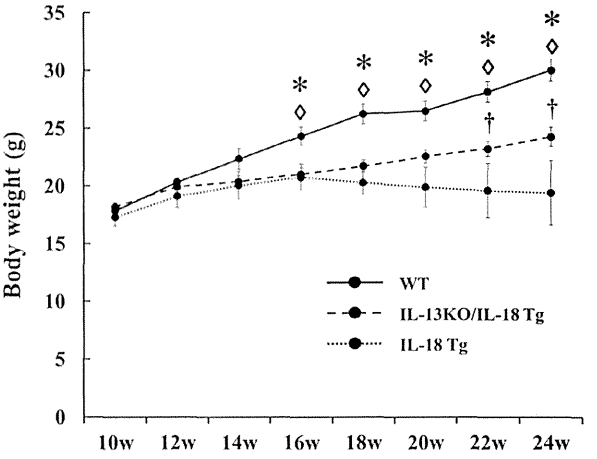

Fig. 1. Aging-related decrease of body weight in IL-18-transgenic (Tg) mice. We examined the body weight of female WT, IL-13 KO/IL-18 Tg, and IL-18 Tg mice every week from 7 to 24 weeks from birth ($n = 4$–5 in each group). $^{*}P < 0.05$: WT mice vs. IL-18 Tg mice. $^{\diamond}P < 0.05$: WT mice vs. IL-13KO/IL-18 Tg mice. $^{\dagger}P < 0.05$: IL-18 Tg mice vs. IL-13KO/IL-18 Tg mice.

3.2. Decrease of quadriceps femoris and gastrocnemius muscle weight in IL-18 Tg mice

At 7 week of age, the quadriceps femoris muscle of WT mice was significantly heavier than that in IL-18 Tg and IL-13 KO/IL-18 Tg mice, although body weight did not differ significantly among the three groups (Fig. 1). There was no significant difference in muscle weight between IL-18 Tg and IL-13 KO/IL-18 Tg mice. At 16 weeks, the quadriceps femoris in WT mice was also significantly heavier than in IL-18 Tg and IL-13KO/IL-18 Tg mice, and was significantly heavier in IL-13KO/IL-18 Tg mice than in IL-18 Tg mice. Interestingly, at 25 weeks, the quadriceps femoris was significantly heavier in WT and IL-13KO/ IL-18 Tg mice than in IL-18 Tg mice. There was no significant difference in quadriceps femoris weight between WT and IL-13KO/IL-18 Tg mice (Fig. 2).

At 7 weeks of age, the gastrocnemius muscle was significantly heavier in WT mice than in IL-18 Tg and IL-13KO/IL-18 Tg mice, but there was no significant difference in the weight of this muscle between IL-18 Tg and IL-13KO/IL-18 Tg mice. At 16 weeks and 25 weeks, the gastrocnemius was significantly heavier in WT and IL-13KO/IL-18 Tg mice than in IL-18 Tg mice, but showed no significant difference between WT and IL-13KO/IL-18 Tg mice (Fig. 2).

3.3. Decrease of bone mineral density in aged IL-18 Tg mice

Next, we examined bone mineral density in mice at 24 weeks of age. That in IL-18 Tg mice was significantly decreased in comparison with WT and IL-13KO/IL-18 Tg mice. However, there was no significant difference in bone mineral density between WT and IL-13KO/IL-18 Tg mice (Fig. 3).

3.4. Impaired glucose tolerance in aged IL-18 Tg mice

We examined glucose tolerance in mice at 20 weeks of age. At 30 and 60 min after glucose administration, blood glucose levels showed no significant difference in IL-18 Tg and WT mice. However, at 120 min after glucose administration, blood glucose levels were significantly higher in IL-18 Tg mice than in WT mice (Fig. 4).

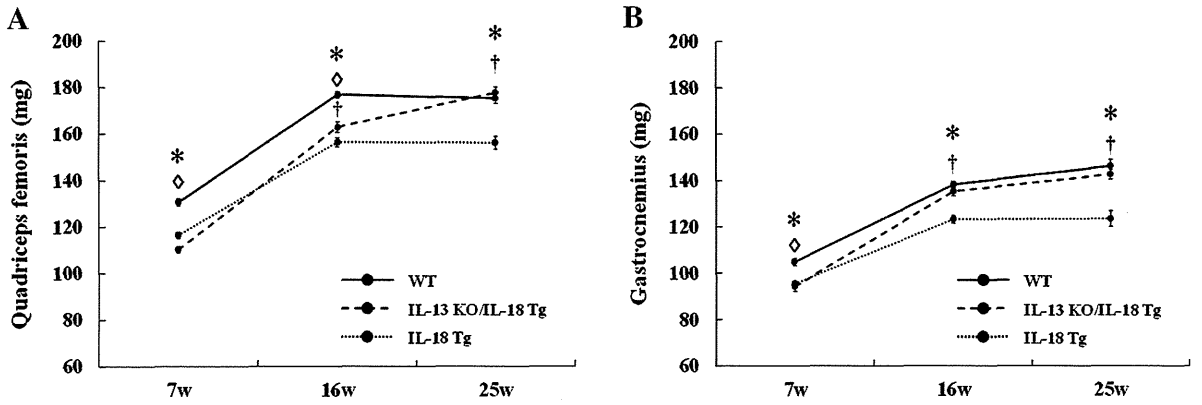

Fig. 2. Decrease in the weight of the quadriceps femoris and gastrocnemius muscles in IL-18 Tg mice. Mice ($n = 12$ each group) were sacrificed at 7, 16, and 25 week after birth, and the weights of the quadriceps femoris and gastrocnemius muscles were measured. (A) quadriceps femoris and (B) gastrocnemius. *$P < 0.05$: WT mice vs. IL-18 Tg mice. $^{\diamond}P < 0.05$: WT mice vs. IL-13KO/IL-18 Tg mice. $^{\dagger}P < 0.05$: IL-18 Tg mice vs. IL-13KO/IL-18 Tg mice.

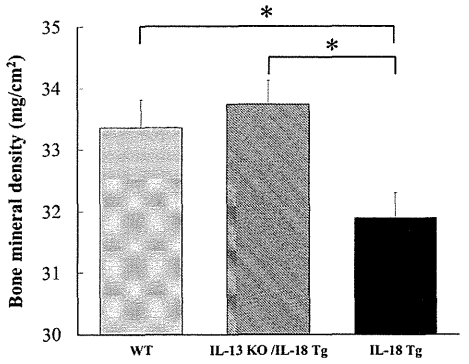

Fig. 3. Decrease of bone mineral density in aged IL-18 Tg mice. Mice were sacrificed at 24 weeks of age (WT: $n = 7$, IL-18 Tg: $n = 7$, IL-13 KO/IL-18 Tg: $n = 8$). Bone mineral density was measured as described in Section 2. *$P < 0.05$.

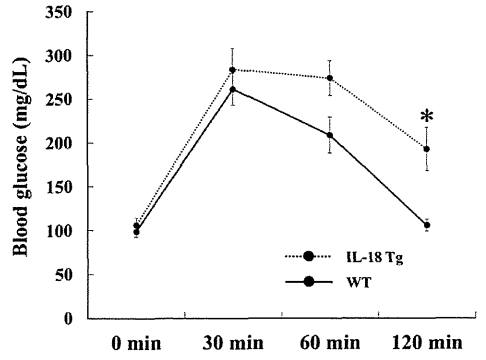

Fig. 4. Impaired glucose tolerance in aged IL-18 Tg mice. Glucose tolerance tests were performed in mice at 20 weeks of age (WT: $n = 5$, IL-18 Tg: $n = 5$), as described in Section 2. *$P < 0.05$ vs. WT mice.

4. Discussion

COPD is characterized by an intense inflammatory process in the airways, parenchyma, and pulmonary vasculature. COPD is also associated with systemic inflammation [3,24]. For instance, the presence of systemic inflammation in COPD has been linked with a variety of complications including weight loss [25–27], cachexia [28,24], osteoporosis [29–31], cardiovascular disease [32–34], diabetes mellitus [35,36], sleep disorder and depression [37,38]. It is has been reported that inflammatory cytokines including TNF-α and IFN-γ may be involved in systemic inflammation in COPD [3]. However, the precise mechanisms of systemic inflammation in severe COPD are still uncertain. We showed previously that IL-18 was overexpressed in the lungs and serum of patients with very severe COPD [15]. Therefore, inflammatory cytokines including IL-18, TNF-α, and IFN-γ overexpressed in lung tissues may "spill" over into the systemic circulation, promoting a generalized inflammatory reaction in COPD.

We previously reported that constitutive overproduction of IL-18 in the lungs resulted in increased production of both Th1 and Th2 cytokines (including IFN-γ and IL-13), emphysematous changes, and severe pulmonary inflammation in the lungs of mice [18]. It has been reported that IL-18 produced by osteoblasts is a powerful osteoclast-inhibitor [39]. Both IL-18 and IL-12 reduce the absorptive activity of osteoclasts through the production of IFN-γ [40]. Our present results showed that bone mineral density in IL-18 Tg mice was significantly decreased in comparison with WT and IL-13-deficient Tg mice, suggesting that IL-18 may inhibit the activities of osteoclasts partly through the production of IL-13 in IL-18 Tg mice.

It has been reported that IL-18 mRNA was highly expressed in biopsy samples of skeletal muscle from COPD patients, relative to those from healthy controls [41]. There is some evidence that patients with COPD have increased skeletal muscle apoptosis [42]. Previous studies have shown that IL-18 causes apoptosis in various cell types including muscle cells and lymphocytes [43]. Here we showed that the weight of the quadriceps and gastrocnemius muscles was significantly decreased in aged IL-18 Tg mice, when compared with control WT and IL-13-deficient Tg mice, suggesting that overexpression of IL-18 may induce apoptosis in muscle cells via IL-13. However, we were unable to detect apoptotic muscle cells to any significant degree in either the quadriceps of the gastrocnemius (data not shown). Further analysis will be needed to verify this issue.

It has been reported that 47% of COPD patients present 3 or more determinants of metabolic syndrome, including cardiovascular disease, diabetes mellitus, hyperlipidemia, and hypertension [35]. Expression of IL-18 is increased in the retinas of diabetic OLETF rats, a model of type 2 diabetes mellitus, and chronic hyperglycemia accelerates the release of IL-18 and IFN-γ from inflammatory cells [44]. It is widely believed that IL-18 can exacerbate type 2 diabetes mellitus and cardiovascular disease [45]. Therefore, overexpression of IL-18 may induce hyperglycemia in patients with very severe COPD.

Our present findings suggest that IL-18 and IL-13 may play important roles in the pathogenesis of comorbidity in COPD

patients, and raise the possibility that blockade of IL-18 may be a feasible treatment for COPD. Caspase-1 inhibitors, antibodies against IL-18 and its receptor, IL-18 binding protein, or inhibitors of genes downstream of the IL-18 signal transduction pathway, such as those encoding MyD88, IL-1 receptor associated kinase, tumor necrosis factor receptor-associated factor 6, nuclear factor-kB, C-jun N-terminal kinase, and p38 mitogen-activated protein kinase, as well as IL-13 inhibitors, may be of clinical benefit in the treatment of patients with severe COPD who have comorbidities and a poor clinical prognosis.

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Original article

COPD assessment tests scores are associated with exacerbated chronic obstructive pulmonary disease in Japanese patients

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ABSTRACT

Background: Guidelines recommend chronic obstructive pulmonary disease (COPD) assessment tests (CATs) for evaluation of symptoms and management risks. To investigate whether CAT can predict moderate or severe exacerbations in Japanese COPD patients, a single-blinded prospective study was performed.

Methods: A 123 Japanese COPD patients were classified into high-CAT ($n=64$) and low-CAT ($n=59$) groups. The frequencies and periods of moderate or severe exacerbation and hospitalization were compared between the two groups. Multivariate logistic regression analysis was performed to investigate whether CAT could predict exacerbations. A receiver operating characteristic (ROC) curve analysis was employed to find an appropriate CAT score for exacerbation.

Results: The high-CAT group was significantly older, had a lower body mass index, and had a lower airflow obstruction as compared to the low CAT group. The frequency of moderate or severe exacerbation (1.3 ± 1.3 events per patient per year, $p<0.0001$) and hospitalizations (0.2 ± 0.4, $p=0.0202$) in the high-CAT group was significantly higher than in the low-CAT group (0.4 ± 0.7 and 0.0 ± 0.1, respectively). Multivariate logistic regression analysis showed that both high CAT score and low airflow obstruction were independently predictive of frequent moderate or severe COPD exacerbation. ROC analysis showed that the best cut-off CAT score for moderate or severe COPD exacerbation was 8 points.

Conclusion: Our present results indicate that COPD Japanese patients showing high CAT scores have a poor prognosis, and that the CAT score is able to predict exacerbation in Japanese COPD.

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1. Introduction

Exacerbation of chronic obstructive pulmonary disease (COPD) is associated with poorer health-related quality of life (HRQOL), hospitalization, and mortality [1–4]. HRQOL is an independent predictor of exacerbation and hospitalization in patients with COPD [5,6], and is associated with the severity of symptoms including chronic cough, sputum production, and dyspnea [7,8]. Chronic cough and sputum production in COPD patients are associated with progressive airflow obstruction, frequent exacerbation and hospitalization, and mortality [9–11]. Dyspnea is also a factor predictive of mortality in such patients [12–14]. Previous studies have demonstrated that evaluation of HRQOL using St. George's Respiratory Questionnaire (SGRQ) is useful for prognostication [4,15].

The COPD assessment test (CAT) has been shown to be a useful and readily applicable tool for optimization of the HRQOL in patients with COPD [16]. It is known that there is a good correlation between CAT score and total SGRQ score in patients with stable COPD, and that CAT evaluation can indicate the severity of exacerbation in progressive COPD [17]. Recently, we validated the Japanese version of the CAT for Japanese COPD patients [18]. However, it is still unknown whether the CAT score can be used as an indicator for prediction of exacerbation and hospitalization. Thus, the primary endpoint of the present single-blinded (investigator-blinded), prospective observation study was to investigate whether the CAT could be used to predict outcome in Japanese patients with COPD.

2. Materials and methods

2.1. Patients

Patients with COPD who had regularly attended each participating hospital for at least one year between September 2011 and August 2013 at the Chest Disease Center of Kurume University Hospital (Kurume, Japan), the Chikugo City Hospital (Chikugo, Japan), and Nagata Hospital (Yanagawa, Japan) were enrolled in this study. However, patients were excluded if they had a main diagnosis of bronchiectasis, asthma, interstitial pneumonia and pneumoconiosis based on medical history and chest high-resolution computed tomography (HRCT); active malignancies; and severe diseases of other organs such as dementia, cerebro- or cardio-vascular disease, hepatitis and cirrhosis, chronic kidney disease, and psychological disease. All patients were Japanese.

2.2. Study design

Each patient had been in stable condition with no history of exacerbation while receiving systemic antibiotics and corticosteroids, or had been hospitalized, for 4 weeks prior to study entry. Demographic data included body mass index, details of smoking habits, smoking index (packs per year), comorbidities, previous exacerbations and hospitalizations during the previous year, and medications were collected at baseline. Other baseline data included chest X-ray, chest HRCT, electrocardiography, and spirometry. The total CAT scores [16] for the previous 2 weeks were obtained from each patient through self-completed reports. For final analysis, the reports were kept in a designated box by special nurses and technicians in an investigator (single)-blinded manner.

The diagnosis of COPD was based on forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC)<0.7 after bronchodilator administration, and the classification of airflow obstruction after bronchodilator administration in COPD was in accordance with the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines 2009 [19]. Spirometry was performed in accordance with the American Thoracic Society (ATS)/European Respiratory Society (ERS) task forces [20].

This prospective observation study was performed for one year after consent was obtained. Each patient self-reported his/her own condition in a daily journal and visited the chief physician monthly. Each physician then entered the monthly conditions, contents and periods of medication including treatments initiated for exacerbation, incidence of death, pulse oximetry oxygen status, and causes of death, hospitalization, or exacerbations, into the medical records. Exacerbation was defined on the basis of symptom-based diagnosis such as increased cough and sputum production, a change of sputum color, and worsening of dyspnea from a stable state and beyond-normal day-to-day variations, i.e., showing acute onset and necessitating a change in regular medication, in accordance with a previous report [21]. Moderate exacerbations required a prescription for antibiotics and/or systemic corticosteroids, and severe exacerbations required hospitalization [22]. COPD-related death was also counted as severe exacerbation. Mortality was also investigated for one year. The frequency of moderate or severe exacerbation and hospitalization due to

Abbreviations: ATS, American Thoracic Society; BMI, body mass index; CAT, COPD assessment test; COPD, chronic obstructive pulmonary disease; ERS, European Respiratory Society; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease; HRCT, high-resolution computed tomography; HRQOL, health-related quality of life; ICS, inhaled corticosteroid; LABA, long-acting beta$_2$ agonist; LAMA, long-acting muscarinic receptor antagonist; SD, standard deviation; SGRQ, St. George's Respiratory Questionnaire; $\%FEV_1$, percentage of predicted forced expiratory volume in 1 s; %FVC, percentage of predicted forced vital capacity

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severe COPD exacerbation per patient per year and the time until a first moderate or severe exacerbation and hospitalization from the baseline were obtained from each medical record and daily journal. The contents of the prescriptions did not change during the period of observation.

The study was conducted in accordance with the Good Clinical Practice guidelines and approved by the Ethics Committee of Kurume University and Chikugo City Hospital (Approval date: September 2011; Approved #: 11127). All of the study patients provided informed written consent.

2.3. Statistical analysis

An intention-to-treat analysis was performed in the study. All data were expressed as mean±standard deviation (SD). Characteristics of COPD patients with low (<10 points) and high CAT (>10 points) scores in accordance with GOLD documents [19] at baseline were compared using Student's t-test. Differences in qualitative variables (e.g., gender, smoking status, comorbidities, GOLD classification, and treatments) were analyzed by χ^2 test. The period until the first moderate or severe COPD exacerbation and hospitalization after obtaining informed consent was analyzed using logistic multivariate regression tests. The odds ratio and 95% confidence interval (CI) of the predictive risk factors for moderate or severe COPD exacerbations and hospitalizations were analyzed by univariable and multivariable tests. The best sensitivity, specificity [sensitivity–(1–specificity)] and area under the receiver operating characteristic (ROC) curve (AUC) for the CAT score indicative of annual at least one moderate or severe COPD exacerbations and hospitalizations were determined by ROC analysis. Differences at $p<0.05$ were considered statistically significant. Kaplan–Meier analyses were performed using the statistical software package JMP version 9.0® (SAS Institute Japan Inc., Tokyo, Japan). However, hospitalizations except for COPD exacerbations were not counted in the analysis.

3. Results

3.1. Patient characteristics

In all, 154 patients with COPD provided informed consent, of which 139 patients were enrolled for this study (Fig. 1).

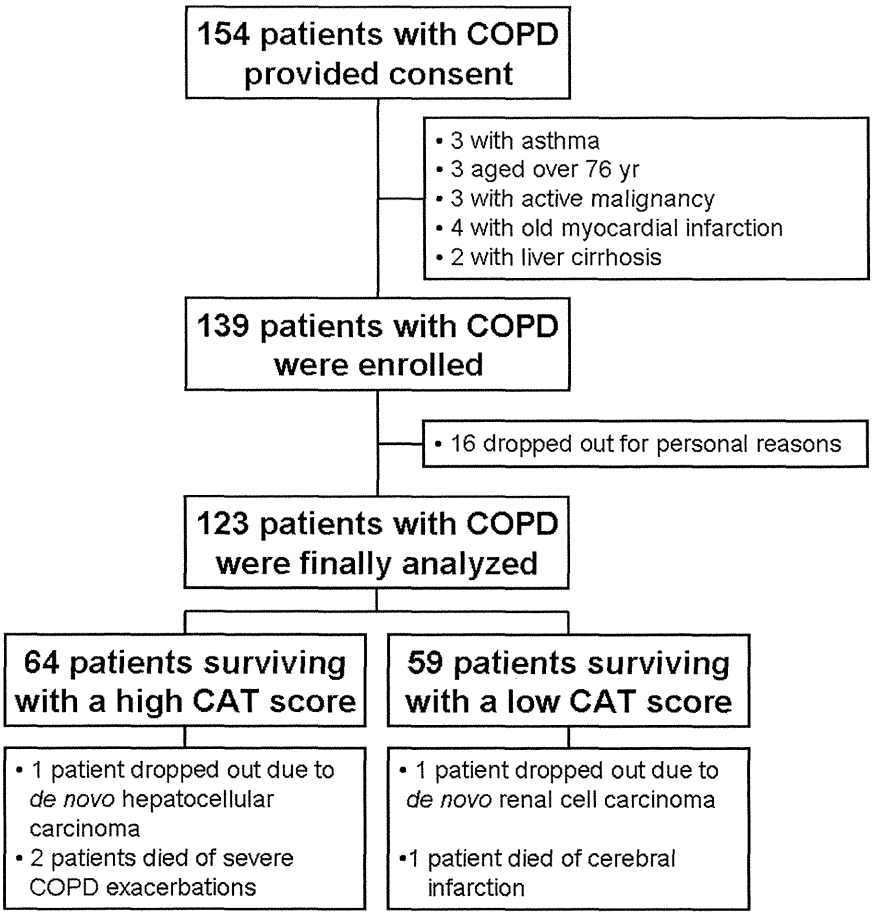

Fig. 1 – Study design. Informed consent was obtained from 154 patients with COPD. However, three patients with asthma were excluded. Three patients aged 76 years and older were also excluded. Three patients with active malignancy required anti-cancer drugs, 4 with old myocardial infarction, and 2 with liver cirrhosis due to hepatitis C virus infection were also excluded because of severe disease involving organs other than the lungs. Thus, 139 patients with COPD were enrolled. However, 16 patients for personal reasons and 2 who had de novo active malignancies were dropped out within 1 year after enrollment. CAT, COPD assessment test; COPD, chronic obstructive pulmonary disease.

Sixteen patients were excluded from the final analysis because of withdrawal of consent for personal reasons during the observation period. Thus, 123 patients were included in the final analysis. Two patients were dropped out due to de novo malignancies. Two patients with a high CAT score died due to severe COPD exacerbations whereas a patient with a low CAT score died due to cerebral infarction without COPD exacerbation during the observation period (Fig. 1).

The baseline characteristics of the 123 patients are shown in Table 1. The numbers of GOLD stages I, II, III, and IV patients were 29, 57, 28, and 9, respectively. The respiratory medicines [long-acting muscarinic antagonists (LAMA), long-acting β_2 agonists (LABA), and/or inhaled corticosteroids (ICS)] were prescribed for 104 (84.6%) of the patients. Eighteen (14.6%) of the patients had inoculated the pneumococcal vaccination within 5 years prior to providing consent. All patients required influenza virus vaccinations.

In a comparison between the high- and low-CAT groups, the high-CAT group was significantly older ($p<0.0001$), had a lower body mass index (BMI) ($p<0.0001$), had a lower %FEV_1 predicted value ($p=0.006$) and FEV_1/FVC ($p=0.005$), and had a severe COPD stage ($p=0.035$) than the low-CAT group (Table 1). The number of patients who received LABA ($p=0.011$) and ICS ($p=0.015$) in the high-CAT group was significantly higher than in the low-CAT group, whereas there was no significant difference in the number of patients who received LAMA between the two groups. The numbers of the patients who received long-term oxygen therapy in high- and low-CAT groups were 3 and 1, respectively. None of the patients received noninvasive positive pressure ventilation at home.

3.2. *Association of CAT with annual exacerbation and hospitalization*

According to GOLD 2011 guidelines [19], we found that the high-CAT group had significantly higher annual frequencies of moderate or severe exacerbations (1.3±1.3 events per patient, $p<0.0001$) and hospitalizations (0.2±0.4 events per patient, $p=0.0202$) than the low-CAT group (0.4±0.7 and 0.0±0.1, respectively) (Fig. 2).

The Kaplan–Meier analysis showed that the high-CAT group had a significantly shorter time until the first moderate

Table 1 – Baseline characteristics of the study patients.

Characteristics	High-CAT group ($n=64$)	Low-CAT group ($n=59$)	p Value
Total CAT score,[a] points	17.9±8.0	4.9±3.0	<0.0001
Age, year	69.4±5.3	65.1±6.1	<0.0001
Male gender,[b] n (%)	54 (84.4)	53 (89.8)	0.4
Body mass index, kg/m^2	21.3±3.5	22.9±3.1	0.008
Current smoker,[b] n (%)	23 (35.9)	17 (28.8)	0.4
Smoking index, packs per year	60.1±25.4	52.2±27.8	0.1
Hypertension,[b] n (%)	10 (15.6)	8 (13.6)	0.8
Hyperlipidemia,[b] n (%)	3 (4.7)	3 (5.1)	1.0
Diabetes,[b] n (%)	17 (26.6)	16 (27.1)	1.0
Periods since COPD diagnosis,[a] year	6.3±4.1	4.9±4.0	0.06
Spirometry after bronchodilation			
FVC, L	3.3±0.8	3.8±0.8	0.005
%FVC predicted value, %	96.1±19.4	101.7±17.9	0.1
FEV_1, L	1.6±0.7	2.0±0.7	0.0004
%FEV_1 predicted value, %	58.2±22.0	69.2±21.3	0.006
FEV_1/FVC, %	46.0±13.8	52.9±13.0	0.005
GOLD stage I/II/III/IV,[b] n	10/31/15/8	19/26/13/1	0.035
Use of respiratory medicine,[c] n (%)			
Any	55 (85.9)	49 (83.1)	0.8
Long-acting beta agonists	21 (33.8)	7 (11.9)	0.011
Long-acting muscarinic agonists	49 (76.6)	48 (81.4)	0.7
Inhaled corticosteroids	14 (21.9)	3 (5.1)	0.015
Previous pneumococcal vaccination within 5 years, n (%)	13 (20.3)	5 (8.5)	0.1

All data are expressed as mean±standard deviation and compared between two groups using Student's t-test.
CAT, COPD assessment test; COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 s; FVC, forced expiratory capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease.
[a] Data were compared between groups by non-parametric Wilcoxon test.
[b] Data were compared between groups using χ^2 test.
[c] This agent was used either alone or as a fixed combination.

or severe exacerbation ($p<0.0001$), but not hospitalization ($p=0.064$), than the low-CAT group (Fig. 3).

3.3. *High CAT score as an independent predictor of exacerbation, but not hospitalization*

In univariable analysis for moderate or severe COPD exacerbation and hospitalization (Table 2), the odds ratio (95% CI) of the patients who had age >65 years, total CAT score >10 points, and GOLD stages III and IV, with at least one moderate or severe COPD exacerbation were 3.0 (1.4–6.3) ($p=0.006$), 5.1 (2.4–11.1) ($p<0.0001$), and 5.8 (2.4–13.9) ($p<0.0001$), respectively. The odds ratio (95% CI) of the patients who had age >65 years, total CAT score >10 points, and GOLD stages III and IV, with at least one hospitalization were 2.9 (0.8–10.7) ($p>0.05$), 4.3 (1.2–16.1) ($p=0.027$), and 4.3 (1.4–13.1) ($p=0.014$), respectively.

In multivariable analysis (Table 3), the odds ratio (95% CI) of the patients who had age >65 years, total CAT score >10 points, and GOLD stages III and IV, with at least one COPD exacerbation were 1.6 (0.6–4.0) ($p>0.05$), 4.5 (1.9–11.3) ($p=0.0005$), and 5.7 (2.3–15.3) ($p=0.0001$), respectively. The odds ratio (95% CI) of the patients who had total CAT score >10 points and GOLD stages III and IV with at least one hospitalization were 3.8 (1.1–17.7) ($p=0.035$) and 3.8 (1.2–12.6) ($p=0.020$), respectively.

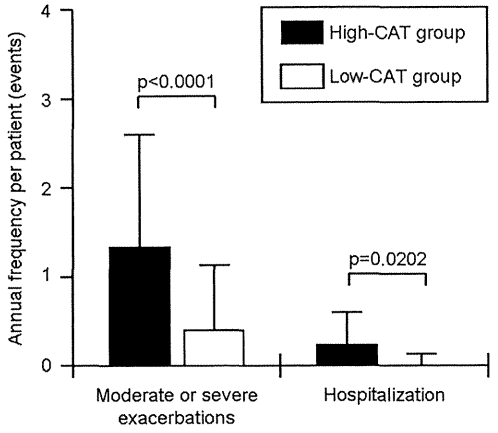

Fig. 2 – Annual frequency of exacerbation and hospitalization in COPD patients. All data are expressed as the annual frequency of moderate or severe COPD exacerbations and hospitalizations due to COPD exacerbations per patient per year (error bars=standard deviation). CAT, COPD assessment test; COPD, chronic obstructive pulmonary disease.

3.4. *Cut-off points for exacerbation and hospitalization*

The AUC of the CAT score for patients with annual moderate or severe COPD exacerbations and hospitalizations was 0.77 and 0.79, respectively (Fig. 4). The best sensitivity and specificity for moderate or severe exacerbations were 0.90 and 0.47, respectively, when the cut-off CAT score was 8 points. The best sensitivity and specificity for hospitalizations were 0.53 and 0.49, respectively, when the cut-off CAT score was 29 points.

4. Discussion

In Japanese patients with COPD, the CAT score, but not hospitalization, is an independent predictor of annual moderate or severe COPD exacerbation. GOLD 2011 guidelines [19] recommended a CAT score of 10 points or higher as one of the indicators for appropriate management of patients with COPD. The patients with severe airflow obstruction such as GOLD stages II and III also had an independent predictive risk factor of moderate or severe COPD exacerbations and hospitalizations. In this study, we found that COPD patients with a high CAT score (>10 points) were significantly older, had a lower BMI, and had a severe airflow obstruction than those with a low CAT score (<10 points), despite the use of more advantaged respiratory medicines such as LABA and ICS in

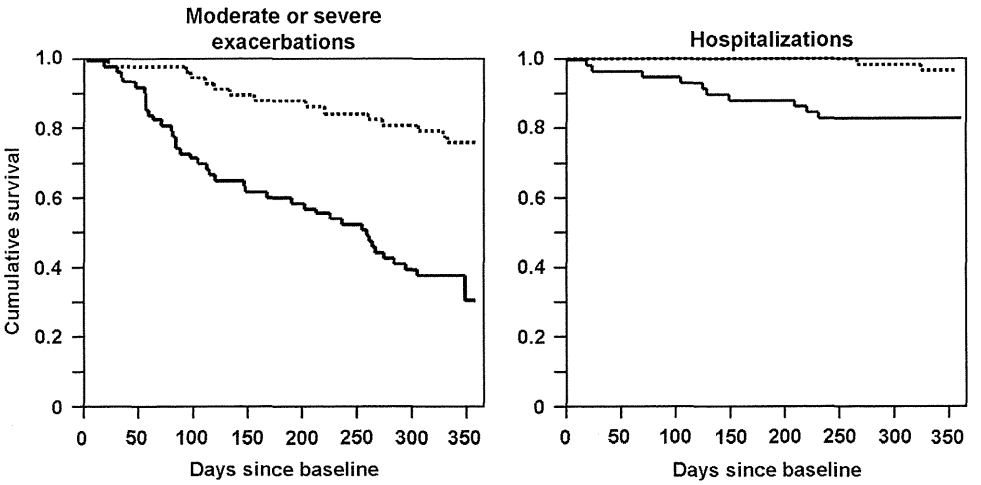

Fig. 3 – Cumulative survival curves for COPD patients with first exacerbation and hospitalization. Cumulative survival curves for patients with first moderate or severe exacerbation and hospitalization during one year. Solid line=high-CAT group; dashed line=low-CAT group. CAT, COPD assessment test; COPD, chronic obstructive pulmonary disease.

the high-CAT group compared to the low-CAT group (Table 1). Moreover, the high-CAT group had a significantly higher frequency of moderate or severe COPD exacerbations and hospitalization, and a shorter period until the next moderate or severe COPD exacerbation than the low-CAT group.

Four stratifications, namely categories A, B, C, and D, in GOLD 2011 guidelines were well defined by identifying individuals at risk of exacerbation [19,23]. Using ROC curve analysis, we sought the optimal cut-off point that would serve as a predictor of exacerbation and hospitalization, as there has been little evidence that a CAT score of 10 points is a reliable cut-off point for this purpose. We found that the best predictive cut-off points for moderate or severe COPD exacerbations and hospitalization due to COPD exacerbations were 8 and 29 points, respectively. All of the enrolled patients

Table 2 – Univariable analysis for exacerbations and hospitalizations in COPD patients.

Characteristics	Exacerbation		Odds ratio (95% CI)	*p* Value	Hospitalization		Odds ratio (95% CI)	*p* Value
	(+) n/N	(−) n/N			(+) n/N	(−) n/N		
Age, ≥ 65 years	43/58	32/65	3.0 (1.4–6.3)	0.006	12/15	63/108	2.9 (0.8–10.7)	0.2
Total CAT score ≥ 10 points	42/58	22/65	5.1 (2.4–11.1)	<0.0001	12/15	52/108	4.3 (1.2–16.1)	0.027
GOLD stage III and IV	28/58	9/65	5.8 (2.4–13.9)	<0.0001	9/15	28/108	4.3 (1.4–13.1)	0.014

The number of patients and total number of patients with at least one moderate or severe COPD exacerbation or hospitalization due to COPD exacerbations are expressed as *n* and *N*, respectively.
CAT, COPD assessment test; CI, confidence interval; COPD, chronic obstructive pulmonary disease; GOLD, Global Initiative for Chronic Obstructive Lung Disease.

Table 3 – Multivariable analysis for exacerbations and hospitalizations in COPD patients.

Characteristics	Exacerbation [odds ratio (95% CI)]	*p* Value	Hospitalization [odds ratio (95% CI)]	*p* Value
Age ≥ 65 years	1.6 (0.6–4.0)	0.3	–	–
Total CAT score ≥ 10 points	4.5 (1.9–11.3)	0.0005	3.8 (1.1–17.7)	0.035
GOLD stages III and IV	5.7 (2.3–15.3)	0.0001	3.8 (1.2–12.6)	0.020

CAT, COPD assessment test; CI, confidence interval; COPD, chronic obstructive pulmonary disease; GOLD, Global Initiative for Chronic Obstructive Lung Disease.

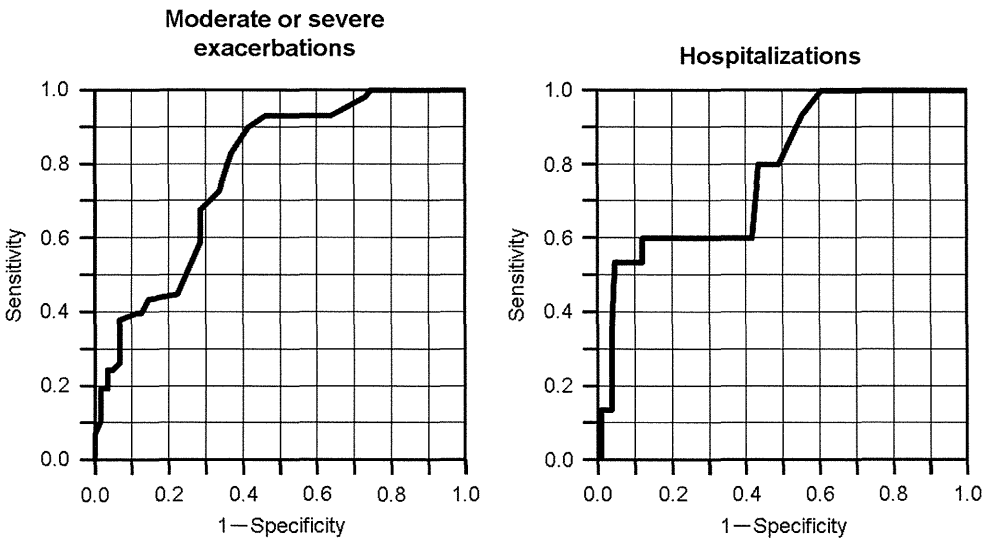

Fig. 4 – Receiver operating characteristic curves of the CAT scores for exacerbations and hospitalizations. The receiver operating characteristic curves of the CAT scores for annual moderate or severe COPD exacerbations and hospitalizations. CAT, COPD assessment test; COPD, chronic obstructive pulmonary disease.

were Japanese. Previous studies have demonstrated that the frequency of annual moderate or severe exacerbations per patient in Japanese individuals (0.54–0.61 events) may be lower than that in the United States and Europe (0.81–0.88 events) [24,25]. Japanese people have significantly lower prevalence of obesity than Westerners [26]. Japanese patients with COPD have significantly lower BMI and less frequent chronic bronchitis with chronic coughing and sputum than Westerners [24,25,27]. All patients with COPD had emphysematous areas by HRCT in our study. Hence, the discrepancy in the best CAT score cut-off point between our results and GOLD 2011 guidelines [19] for exacerbations may have been due to differences in race or ethnicity. Our findings also suggest that CAT may be more suitable for predicting exacerbation rather than hospitalization in COPD patients. Further analysis will be needed to verify this hypothesis.

The CAT was originally developed as a tool to allow communication between physicians and patients about the impact of COPD [16]. The CAT has been shown to correlate well with HRQOL measured by the SGRQ and is simpler and easier to use than the latter [17]. The CAT can also help with diagnosis of COPD, its exacerbations, and their severity [26–31]. In this study, we found that the CAT score was an independent predictor of moderate or severe COPD exacerbations, but not hospitalizations due to COPD exacerbations, and was also predictive of a shorter period until the next moderate or severe COPD exacerbation in patients with COPD. Thus, the CAT score may be useful for devising changes in interventions to prevent exacerbations, as has been reported recently [32,33]. In this study, the reproducibility of the CAT has a limitation, because the CAT score was only measured one time in each patient. Further and longer trials will be necessary to clarify the reproducibility of the CAT and to investigate whether CAT can be used as a predictor of lung function decline and mortality in patients with COPD. In addition, investigations of future risks by using classification of category ABCD should be conducted in Japanese patients with COPD.

5. Conclusion

Our present results indicate that Japanese patients with COPD showing high CAT scores have a poor prognosis and that the CAT score is a promising tool to predict exacerbation in Japanese COPD.

Statement indicating the role of each author

Dr. M. Suetomo contributed to protocol design, data collection, analysis, and writing of the manuscript.
Dr. T. Kawayama contributed to protocol design and editing of the manuscript.
Dr. T. Kinoshita contributed to data collection.
Dr. S. Takenaka contributed to data collection.
Dr. M. Matsuoka contributed to data collection.
Dr. K. Matsunaga contributed to data collection.
Dr. T. Hoshino supervised the protocol design and edited the manuscript.

Conflict of interest

Tomotaka Kawayama received lecture fees from Novartis Pharmaceuticals Japan. Tomoaki Hoshino received a grant from GSK, Japan. There are no current funding sources, nor competing financial interests, associated with this work.

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PLOS ONE

Overexpression of CD163, CD204 and CD206 on Alveolar Macrophages in the Lungs of Patients with Severe Chronic Obstructive Pulmonary Disease

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Abstract

We have previously reported that the lungs of patients with very severe chronic obstructive pulmonary disease (COPD) contain significantly higher numbers of alveolar macrophages than those of non-smokers or smokers. M1 and M2 macrophages represent pro- and anti-inflammatory populations, respectively. However, the roles of M1 and M2 alveolar macrophages in COPD remain unclear. Immunohistochemical techniques were used to examine CD163, CD204 and CD206, as M2 markers, expressed on alveolar macrophages in the lungs of patients with mild to very severe COPD (Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage I (mild) n = 11, II (moderate) n = 9, III (severe) n = 2, and IV (very severe) n = 16). Fifteen smokers and 10 non-smokers were also examined for comparison. There were significantly higher numbers of alveolar macrophages in COPD patients than in smokers and non-smokers. The numbers and percentages of $CD163^+$, $CD204^+$ or $CD206^+$ alveolar macrophages in patients with COPD at GOLD stages III and IV were significantly higher than in those at GOLD stages I and II, and those in smokers and non-smokers. In patients with COPD, there was a significant negative correlation between the number of $CD163^+$, $CD204^+$ or $CD206^+$ alveolar macrophages and the predicted forced expiratory volume in one second. Overexpression of CD163, CD204 and CD206 on lung alveolar macrophages may be involved in the pathogenesis of COPD.

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Introduction

Chronic obstructive pulmonary disease (COPD) is an important pulmonary inflammatory disease for which the prevalence and associated mortality rates have been predicted to rise. Smoking is recognized as the largest risk factor for COPD, and quitting smoking is thought to be important for prevention and control of COPD [1,2]. However, there is no effective treatment for COPD-related pulmonary inflammation. We have previously demonstrated persistent and severe inflammation of small airways in the lungs of ex-smokers with very severe COPD. Furthermore the number of macrophages in the lungs of patients with very severe COPD was increased [3]. Increased numbers of $CD8^+$ T-cells, alveolar macrophages and neutrophils are characteristic pathological features of the lungs in COPD [4,5]. However, the effects of smoking on macrophage phenotypes in COPD are incompletely understood.

Macrophages display polarized phenotypes by which they can be divided into certain subpopulations. Proinflammatory or classically activated macrophages (M1) display pro-inflammatory and cytotoxic properties and can eradicate intracellular pathogens. In contrast, anti-inflammatory or alternatively activated macrophages (M2) display anti-inflammatory properties and are implicated in tissue repair [6,7]. Granulocyte-macrophage colony stimulating factor (GM-CSF) and IFNs can generate M1 in vitro from human peripheral blood monocytes, and macrophage colony stimulating factor (M-CSF), IL-4 and IL-10 can generate M2 [8]. M1 macrophages secrete pro-inflammatory cytokines such as interleukin (IL)-12 and tumor necrosis factor (TNF)-α, have good antigen-presenting capacity, and promote Th1 immunity. In contrast, M2 macrophages secrete anti-inflammatory mediators such as IL-10, show poor antigen-presenting capacity, and promote the development of T-regulatory cells [8–10]. Alveolar macrophages show anti-inflammatory M2-characteristics [11–13], which can be distinguished from pro-inflammatory macrophages using M2 markers such as the scavenger receptors CD163 and CD204 [14,15]. Although it has been unclear which phenotype has more phagocytic activity, the phagocytic capacity of alveolar macrophages is reported to be decreased in COPD patients who smoke, whereas it improves when patients quit smoking [16]. This

Table 1. Clinical characteristics of non-smokers, smokers and chronic obstructive pulmonary disease (COPD) patients examined by immunohistochemical analysis.

	Non-smoker	Smoker	COPD
Patients	10	15	38
Age yrs	63.6±8.8	64.7±4.8	68.7±5.2
Sex			
Male	7	6	36
Female	3	9	2
GOLD stage			
I (mild)	0	0	11
II (moderate)	0	0	9
III (severe)	0	0	2
IV (very severe)	0	0	16
Smoking history			
Current	0	15	8
Ex-smoker n (mean±sem yrs since smoking cessation)	0	0	30 (4.5±0.7)
Pack-yrs	0	37.0±7.8	27.7±6.1
BMI kg/m^2	24.5±0.9	23.0±1.0	23.3±1.3
%VC	121.2±4.03	109.7±1.8	92.15±3.7
%FEV$_{1.0}$	114.5±4.3	95.6±6.0	54.8±3.2
FEV$_{1.0}$/FVC%	75.1±1.8	74.0±1.6	45.7±1.6
Treatment			
Systemic steroids	0	0	0
Inhaled corticosteroids	0	0	7
Bronchodilators	0	0	22(57.9)
No drug treatment	10(100)	15(100)	16(42.1)

Data are presented as n, n(%) or mean±SEM, unless otherwise stated. GOLD: Global Initiative for Chronic Obstructive Lung Disease; BMI: body mass index; VC: vital capacity; FVC: forced VC; % pred: % predicted; FEV$_{1.0}$: forced expiratory volume in one second.
doi:10.1371/journal.pone.0087400.t001

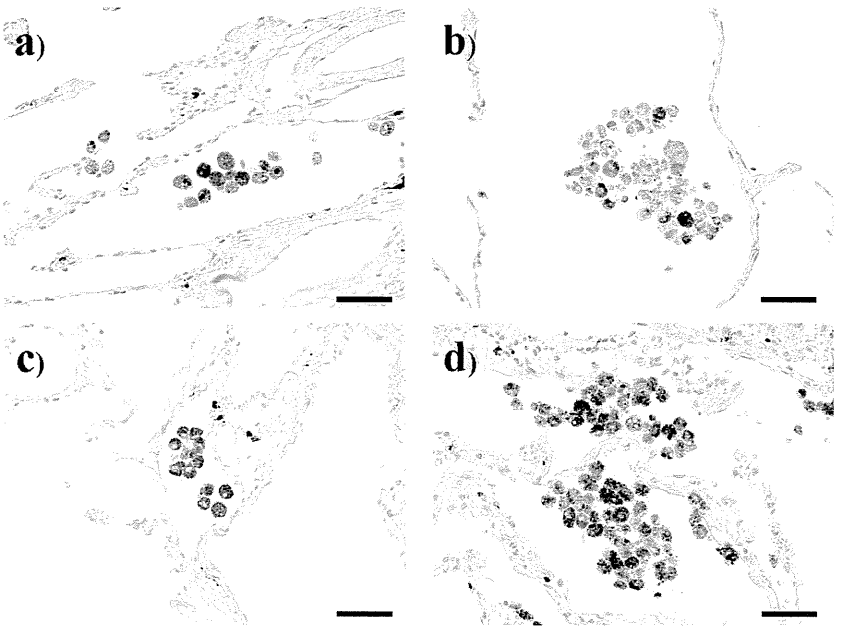

Figure 1. CD68 immunohistochemical staining of lung tissue samples from a) a non-smoker, b) a smoker, c) a mild chronic obstructive pulmonary disease patient (COPD), and d) a very severe COPD. Bar: 50 μm.
doi:10.1371/journal.pone.0087400.g001

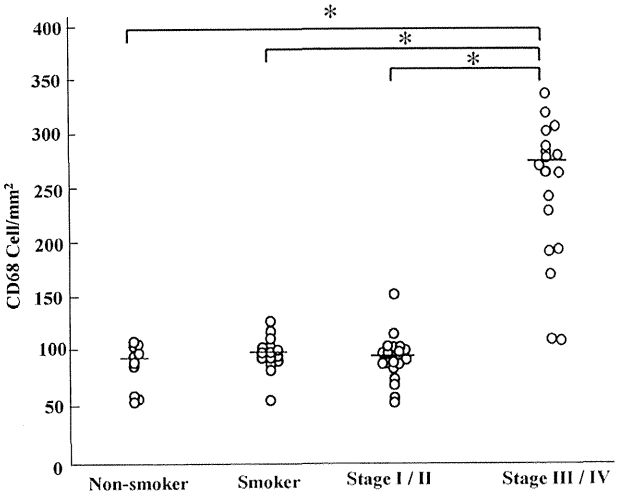

Figure 2. The number of CD68 positive cells in non-smoker, smoker and chronic obstructive pulmonary disease patient (COPD). *: $p<0.01$. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) clinical criteria for the diagnosis and severity of COPD stage I: mild, II: moderate, III: severe, IV: very severe.
doi:10.1371/journal.pone.0087400.g002

suggests that a phenotypic alteration has occurred in COPD. Phenotypic changes in macrophages are considered to be involved in progression of diseases such as tumors [17], atherosclerosis [18] and renal disease [19].

The aim of the present study was to clarify the phenotypes of macrophages in the lungs of COPD patients in order to evaluate the role of macrophages in the pulmonary function of COPD patients.

Materials and Methods

Subjects

A total of 38 COPD patients (36 males and 2 females) were monitored at Kurume University Hospital (Kurume, Japan), Fukuoka University Hospital (Fukuoka, Japan). All were diagnosed on the basis of clinical history, physical examination, chest radiography, chest computed tomography and pulmonary function tests in accordance with the Global Initiative for Chronic Obstructive Lung Disease (GOLD) clinical criteria for the diagnosis and severity of COPD [20]. Exclusion criteria included chronic lung conditions such as asthma, bronchiectasis and interstitial lung disease, cardiac, hepatic or renal failure, and current oral steroid therapy. Lung tissues were obtained from 16 patients with very severe COPD (GOLD stage IV) who had undergone lung volume reduction surgery (LVRS) at Fukuoka University Hospital. Other lung samples were obtained from normal tissues around preserved cancer specimens that had been obtained surgically from 11 patients with mild COPD (GOLD stage I), 9 patients with moderate COPD (GOLD stage II), 2 patients with severe COPD (GOLD stage III), and 10 non-smokers and 15 smokers who had undergone resection of lung cancer at Kurume University Hospital. Lung diseases (e.g. sarcoidosis, infectious diseases) and collagen vascular diseases were carefully excluded from all subjects, and ex-smokers were carefully excluded from the group of non-smokers (Table 1). Informed written consent was obtained from all subjects, and sample collection and all procedures were approved by the ethics committees of Kurume University and Fukuoka University.

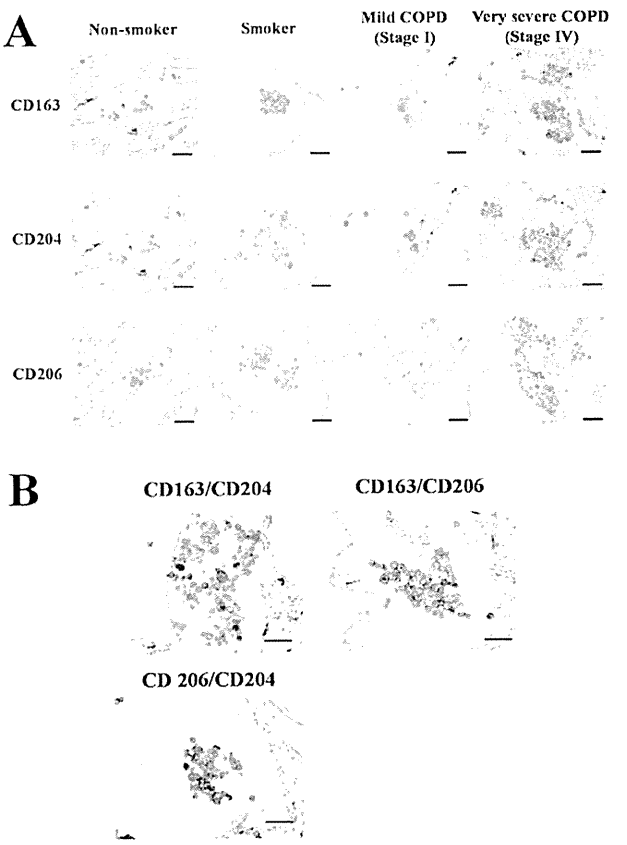

Figure 3. CD163, CD204 and CD206 immunohistochemical staining of lung tissue samples from a non-smoker, a smoker, a mild chronic obstructive pulmonary disease patient (COPD), and a very severe COPD. (A) Positive reactivity was identified by 3–3′-diaminobenzidine-4HCl (DAB). Bar: 50 μm. (B) Double immunohistochemical analysis for CD163/CD204, CD163/CD206 and CD206/CD204. Lung tissues were obtained from a very severe COPD. CD163 (brown)/CD204 (green), CD163 (brown)/CD206 (green) and CD206 (brown)/CD204 (green) were double stained with DAB (brown) and HistoGreen (green), respectively. Bar: 50 μm.
doi:10.1371/journal.pone.0087400.g003

Pulmonary Function Tests

Predicted normal Japanese values were used to calculate the vital capacity (VC), the forced vital capacity (FVC), the forced expiratory volume in one second ($FEV_{1.0}$) and each % predicted, which met the Japanese Pulmonary Function Standard in the Japanese Respiratory Society Statement [21].

Morphometric Analysis

The cross-sectional area occupied by the alveolar wall was quantified as the ratio of the total cross-sectional area, and the cross-sectional area occupied by the luminal mucosa was quantified as the ratio of the total cross-sectional area using a computer image analysis system, as reported by Hogg et al [22]. Digitized video images of the entire lung field were analyzed using a computerized color image analysis software system (Win Roof Version 5.0; Mitani Co., Fukui, Japan) as reported recently elsewhere [3,23].

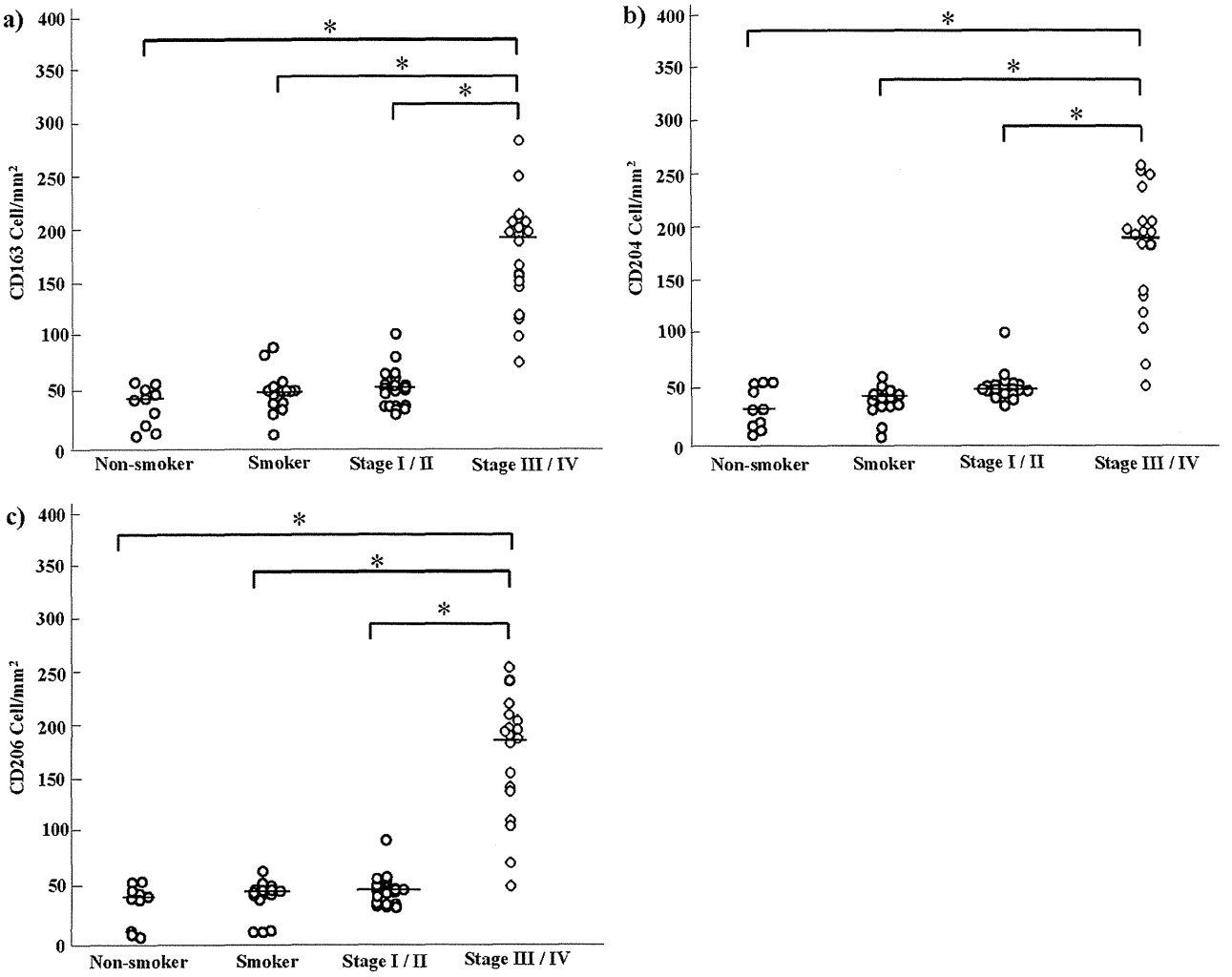

Figure 4. The number of CD163, CD204 and CD206 positive cells in non-smoker, smoker and chronic obstructive pulmonary disease patient (COPD). a) CD163, b) CD204, c) CD206, *: $p<0.01$. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) clinical criteria for the diagnosis and severity of COPD stage I: mild, II: moderate, III: severe, IV: very severe.
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Immunohistochemical Staining

For blockade of endogenous peroxidase activity, deparaffinized sections 3 μm thick were incubated with 1% H_2O_2 for 30 min. To detect macrophages, the tissues were reacted overnight at 4°C with anti-CD68 (ED1; Serotec, Oxford, UK), anti-CD163 (ED2; Serotec), anti-CD204 (SRA-E5; Transgenic, Kumamoto, Japan), anti-CD206 (Acris Antibodies, San Diego, CA), and anti-MMP-9 (Santa Cruz, Dallas, Texas). The samples were then washed extensively, and further incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After the removal of non-reacted secondary antibodies, the samples were incubated with 3,3′-diaminobenzidine-4HCl (DAB, Dako, Tokyo, Japan)-H_2O_2 solution to visualize the immunolabeling. Some sections were then counterstained with hematoxylin and eosin, and mounted with a coverslip. Double immunohistochemical analysis was performed as we previously reported [3]. CD163/CD204, CD163/CD206 and CD206/CD204 were double stained with DAB (brown) and HistoGreen (green) (Cosmobio, Tokyo, Japan), respectively.

Quantitative Assessment of $CD68^+$, $CD163^+$, $CD204^+$, and $CD206^+$ Alveolar Macrophages in Lung Tissue

Quantitative assessment of macrophages was performed as reported previously with minor modification [3]. Briefly, immunostaining of CD68, CD163, CD204, and CD206 was performed using serial sections of lung tissues obtained from COPD patients who had undergone LVRS [24,25]. Initially, nine square fields in which small-airway inflammation appeared most severe were selected. The numbers of CD68-positive cells, as alveolar macrophages (AMs), in the lung tissues were counted within these nine square fields and expressed as the number per mm^2. The total numbers of AMs in non-smokers, smokers and COPD patients were expressed as mean ± SEM cells/mm^2. Then $CD163^+$, $CD204^+$, and $CD206^+$ cells were counted in the same fields as $CD68^+$ cells. Two pathologists examined these sections independently in a blinded manner, without prior knowledge of the patients' clinical status.