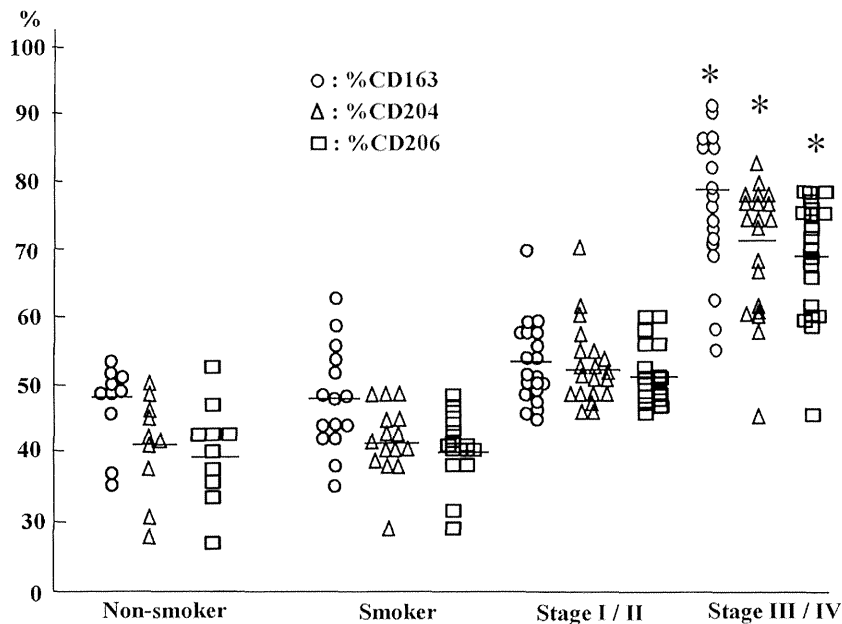

Figure 5. The percentage of CD163, CD204 and CD206 positive cell in non-smoker, smoker and chronic obstructive pulmonary disease patient (COPD). *: $p<0.01$ versus nonsmoker, smoker and stage I/II. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) clinical criteria for the diagnosis and severity of COPD stage I: mild, II: moderate, III: severe, IV: very severe.
doi:10.1371/journal.pone.0087400.g005

Statistical Analysis

Results are expressed as means ± standard error of the mean (SEM). ANOVA was used to compare differences between groups. Nonparametric tests (Kruskal–Wallis and Mann–Whitney U-tests) were used to compare differences between the groups. Correlations were analyzed by simple regression. SAS 9.1.3 software, Japanese edition (SAS Institute, Cary, NC, USA), was used for statistical analysis. Differences at $p<0.05$ were considered to be statistically significant.

Results

Clinical Findings

Table 1 provides details (number, age, sex, GOLD stage, smoking history, body mass index, pulmonary function and treatment) of all individuals whose samples were subjected to immunohistochemical staining. All of the COPD patients who had undergone LVRS had stopped smoking 2–21years previously (mean 8.9±1.8 yr). Three COPD patients who had undergone LVRS had received inhaled beclomethasone dipropionate 400 μg/day and three were receiving 800 μg/day. Twenty-two COPD patients were receiving bronchodilators, such as β_2-agonists, anticholinergics and/or methylxanthines. All the COPD patients analyzed were clinically stable and had experienced no disease exacerbations during the previous 3 months. We have analyzed the data to determine if there are any correlations between the number of alveolar macrophages and rate of exacerbation, infections and hospitalization. However there were no statistically significant correlations between those groups.

Increased Number of CD68$^+$ Alveolar Macrophages in Stage III/IV COPD Patients

First, immunostaining for CD68 was performed using pathological sections to examine the distribution of all alveolar macrophages. As shown in Figs. 1 and 2, although CD68 staining intensity did not differ between non-smokers and smokers, or between different stages, the number of CD68$^+$ alveolar macrophages was increased among stage III and stage IV patients (stage III/IV). Since there were no significant differences between the number of stage III and stage IV patients, and between the number of stage I and stage II patients, the patients with COPD were divided into two groups: stage I/II, and stage III/IV.

Increased Numbers of CD163$^+$, CD204$^+$, and CD206$^+$ Alveolar Macrophages in Stage III/IV COPD Patients

It is well known that CD163, CD204, and CD206 are specifically expressed on macrophages and are useful as M2 macrophage markers. Therefore we performed immunostaining of CD163, CD204, and CD206 using serial sections. Similarly to the number of CD68$^+$ macrophages, the numbers of macrophages positive for CD163, CD204, and CD206 were significantly increased in stage III/IV patients (Figs. 3 and 4). Double immunohistochemical analysis revealed that as shown CD163, CD204, and CD206 positive cells in stage III/IV COPD were increased (Fig. 3B).

Increased Percentages of Cells Positive for CD163, CD204 or CD206 in the Lungs of COPD Patients Relative to Those in Non-smokers and Smokers

We then calculated the percentages of CD163$^+$, CD204$^+$, and CD206$^+$ cells among CD68$^+$ alveolar macrophages. CD163$^+$, CD204$^+$ and CD206$^+$ cells accounted for approximately 40–45% of alveolar macrophages in the lungs of non-smokers and smokers, whereas they accounted for 80–70% of alveolar macrophages in patients with stage III/IV COPD. There were no significant differences in the percentages of CD163$^+$, CD204$^+$ or CD206$^+$ cells between non-smokers and smokers or between non-smokers or smokers and COPD stage I/II patients (Fig. 5).

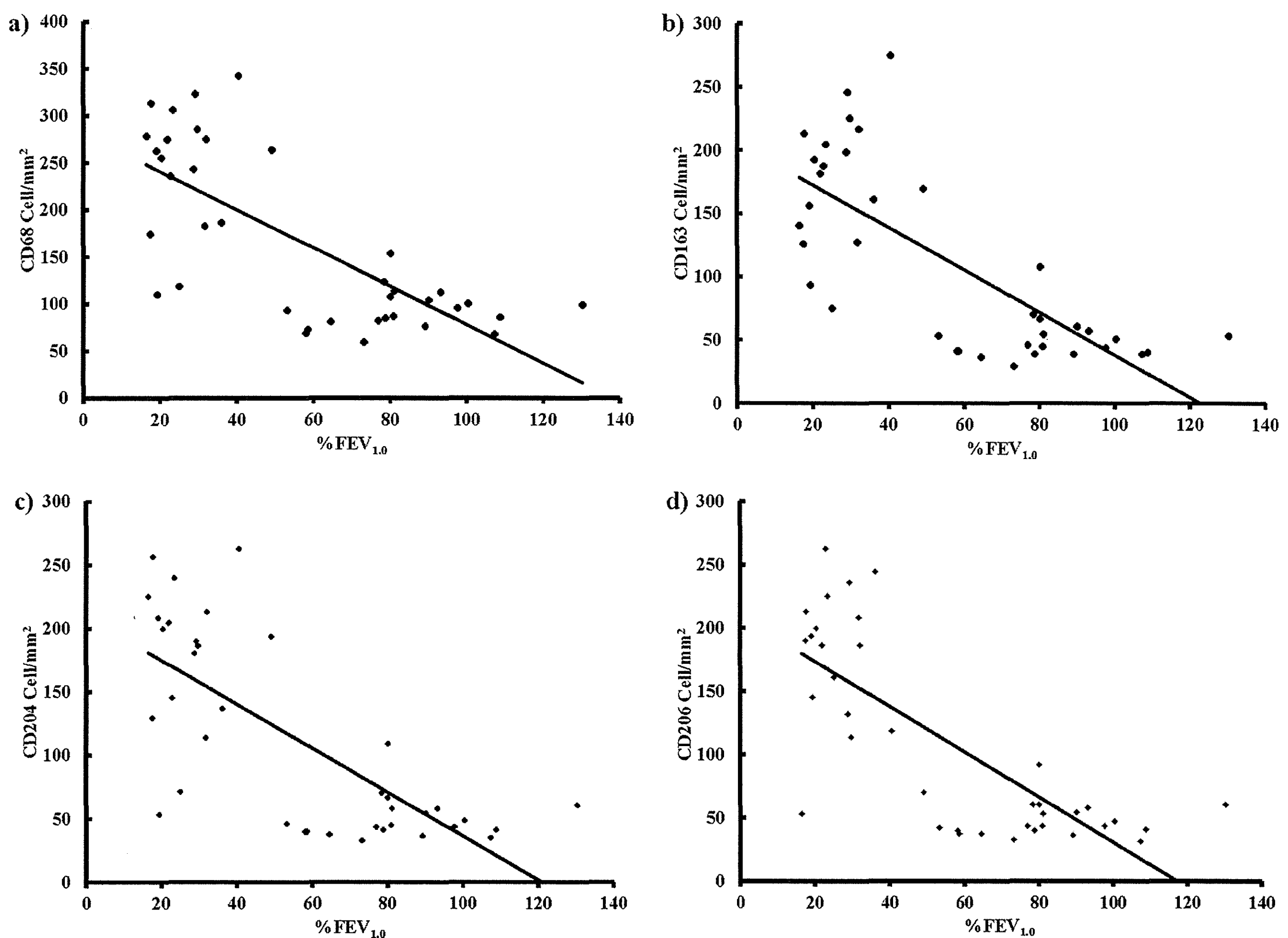

Figure 6. Correlation between the number of CD68, CD163, CD204 or CD206 positive cells and lung function measured as forced expiratory volume in one second % predicted (%$FEV_{1.0}$) in chronic obstructive pulmonary disease patients (n = 38). a) The number of CD68 positive cells and %$FEV_{1.0}$ ($r = 0.729$ and $p < 0.01$), b) The number of CD163 positive cells and %$FEV_{1.0}$ ($r = 0.739$ and $p < 0.01$), c) The number of CD204 positive cells and %$FEV_{1.0}$ ($r = 0.732$ and $p < 0.01$), d) The number of CD206 positive cells and %$FEV_{1.0}$ ($r = 0.765$ and $p < 0.01$).
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Negative Correlation between the Numbers of Cells Positive for CD68, CD163, CD204 or CD206 and %$FEV_{1.0}$ in COPD Patients

We also analyzed the correlation between the number of $CD163^+$, $CD204^+$ or $CD206^+$ cells and pulmonary function in non-smokers, smokers and COPD patients. In COPD patients, there was a significant negative correlation between the number of $CD163^+$, $CD204^+$ or $CD206^+$ cells and %$FEV_{1.0}$, but not the $FEV_{1.0}$/FVC% ratio ($r = 0.729$, $r = 0.739$, $r = 0.732$, $r = 0.765$; Fig. 6). In contrast, there were no significant correlations between the numbers of $CD68^+$, $CD163^+$, $CD204^+$ or $CD206^+$ cells and %$FEV_{1.0}$ in non-smokers or smokers.

MMP-9 Expressed in Alveolar Macrophages in the Lungs of COPD Patients

We analyzed the expression of matrix metalloproteinases (MMPs) in alveolar macrophages in the lungs of non-smokers, smokers and COPD patients by using immunohistochemical assay. MMP-9 was strongly expressed in alveolar macrophages in the lungs of non-smokers, smokers and COPD patients. Moreover, MMP-9 positive alveolar macrophages were increased in the lungs of very severe COPD (Fig. 7).

Discussion

The present study demonstrated that $CD163^+$, $CD204^+$ and $CD206^+$ macrophages were abundant in the lungs of ex-smokers with sever and very severe COPD (GOLD stage III/IV). In addition, there was a negative correlation between the number of $CD163^+$, $CD204^+$ or $CD206^+$ cells and %$FEV_{1.0}$ in COPD patients. These results suggest that cigarette smoke had activated alveolar macrophages and led to overexpression of CD163, CD204 and CD206. Both the lung tissues of non-smokers and smokers were obtained from the normal lung tissues derived from lung cancer patients. Thus, the effect of smoking versus non-smoking might be influenced by alveolar macrophage induction by the tumor environment. Further analysis is needed to test this possibility.

Previous studies have confirmed persistent and severe airway inflammation in lung tissues resected from ex-smokers with very severe COPD [26,27]. Our previous results showed that cessation of smoking cannot prevent pulmonary inflammation in patients with severe COPD [3]. In this study, we found that the numbers of CD68-positive cells in the lungs of patients with severe and very severe COPD who had stopped smoking more than 2 years previously were significantly higher than in non-smokers, smokers

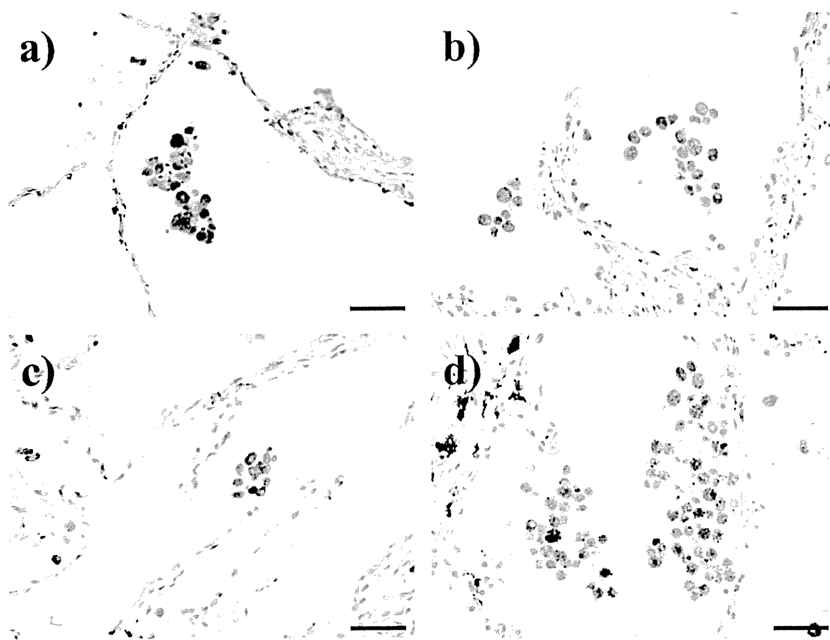

Figure 7. MMP-9 immunohistochemical staining of lung tissue samples from a) a non-smoker, b) a smoker, c) a mild chronic obstructive pulmonary disease patient (COPD), and d) a very severe COPD. Bar: 50 μm.
doi:10.1371/journal.pone.0087400.g007

and patients with mild or moderate COPD. Therefore, CD68-positive alveolar macrophages may be involved in the persistent and severe pulmonary inflammation seen in severe COPD.

The CD163, CD204, and CD206 antigens are known to be upregulated in M2-type macrophages, and are considered to be M2 markers [28,29]. CD163 and CD204 (macrophage scavenger receptor class A) are receptors of the hemoglobin/haptoglobin complex and modified LDL, respectively, although recent studies have demonstrated that these receptors bind many ligands such as bacteria [30,31]. CD206 (macrophage mannose receptor, C type I) is a receptor involved in phagocytosis of bacteria and fungi [32]. The mechanisms and roles of CD163 and CD206 in macrophage phenotypic changes have been unclear. Studies using CD204-deficient mice have shown that CD204 expression plays an important role in macrophage M2 polarization by inhibition of TLR signaling [33,34]. Thomsen demonstrated that a variant of the CD204 gene, Arg293X, is related to increased risk of COPD [35]. Ohar demonstrated that CD204 overexpression induced by *MSR1* gene SNP is associated to high susceptibility to COPD [36]. These data suggest that CD204-induced M2 polarization of alveolar macrophages is involved in pathogenesis of COPD. M2 macrophages are known to preferentially secrete matrix metalloproteinases, which induce collagen destruction [37,38]. Our data has shown that there are increased numbers of $CD163^+$ $CD204^+$ $CD206^+$ M2 alveolar macrophages in stage III/IV COPD patients, and MMP-9 positive alveolar macrophages, presumably M2 macrophages, were increased in the lungs of very severe COPD. These findings suggest that mechanisms leading to accumulation of $CD163^+$ $CD204^+$ $CD206^+$ M2 macrophages in the lung could contribute to severe emphysema and COPD. In this study, we divided COPD patients into two subgroups - into stages I/II and III/IV. The I/II group has 11 mild and 9 moderate patients while the III/IV group has 2 severe and 16 very severe patients. The difference in participant numbers in stage III ($n = 2$) and stage IV ($n = 16$) may influence the statistical evaluation of the results. Further analysis is needed to evaluate this issue. However, it has always been unclear which molecules are related to M2-induced COPD, and therefore further studies are necessary to clarify this association in more detail.

In this study, we showed that the numbers of M2 alveolar macrophages in stage III/IV COPD patients were significantly greater in non-smokers, smokers and stage I/II COPD patient. Our previous study has shown that IL-18 was strongly expressed in alveolar macrophages in the lungs of patients with very severe COPD [3]. These results suggest that overproduction of IL-18 from M2 alveolar macrophages in the lungs may be involved in the pathogenesis of COPD.

The treatment strategy for COPD consists mainly of bronchodilators, such as β2-agonists, theophylline and anticholinergics [20]. It is believed that there is no effective therapy for reducing the persistent pulmonary inflammation in patients with COPD and for improving their outcome, even in those who use inhaled corticosteroids [39,40]. Therefore, the disease is being targeted with new anti-inflammatory treatments. The present study demonstrated macrophages showing overexpression of CD163, CD204 or CD206 in the lungs of patients with severe COPD. The present results raise the possibility that blockade of $CD163^+$, $CD204^+$ or $CD206^+$ macrophages be a feasible treatment for COPD.

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Author Contributions

Conceived and designed the experiments: YK HI YM YK MT TH. Performed the experiments: YK HI KO HO ST MM. Analyzed the data: YK HI TK TH. Contributed reagents/materials/analysis tools: YK HI MT TH. Wrote the paper: YK HI YK.

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CASE REPORT

An autopsy case of refractory vasculo-Behçet's disease

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Abstract Pulmonary vascular involvement in Behçet's disease is a rare complication with a poor prognosis. We present an autopsy case of vasculo-Behçet's disease complicated by pulmonary hemorrhage, possibly caused by rupture of pulmonary artery aneurysms. The patient was treated with a combination of high-dose steroids and pulse cyclophosphamide, but he died from massive hemoptysis. This case highlights the need for potent new therapies for patients with vasculo-Behçet's disease refractory to conventional immunosuppressive therapy, such as a combination of steroids and cyclophosphamide.

Keywords Immunosuppressive therapy · Pulmonary artery aneurysm · Vasculitis · Vasculo-Behçet disease

Introduction

Behçet's disease is a recurring illness characterized by the triple-symptom complex of aphthous stomatitis, genital ulcerations, and uveitis [1]. Various organs, including those of the mucocutaneous, pulmonary, neurological, and articular systems, can also be involved. Behçet's disease associated with lesions in the large vessels is referred to as vasculo-Behçet's disease, which includes venous or arterial occlusion and aneurysm formation. Pulmonary vascular problems, such as pulmonary artery aneurysms (PAAs), are rare complications [2]. However, when present, PAAs are the main cause of mortality, especially bleeding PAAs. Patients with vasculo-Behçet's disease are treated with anticoagulants and immunosuppressants, but the vascular involvement is often refractory to these treatments [2, 3].

We report the case of a patient with vasculo-Behçet's disease who was refractory to conventional immunosuppressive therapy and died suddenly from a massive hemoptysis.

Case report

In 2009, a 42-year-old man presented with fever, cough, oral aphthae, scant hemoptysis, and erythema nodosum on the leg. The patient did not meet the Japanese criteria for the diagnosis of Behçet's disease (revised in 2003) due to the absence of genital ulcers and eye involvement. However, he did show initial manifestations of vascular involvement, such as deep vein and pulmonary thrombosis, leading us to suspect vasculo-Behçet's disease based on the characteristic venous complications. We also could not identify any condition other than Behçet's disease that fit this patient's clinical features. Treatment with colchicine and anticoagulants was initiated. However, colchicine was stopped because of no therapeutic benefit. Treatment with steroids was initiated from January 2010, and the symptoms, including fever, cutaneous involvement, and small hemoptysis, were relieved. The steroid treatment was then gradually reduced. Five months after the initiation of

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steroid therapy, hemoptysis recurred, and azathioprine was prescribed; however, this immunosuppressant was discontinued because of liver toxicity. In 2010, the patient was admitted to the hospital on an emergency basis because of massive hemoptysis. He was intubated, ventilated, and transferred to the intensive care unit. Axial chest enhanced computed tomography (CT) examination revealed the rupture of left PAAs. Anticoagulants were stopped, and his condition improved with conservative treatment. As surgical treatment was considered to be too risky, the patient was treated with a combination therapy of high-dose corticosteroids and intravenous pulse cyclophosphamide (IVCY). A total of six courses of IVCY (750 mg) were administered but the patient showed repeated recurrence of scanty hemoptysis. He was transferred to our jurisdiction and followed up in our hospital from September 2011 onwards. At the end of September 2011, he admitted once again on an emergency basis to a local hospital because of massive hemoptysis. After the condition of the patient improved following the initiation of conservative treatment, he was transferred to our hospital for further examination and treatment. His clinical course is shown in Fig. 1.

Upon admission to our hospital, he presented with aphthous stomatitis and erythema nodosum on both lower extremities. Vital signs were normal. The palpebral conjunctiva was slightly anemic. Rhonchi were audible on the lower lung field. Bruit and venous dilatation were absent. Laboratory data revealed mild anemia (hemoglobin, 9.8 g/dl) and elevated levels of C-reactive protein (1.87 mg/dl), fibrin degradation products (5.5 μg/ml), and D-dimer (2.5 mg/dl). The levels of Protein C and S were normal. Hypoxemia was detected (PaO_2, 58.1 mmHg). An immunological test revealed that the patient was negative for anti-nuclear antibodies, anti-phospholipid antibodies, and anti-neutrophil cytoplasmic antibodies. Human leukocyte antigen (HLA) testing revealed positivity for HLA-A26, which is more common in patients with Behçet's disease in Asia, especially in Japan and Taiwan, than among those in other areas [4]. Laboratory data on admission are shown in Table 1.

An axial chest enhanced CT scan, obtained in the previous hospital, revealed the presence of bilateral patchy shadows that represented intra-alveolar hemorrhage (Fig. 2a, left). A thoracic CT scan obtained after admission to our hospital showed little evidence of this patchy shadow, but there an increase in thrombosis was clearly evident in the left pulmonary artery (Fig. 2a, right; 2b, left, arrow). A thrombus was also detected in an inferior left pulmonary vein (Fig. 2b, right, arrowhead), and the lingular branch and lower lobe branch of the left pulmonary artery were occluded (images not shown). We cautiously initiated anticoagulant therapy using heparin because of increasing thrombosis in the left pulmonary artery. However, because of the recurrence of scanty hemoptysis, anticoagulant therapy was stopped immediately. The activated partial thromboplastin time (APTT) was slightly longer at 44.7 s (normal range 27–43 s). After discontinuation of heparin, however, the APTT was within the

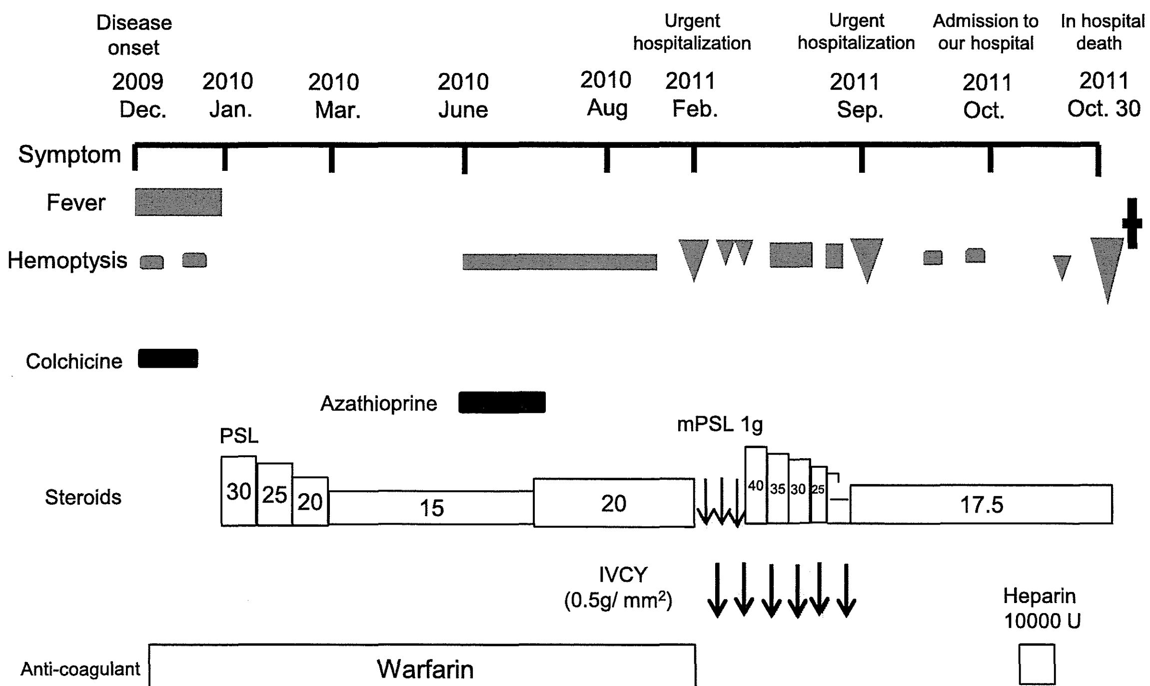

Fig. 1 Clinical course of the patient. *mPSL* Methylprednisolone, *PSL* prednisolone, *IVCY* intravenous pulse cyclophosphamide

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Table 1 Laboratory data on admission

Hematology		Blood chemistry tests		Serological tests	
Target	Value	Target	Value	Target	Value
Red blood cells	374 × 10^4/μl	Total protein	6.53 g/dl	C-reactive protein	1.87 mg/dl
Hemoglobin	9.8 g/dl	Albumin	3.72 g/l	Immunoglobulin (Ig)G	1229 mg/dl
Hemocrit	31 %	Total bilirubin	1.06 mg/dl	IgA	267 mg/dl
Mean corpuscular volume	83 fl	Lactate dehydrogenase	259 mg/dl	IgM	103 mg/dl
Mean corpuscular hemoglobin	26.2 g/dl	Aspartate transaminiase	12 U/l	C3	152 μg/ml
Mean corpuscular hemoglobin concentration	31.6 g/dl	Alanine transaminase	10 U/l	C4	52 μg/ml
White blood cells	8,600/μl	Alkaline phosphatase	237 U/l	Rheumatoid factor	>1 IU/ml
Neutrophils	79.2 %	Gamma-glutamyltransferase	81 U/l	Anti-nuclear antibodies	>40 fold
Lymphocytes	13.5 %	Blood urine nitrogen	12.6 mg/dl	Myeloperoxidase–anti-neutrophil cytoplasmic antibodies (ANCA)	>10 EU
Monocytes	6.8 %	Creatinine	0.63 mg/dl	Proteinase 3–ANCA	>10 EU
Eosinophils	0.4 %			Lupus anticoagulant	(–)
Platelets	28.6 × 10^4/μl			Anti-cardiolipin-β2 GPI antibodies	(–)

Hemostatic data		Air blood gas		Urinalysis	
Target	Value	Target	Value	Target	Value
Prothrombin time-International Normalized Ratio	1.03	pH	7.425	Protein	(–)
Activated partial thromboplastin time	26.1 s	Oxygen partial pressure (PO_2)	58.1 mmHg	Occult blood	(–)
Fibrin degradation products	5.5 μg/ml	Arterial carbon dioxide tension ($PaCO_2$)	38.5 mmHg		
D-Dimer	2.5 mg/dl	HCO_3	24.7 mmol/l		
Protein C	99 %	Oxygen saturation (SpO_2)	90.6 %		
Protein S	106 %				
Human leukocyte antigen (HLA) tests		Feces			
Target	Value	Target	Value		
HLA-A	A1 A26	Occult blood	(–)		
HLA-B	B37 B62				

normal range. As disease activity could not be controlled with prednisolone and IVCY, we considered anti-tumor necrosis factor-alpha as a therapeutic option. However, the patient again developed massive hemoptysis, with a total blood loss estimated at 3,600 mg. He died suddenly, 20 min after massive hemoptysis. A pathological autopsy was performed 20 h after his death.

Postmortem examination showed diffuse hemorrhages in both lungs and a thrombus in the left pulmonary artery (Fig. 3a). Microscopic examination clearly revealed diffuse alveolar hemorrhage in both lungs (Fig. 3b). Thrombus formation and prominent destruction of the elastic interna were observed in the pulmonary artery (Fig. 3c, arrow). Infiltration of mononuclear inflammatory cells was detected (Fig. 3c, right). In addition, aggregation of lymphocytes mimicking lymphoid follicles was induced by the recurrence of artery-wall collapse (Fig. 3c, "L"). Small pulmonary arteries were also damaged and had penetrated through to the alveolus (Fig. 3d, arrowhead), and recanalization was observed in the pulmonary arteries (Fig. 3e).

Discussion

Behçet's disease is a multi-system vasculitis of uncertain etiology. It affects virtually all sizes of arteries and veins with large artery involvement. The frequency of vascular involvement varies by ethnicity and region. Studies performed in Turkey, Iran, Japan, and Europe report that the prevalence of vascular involvement in Behçet's disease in their respective countries is 17, 9, 9, and 10–37 % [2]. Recent studies have revealed that the frequency of vascular involvement in patients with Behçet's disease in Japan is 6.3 %, which is lower than that in other ethnic populations [5]. The primary complications associated with mortality are PAAs, Budd–Chiari syndrome, and vena cava thrombosis [6]. A retrospective study in Turkey revealed that 34 of approximately 2,500 patients developed PAAs [7]. A recent publication from a Japanese research group reported that PAAs did not cause complications in any of the 412 patients with Behçet's disease enrolled in their study [5]. It can therefore be concluded that PAA involvement in patients with Behçet's disease is very rare in Japan.

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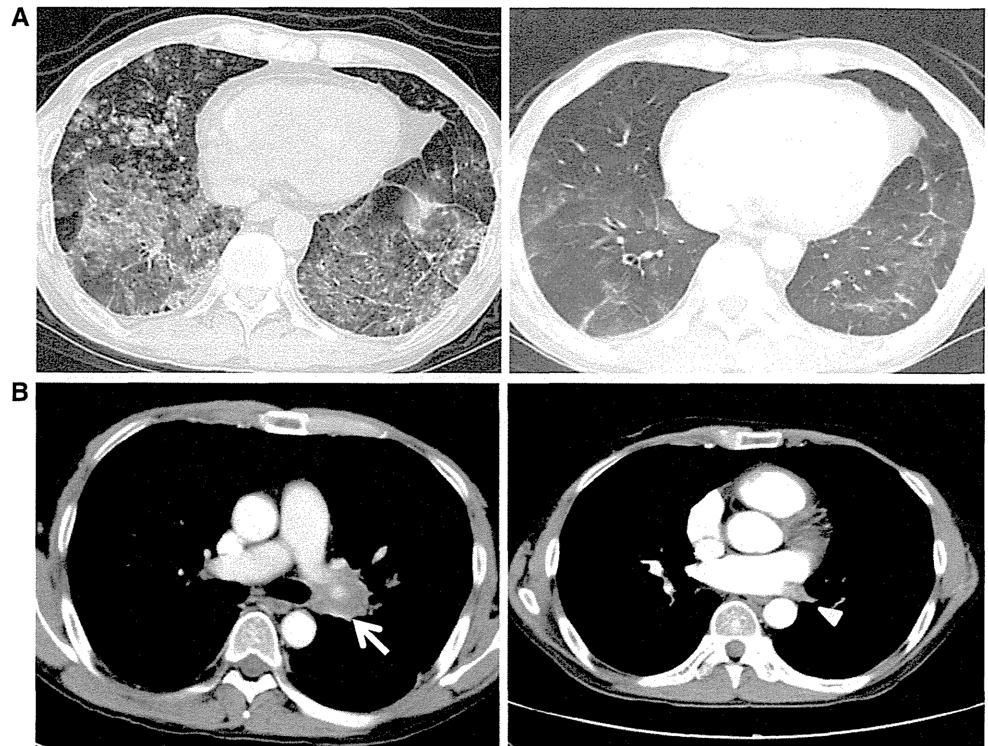

Fig. 2 Pulmonary manifestations in the present case. **a** Thoracic computed tomography scans shows bilateral patchy shadows (*left*) and the subsequent disappearance of these abnormal shadows (*right*). **b** Thrombus is detected in the left pulmonary artery (*left*, *arrow*) and in an inferior left pulmonary vein (*right*, *arrowhead*)

Previous studies showed that young men with thrombophlebitis were prone to PAAs, as was observed in our case [3]. Thromboembolism is uncommon, probably because the thrombi adhere tightly to the vessel walls [8]. Thrombosis usually develops as a complication of underlying extensive vasculitis, and systemic immunosuppressive agents are used to reduce this inflammation. We used heparin because of the increasing thrombosis in the left pulmonary artery; however, it may have induced scanty hemoptysis. The European League Against Rheumatism has discouraged the use of anticoagulants in such cases because it may result in fatal bleeding when there is coexisting pulmonary involvement, particularly in cases with PAAs [9]. However, the risk of anticoagulation therapy-associated bleeding events may differ among different ethnic groups, and the risks and benefits of anticoagulant therapy remain controversial [5, 10].

Although PAAs were detected in our patient by thoracic CT performed in the first hospital to which he was admitted, these were not confirmed by macroscopic or microscopic examination. We were unable to exclude the possibility of massive pulmonary bleeding resulting from acute injury of large vessels, such as the rupture of PAAs, because the patient died suddenly from hemorrhagic shock. As demonstrated in Fig. 3c, d, the destruction of the vascular structure was evident, indicating that it contributed to pulmonary hemorrhage. Recanalization was observed (Fig. 3e) and could also have been involved. Infiltration of mononuclear cells was detected in the lungs (Fig. 3c, right image). Previous studies indicated that inflammatory infiltration in and around vessel walls consists mostly of mononuclear inflammatory cells, predominantly lymphocytes [11, 12]. Infiltration of mononuclear cells appears to result in fragility of the vessel walls, leading to the collapse of the vascular structure.

Pulmonary artery aneurysms may cause major bleeding and are often associated with a poor prognosis. In one study, 12 of 24 patients with PAA died at a mean of 10 months after onset of hemoptysis [3]. In another study, patients with PAA had a poor prognosis, with a 2-year survival rate of less than 50 % [2]. However, several clinical studies have found that early identification and prompt treatment of PAAs, including therapy with combined high-dose steroids and cyclophosphamide, may improve prognosis [13, 14]. In one study there were significantly fewer deaths (23 %) during a mean 4-year period when therapy consisted of a combination of steroids and cyclophosphamide [13]. Another study demonstrated that 35 of 46 PAAs (76 %) completely disappeared after the patients received a combination therapy of steroids and cyclophosphamide, suggesting the efficacy of this combination therapy [14]. High-dose steroids and IVCY were given to our patient 15 months after the onset of hemoptysis. Therefore, it is necessary to diagnose pulmonary involvement within a significantly short period and seriously consider the early immunosuppressive therapy with steroids and cyclophosphamide. Moreover, there is an

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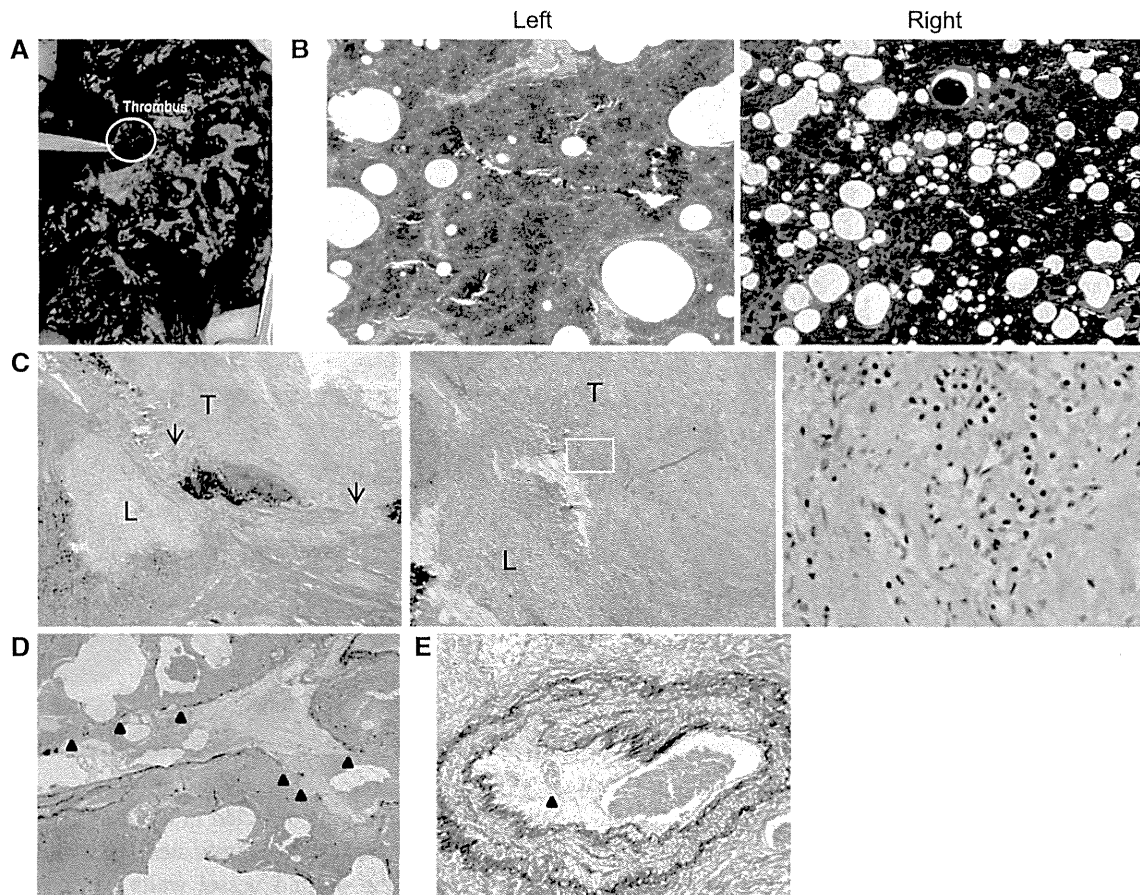

Fig. 3 **a** Gross findings of the left lung. A thrombus was confirmed in the pulmonary artery (*encircled*). **b** Microscopic findings of both lungs. Diffuse alveolar hemorrhage was evident [hematoxylin and eosin (H&E) stain; *left* ×40, *right* ×20). **c**, **d** Microscopic findings of the right lung. **c** *Left*, *middle* images thrombus (*T*) and destruction of the elastic interna (*arrow*), with aggregation of lymphocytes mimicking lymphoid follicles (*L*) around the pulmonary artery; *right image* higher magnification of the area indicated in the middle image, with infiltration of mononuclear cells [elastic-Van Gieson (EVG) stain (*left*) and H&E stain (*middle* and *right*), ×12.5, ×20, ×400, respectively]. **d** Destruction of the small pulmonary arteries and their penetration through to the alveolus (*arrowhead*) (EVG stain, ×40). **e** Recanalization in the pulmonary arteries (*arrowhead*) (EVG stain, ×40)

urgent need for new therapies for patients with vasculo-Behçet's disease refractory to conventional immunosuppressive therapy, such as a combination of steroids and cyclophosphamide.

Although several case reports demonstrate the efficacy of anti-tumor necrosis factor-alpha (TNF-α) agents for Behçet's disease patients with complications of vascular involvement, including PAA, aortitis, and deep-vein thrombosis, only a limited number of studies on these agents have been published [15–18]. In contrast, several studies have raised awareness on the development of thrombophlebitis as a potential side effect of infliximab in patients treated for Behçet's disease or Crohn's disease [19, 20]. Further research is needed to clarify the efficacy and safety of anti-TNF-α agents in the treatment of vascular involvement in Behçet's disease.

Conflict of interest None.

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Predictive factors in patients with EGFR mutation-negative non-small cell lung cancer treated with erlotinib

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Abstract. Factors predicting the efficacy of erlotinib treatment in patients with EGFR mutation-negative non-small-cell lung cancer (NSCLC) have not been well studied. This retrospective study investigates whether patient characteristics, such as site of metastasis, can predict the efficacy of erlotinib treatment in NSCLC patients. In total, 53 EGFR mutation-negative NSCLC patients treated with erlotinib were enrolled, and the associations between clinicopathological characteristics and patient survival were analyzed. The EGFR mutation status was determined using the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp method. Survival curves were obtained using the Kaplan-Meier method. Among the NSCLC patients treated with erlotinib, 27 patients with pulmonary metastasis exhibited significantly longer progression-free survival (PFS) and overall survival (OS) times than those without pulmonary metastasis (median PFS time, 2.9 versus 1.2 months; P=0.0010 and median OS time, 12.4 versus 4.1 months; P=0.0007). Multivariate analyses also revealed that pulmonary metastasis independently correlated with PFS and OS times (hazard ratio, 0.39; P=0.0055 and hazard ratio, 0.33; P=0.0022, respectively). Patients with pulmonary metastasis exhibited significantly longer PFS and OS times than those without pulmonary metastasis. The presence of pulmonary metastasis may be a predictive factor in patients with EGFR mutation-negative NSCLC treated with erlotinib.

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Key words: pulmonary metastasis, non-small cell lung cancer, erlotinib, epidermal growth factor receptor mutation

Introduction

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer mortality worldwide (1). Recently, molecular-targeting therapies such as gefitinib and erlotinib have gained attention due to their potential to improve survival and reduce toxic side effects in patients with NSCLC (2-4). Four phase III trials with gefitinib or erlotinib in patients with epidermal growth factor receptor (EGFR) mutation-positive NSCLC have demonstrated higher response rates and longer progression-free survival (PFS) times than those of patients who received platinum doublets as first-line chemotherapy (5-8). These results indicate that treatment with EGFR-tyrosine kinase inhibitors (TKIs) may now be the standard treatment for EGFR mutation-positive NSCLC patients. However, the clinical role of EGFR-TKI treatment in EGFR mutation-negative patients has not yet been elucidated. A number of researchers have reported that erlotinib may also have efficacy against EGFR-negative NSCLC (9-11).

The factors predicting the efficacy of erlotinib treatment in patients with EGFR mutation-negative NSCLC have not been well studied. In order to improve the survival of patients with EGFR mutation-negative NSCLC receiving EGFR-TKIs including erlotinib, a biomarker that can predict the efficacy of EGFR-TKIs is required.

The presence of pulmonary metastasis and malignant pleural effusion in patients with NSCLC has also been reported to be a predictive factor of EGFR mutations (12,13). However, the association between these characteristics and the efficacy of erlotinib treatment in patients with EGFR mutation-negative NSCLC remains uncertain. These findings prompted the investigation of the correlation between the efficacy of erlotinib treatment and sites of metastasis in patients with EGFR mutation-negative NSCLC in the current study. It was investigated whether metastasis to specific organs, including pulmonary metastasis and malignant pleural effusion, may predict the efficacy and outcome of erlotinib treatment in patients with EGFR mutation-negative NSCLC.

Patients and methods

Patient characteristics. This retrospective study included cases of histologically or cytologically diagnosed NSCLC, which were advanced stage IIIB or IV, according to the International

Association for the Study of Lung Cancer staging system (14) or recurrent at initial diagnosis. In total, 206 NSCLC patients were treated with EGFR-TKIs at Kurume University Hospital (Kurume, Japan) between April 2008 and September 2012. Of these patients, 53 were identified as EGFR mutation-negative and thus, were enrolled in this study. The clinical characteristics of the patients, including age, gender, smoking history, tumor histology, Eastern Cooperative Oncology Group (ECOG) performance status (PS) (15), onset of skin rash following treatment and metastatic sites, were recorded. Tumor nodules in the primary (T3) and in other ipsilateral lobes (T4) were included as pulmonary metastases. Tumor response was examined by computed tomography and evaluated using the Response Evaluation Criteria for Solid Tumors, version 1.0 (RECIST, v 1.0) (16). The present study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Kurume University Hospital (Kurume, Japan).

DNA extraction and peptic nucleic acid-locked nucleic acid (PNA-LNA) polymerase chain reaction (PCR) clamp assay. For *EGFR* mutation analysis, the PNA-LNA PCR clamp method was adopted, using protocols described previously (17). Specific PNA-LNA probe sets for two mutation sites, exon 19 (delE746-A750) and exon 21 (L858R), were developed and these covered >90% of *EGFR* mutations reported previously in Japan. In brief, the genomic DNA was purified from paraffin-embedded tissues using a QIAamp DNA Micro kit (Qiagen, Valencia, CA, USA). The PCR primers employed were synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA), PNA clamp primers and LNA mutant probes were purchased from FASMEC (Kanagawa, Japan) and Integrated DNA Technologies, Inc., (Coralville, IA, USA), respectively. The PNA-LNA PCR clamp assay was performed using a SDS-7500 System (Applied Biosystems Life Technologies, Foster City, CA, USA).

Statistical analysis. Fisher's exact test was used to analyze the significance of associations between patient characteristics and overall response [complete response (CR) and partial response (PR) by RECIST]. The objective response rate (RR) was defined as the proportion of CR or PR. PFS was defined as the period from the date of initiation of erlotinib treatment to the onset of disease progression or mortality from any cause. Overall survival (OS) was measured from the administration of the initial dose of erlotinib until the date of mortality or loss to follow-up. The Kaplan-Meier method was used to assess the survival curves and the log-rank test was used to evaluate the significance of differences between the two groups. The univariate survival analyses were conducted by means of log-rank test, and the multivariate regression was performed using the Cox proportional-hazards regression model. All variables that had P-values of <0.05 were included in the Cox regression model. All tests were two-sided, and P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were conducted using JMP, version 10 (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics. The clinical characteristics of the 53 patients are shown in Table I. Overall, 13 patients were female and 12 were never-smokers; the age range was 35-80 years (median, 64.2 years). In total, 36 patients had adenocarcinoma and 11 had squamous cell carcinoma. The PS was good (ECOG, 0-1) in 44 patients, and poor (ECOG, 2-3) in the remaining nine patients. Erlotinib was used as the first-line therapy in one patient, as a second-line therapy in 13 patients, as a third-line therapy in 29 patients, and as a fourth-line therapy or thereafter in 10 patients. Among the 53 patients who exhibited distant metastasis, 27 (50.9%), 13 (24.5%), 11 (20.8%), 10 (18.9%), 5 (9.4%), 6 (11.3%) and 14 (26.4%) also had pulmonary, brain, bone, extrathoracic lymph node, adrenal gland and liver metastasis, and malignant pleural effusion, respectively.

Table I. Characteristics of the 53 non-small cell lung cancer patients.

Characteristics	Patients, n	%
Age, years		
Median	64	
Range	35-80	
Gender, n		
Male	40	75.5
Female	13	24.5
Smoking history, n		
Never	12	22.6
Former/current	41	77.4
Histology, n		
Adenocarcinoma	36	67.9
Squamous	13	24.5
Adeno-squamous/unidentified	1/3	1.9/5.7
Performance status, n		
0-1	44	83.0
2-3	9	17.0
Metastatic site, n		
Lung	27	50.9
Brain	13	24.5
Bone	11	20.8
Extrathoracic lymph node	10	18.9
Adrenal grand	5	9.4
Liver	6	11.3
Malignant pleural effusion	14	26.4
Others[a]	6	11.3

[a]Skin, 2; spleen, 1; muscle, 1; kidney, 1; peritoneum, 1.

Survival analysis. In total, four patients responded to erlotinib therapy, exhibiting a response rate of 7.5%. All four of these patients also had pulmonary metastasis and malignant pleural effusion with adenocarcinoma. At the time of analysis, the median duration of follow-up was 9.8 months (range, 1.2-31.3 months). The median PFS time for the

Table II. RR, PFS and OS for the patients according to characteristics.

Factor	n	RR, %	P[a]	mPFS, mo	P-value[b]	mOS, mo	P-value[b]
Age, years							
>70	18	11.1	0.2493	1.9	0.1876	5.8	0.1151
<71	35	5.7		3.7		13.1	
Gender, n							
Male	40	5.0	0.2493	2.1	0.1235	5.8	0.1788
Female	13	15.4		3.9		16.6	
Smoking history, n							
Never	12	8.3	1.0000	2.3	0.2893	16.6	0.0975
Former/current	41	7.3		2.2		6.0	
Histology, n							
Adenocarcinoma	35	11.4	0.5619	1.8	0.2847	5.8	0.8179
Squamous	13	0.0		3.7		9.3	
Performance status, n							
0-1	44	9.1	1.0000	2.9	0.0002	8.6	<0.0001
2-3	9	0.0		0.5		1.9	
Skin rash, n							
Present	35	11.4	0.5619	2.9	0.0077	8.6	0.0026
Not present	18	0.0		1.0		2.8	

[a]Determined by Fisher's exact test. [b]Univariate analysis by log-rank test. RR, response rate; mPFS, median progression-free survival; mOS, median overall survival; mo, months.

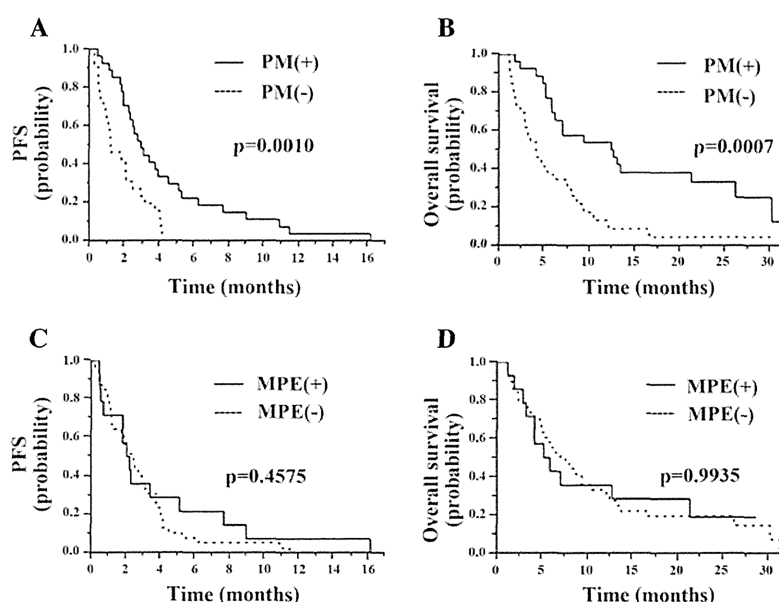

Figure 1. Kaplan-Meier survival curves of (A) PFS and (B) OS according to PM. Kaplan-Meier survival curves of (C) PFS and (D) OS according to MPE. PFS, progression-free survival; OS, overall survival; PM, pulmonary metastasis; MPE, malignant pleural effusion.

patients overall was 2.2 months and the median OS time was 6.2 months. Table II shows the patient demographics, excluding metastatic sites, associated with RR, PFS and OS. The patients with improved PS and skin rash following treatment, exhibited longer PFS and OS times than those with poor PS and without skin rash, as indicated in previous studies (PFS, P=0.0002 and P=0.0077; OS, P<0.0001 and P=0.0026, respectively) (18,19). However, other factors were demonstrated to be unrelated to PFS and OS. The median PFS and median OS times for patients according to metastatic sites are shown in Table III.

Table III. RR, PFS and OS for the 53 patients according to the presence of metastatic sites.

Metastatic site	n	RR, %	P-value[a]	mPFS, mo	P-value[b]	mOS, mo	P-value[b]
Pulmonary metastasis, n							
Yes	27	14.8	0.1110	2.9	0.0010	12.4	0.0007
No	26	0.0		1.2		4.1	
Brain metastasis, n							
Yes	13	7.7	1.0000	1.7	0.0440	5.0	0.0929
No	40	7.5		2.7		7.0	
Bone metastasis, n							
Yes	11	0.0	0.5688	1.2	0.0153	4.9	0.4427
No	42	9.5		2.7		7.0	
Extrathoracic lymph node metastasis, n							
Yes	10	0.0	1.0000	1.9	0.5291	7.0	0.3850
No	43	9.3		2.3		6.2	
Adrenal grand metastasis, n							
Yes	5	20.0	0.3355	1.7	0.3993	5.8	0.3109
No	48	6.3		2.5		7.0	
Liver metastasis, n							
Yes	6	0.0	1.0000	0.7	<0.0001	2.9	0.0004
No	47	8.5		2.5		7.6	
Malignant pleural effusion, n							
Yes	14	26.4	0.0034	2.1	0.4575	5.5	0.9935
No	39	0.0		2.5		7.3	

[a]Determined by Fisher's exact test. [b]Univariate analysis by log-rank test. RR, response rate; mPFS, median progression-free survival; mOS, median overall survival; mo, months.

Table IV. Multivariate analysis of progression-free survival.

Independent factor	Hazard ratio	95% CI	P-value
Pulmonary metastasis	0.39	0.20-0.76	0.0055
Brain metastasis	0.94	0.40-2.06	0.8721
Bone metastasis	2.24	0.96-4.95	0.0616
Liver metastasis	3.82	1.17-11.65	0.0279
Onset of skin rash	0.49	0.25-1.01	0.0522
PS (2-3 vs. 0-1)	3.12	1.20-7.51	0.0214

Multivariate analysis by Cox proportional-hazards regression model. CI, confidence interval; PS, performance status.

Table V. Multivariate Analysis of overall survival.

Independent factor	Hazard ratio	95% CI	P-value
Pulmonary metastasis	0.33	0.16-0.67	0.0022
Liver metastasis	2.65	0.88-7.18	0.0801
Onset of skin rash	0.43	0.20-0.95	0.0381
PS (2-3 vs. 0-1)	3.74	1.36-9.84	0.0115

Multivariate analysis by Cox proportional-hazards regression model. CI, confidence interval; PS, performance status.

The PFS and OS did not depend on the presence or absence of extrathoracic lymph node and adrenal gland metastasis. In patients with brain, bone and liver metastasis, the median PFS times were shorter than for those patients without these metastases. Furthermore, patients with liver metastasis exhibited a shorter OS time than patients without liver metastasis. The median PFS times in the two groups of patients with and without pulmonary metastasis were 2.9 months (95% CI, 1.9-4.5 months) and 1.2 months (95% CI, 0.8-2.1 months), respectively (P=0.001; Fig. 1A). Although no significant differences were identified between the response rate in patients with and without pulmonary metastasis, the response rate tended to be higher in patients with pulmonary metastasis (response rate, 14.8 vs. 0.0%; P=0.1110). The median duration of OS in the two groups of patients with and without pulmonary metastasis was 12.4 months (95% CI, 5.8-26.2 months) and 4.1 months (95% CI, 2.3-7.6 months), respectively (P=0.0007; Fig. 1B). The response rate in patients with malignant pleural

effusion was significantly higher than that of patients without malignant pleural effusion (response rate, 28.6% vs. 0.0%; P=0.0034). However, as shown in Fig. 1C, the median PFS times in the patients with and without malignant pleural effusion were 2.1 and 2.5 months, respectively (P=0.4575). Furthermore, no significant differences were identified in OS between the patients with and without malignant pleural effusion (median OS time, 5.5 months vs. 7.3 months; P=0.9935; Fig. 1D). Of the 13 variables assessed, six were observed to be significantly associated with PFS in univariate analysis: Pulmonary, brain, bone and liver metastasis, plus the onset of skin rash and PS. The multivariate analyses of PFS demonstrated that pulmonary metastasis was an independent and significant predictive factor for PFS (P=0.0055) (Table IV). By contrast, liver metastasis and poor PS were risk factors for an unfavorable PFS following erlotinib therapy (P=0.0279 and P=0.0214, respectively). Additionally, four factors were observed to be significantly associated with OS in the univariate analysis: Pulmonary and liver metastasis plus the onset of skin rash and PS. The presence of pulmonary metastasis was also an independent and significant prognostic factor in the multivariate analysis (P=0.0022).

Discussion

This study demonstrated that the presence of pulmonary metastasis was a predictive marker of the outcome in patients with EGFR-negative NSCLC, receiving erlotinib treatment. Previously, a randomized controlled trial (BR21) investigating the effects of erlotinib versus placebo demonstrated that erlotinib significantly prolonged the median OS, PFS and improved the RR in comparison with the placebo (9). Furthermore, subset analysis in this trial demonstrated that erlotinib treatment was effective in patients with EGFR mutation-negative NSCLC. Several studies have reported that skin rashes following erlotinib treatment tend to correlate with the therapeutic efficacy in patients with NSCLC (18,19). Therefore, the requirement for biomarkers that can predict the efficacy of erlotinib therapy prior to initiation is evident. A number of authors have examined the association between the efficacy of EGFR-TKIs and patient demographics, including gender, tumor histology, smoking history and ECOG-PS. However, few studies have evaluated the efficacy of EGFR-TKIs focusing on metastatic sites as a tumor property. The present study investigated the association between patient characteristics, including metastatic sites and the efficacy of erlotinib treatment in EGFR-mutation negative NSCLC, and demonstrated that pulmonary metastasis was a significant and independent factor associated with PFS and OS. Together, these findings suggest that the presence of pulmonary metastasis may be useful for predicting the efficacy of erlotinib in patients with EGFR mutation-negative NSCLC.

Somatic mutations in the *EGFR* gene have been identified as a major determinant of the clinical response to treatment with EGFR-TKIs, such as gefitinib and erlotinib, in individuals with NSCLC (2,3). In the current study, the four patients who responded to erlotinib treatment had pulmonary metastasis and malignant pleural effusion with adenocarcinoma. Recent studies have suggested that the presence of pulmonary metastasis and malignant pleural effusion is predictive of EGFR mutations, as is the case in adenocarcinoma (12,13). In the current study, a number of cases were reanalyzed for EGFR mutations, including minor mutations, such as exon 20 insertions and G719X in exon 18; however, no EGFR mutations were identified in the reanalyzed samples (results not shown), suggesting that erlotinib may be effective in certain patients with EGFR mutation-negative NSCLC. Erlotinib inhibits the activity of EGFR mutation-negative NSCLC tumor cells at a 50% inhibitory concentration of 2-20 nmol/l. By contrast, three-fold higher concentrations of gefitinib are required in order to block mutation-negative EGFR signaling (20,21). In EGFR mutation-negative NSCLC, it is postulated that erlotinib may bind to the EGFR more readily than gefitinib. These results suggest that erlotinib treatment may be effective in patients with EGFR mutation-negative NSCLC. Patients who responded to erlotinib treatment in the current study exhibited a rapid reduction of tumor size, as was the case for EGFR mutation-positive NSCLC. The mean PFS of the patients in this study was 9.5 months, which was equivalent to that observed in patients with EGFR mutation-positive NSCLC (6-8). These results suggested that erlotinib may inhibit an unknown survival pathway or may act on tumors that have an unknown EGFR mutation status.

A number of limitations were present in the current study: i) The number of patients included was relatively small and, therefore, assessing the significance of differences was challenging and not necessarily representative of a larger population; ii) the retrospective nature of this study did not allow for a standardized measurement of PFS.

In conclusion, the findings suggest that the presence of pulmonary metastasis may be a predictive marker of the response to erlotinib in patients with EGFR mutation-negative NSCLC. Currently, EGFR mutation-negative NSCLC patients have been identified for whom treatment is terminated without receiving erlotinib. However, EGFR mutation-negative NSCLC patients with pulmonary metastasis may benefit from erlotinib treatment. A prospective clinical trial is required to confirm the efficacy of erlotinib treatment in EGFR mutation-negative NSCLC patients with pulmonary metastasis.

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Association of PD-L1 overexpression with activating EGFR mutations in surgically resected nonsmall-cell lung cancer

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Background: Recent clinical trials have shown that immune-checkpoint blockade yields a clinical response in a subset of individuals with advanced nonsmall-cell lung cancer (NSCLC). We examined whether the expression of programmed death–ligand 1 (PD-L1) is related to clinicopathologic or prognostic factors in patients with surgically resected NSCLC.
Patients and methods: The expression of PD-L1 was evaluated by immunohistochemical analysis in 164 specimens of surgically resected NSCLC. Cell surface expression of PD-L1 in NSCLC cell lines was quantified by flow cytometry.
Results: Expression of PD-L1 in tumor specimens was significantly higher for women than for men, for never smokers than for smokers, and for patients with adenocarcinoma than for those with squamous cell carcinoma. Multivariate analysis revealed that the presence of epidermal growth factor receptor gene (*EGFR*) mutations and adenocarcinoma histology were significantly associated with increased PD-L1 expression in a manner independent of other factors. Cell surface expression of PD-L1 was also significantly higher in NSCLC cell lines positive for activating *EGFR* mutations than in those with wild-type *EGFR*. The EGFR inhibitor erlotinib downregulated PD-L1 expression in the former cell lines but not in the latter, suggesting that PD-L1 expression is increased by EGFR signaling conferred by activating *EGFR* mutations. A high level of PD-L1 expression in resected tumor tissue was associated with a significantly shorter overall survival for NSCLC patients.
Conclusions: High expression of PD-L1 was associated with the presence of *EGFR* mutations in surgically resected NSCLC and was an independent negative prognostic factor for this disease.
Key words: PD-L1, activating EGFR mutation, immunohistochemistry, nonsmall-cell lung cancer, EGFR-TKI

introduction

Lung cancer is the leading cause of cancer death worldwide [1]. Recent advances in targeted therapy, however, have led to a major paradigm shift in clinical oncology. Molecularly targeted drugs such as erlotinib and crizotinib have thus greatly improved the clinical course for nonsmall-cell lung cancer (NSCLC) patients with sensitizing epidermal growth factor receptor (EGFR) gene mutations or anaplastic lymphoma kinase (ALK) gene translocations, respectively. Despite these advances, however, the prognosis of individuals with NSCLC who do not harbor an identifiable driver oncogene remains poor [2].

Blockade of immune checkpoints with monoclonal antibodies has also recently emerged as a new therapeutic tool in oncology [3]. Programmed death 1 (PD1), which belongs to the CD28 family of proteins, is a receptor expressed on the surface of T cells that regulates their activation and proliferation. Its ligand, programmed death–ligand 1 (PD-L1), is frequently overexpressed in many types of human cancer [3]. The binding of PD-L1 to PD1 induces apoptosis or exhaustion in activated T cells, and blockade of this interaction has been shown to enhance the antitumor activity of T cells [3]. Recent clinical trials found that inhibition of the PD-L1–PD1 interaction with antibodies specific for these proteins had promising antitumor efficacy in patients with various malignancies including NSCLC [4, 5]. The clinical relevance of PD-L1 expression in NSCLC has remained unclear, however. We have therefore now examined PD-L1 expression in surgically resected NSCLC specimens and analyzed

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its associated clinicopathologic characteristics and prognostic relevance.

materials and methods

Human NSCLC cell lines were maintained under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (PC9, HCC827, NCI-H1975, QG56, 1-87, H1299, H2122, H322, H460, LK2, LK87, H23) or in Dulbecco's modified eagle's medium (A549, H157), each supplemented with 10% fetal bovine serum. Both HCC827 and PC9 cell lines harbor an activating in-frame deletion [del(E746–A750)] in exon 19 of *EGFR*; H1975 harbors an activating mutation (L858R) in exon 21 as well as a secondary mutation (T790M) in exon 20 that contributes to the development of resistance to EGFR–tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib; and all other cell lines are wild type for *EGFR* [6] (supplementary Data 1, available at *Annals of Oncology* online). Erlotinib was obtained from Cayman Chemical (Ann Arbor, MI).

In this retrospective study, we screened 164 NSCLC patients who underwent surgical resection of their tumors between 2000 and 2010 at Kurume University Hospital. *EGFR* mutations were identified in tumor tissue by the peptide nucleic acid–locked nucleic acid polymerase chain reaction clamp method [7]. The present study conforms to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Kurume University Hospital.

Paraffin-embedded tumor tissue was sectioned at a thickness of 4 μm, and the sections were then mounted on glass slides for immunohistochemical analysis of PD-L1 with the use of the BenchMark XT platform (Ventana Automated Systems, Tucson, AZ). In brief, each slide was subjected to heat treatment in Ventana CC1 retrieval solution for 30 min and then incubated for 30 min with rabbit polyclonal antibodies to human PD-L1 (Lifespan Biosciences, Seattle, WA). Immune complexes were detected with the use of an ultraVIEW 3,3′-diaminobenzidine (DAB) detection kit with DAB as the chromogen (Ventana). The intensity of staining was evaluated according to the following scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The proportion of all tumor cells found to express PD-L1 was determined and then multiplied by the staining intensity score to obtain a final semiquantitative *H* score (maximum value of 300 corresponding to 100% of tumor cells positive for PD-L1 with an overall staining intensity score of 3). All immunohistochemical images were evaluated by two experienced observers (AK and MK) who were unaware of the identity of the specimens, and the mean of the two determinations was used for further analysis.

To examine the effect of EGFR signaling on PD-L1 expression, we exposed PC9, HCC827, H1975, or A549 cells to 100 nM erlotinib for 48 h and then stained the cells consecutively with biotinylated mouse monoclonal antibodies to human PD-L1 (eBioscience, San Diego, CA) and phycoerythrin-labeled streptavidin (BD Biosciences, San Jose, CA) for flow cytometric analysis with a FACS Calibur instrument equipped with CELLQuest software (BD Biosciences) [8]. For examination of EGFR phosphorylation, immunoblot analysis was carried out as previously described [9] with rabbit polyclonal antibodies to phosphorylated (pY1086) or total forms of EGFR (Cell Signaling Technology, Danvers, MA).

See supplementary Data 2, available at *Annals of Oncology* online.

results

A total of 164 patients with NSCLC (114 with adenocarcinoma and 50 with squamous cell carcinoma) who underwent surgical resection was included in the present study (Table 1). Ninety-one (55%) patients were male and 95 (58%) were never smokers, and the median age of all patients was 66 years (range, 39–82 years). With regard to *EGFR* mutation status, 30 patients harbored a deletion in exon 19 and 27 patients had an L858R missense mutation in exon 21.

Immunohistochemical staining for PD-L1 was detected at the membrane or in the cytoplasm (or both) of tumor cells and stromal lymphocytes in the surgically resected tumor specimens (Figure 1A). Expression of PD-L1 was significantly higher in tumors from women than in those from men ($P < 0.001$), in those with an adenocarcinoma histology than in those with a squamous cell carcinoma histology ($P < 0.001$), in those from never smokers than in those from smokers ($P < 0.001$), and in those positive for *EGFR* mutations than in those wild type for *EGFR* ($P < 0.001$) (Figure 1B). No significant association was

Characteristic	
Age (years)	
Median	66
Range	39–82
Sex	
Male	91
Female	73
Smoking status	
Never smoker	95
Smoker	69
Histology	
Adenocarcinoma	114
Squamous cell carcinoma	50
p Stage	
IA	34
IB	33
IIA	11
IIB	35
IIIA	34
IIIB	17
EGFR mutation status	
Wild type	107
L858R	27
Exon 19 deletion	30

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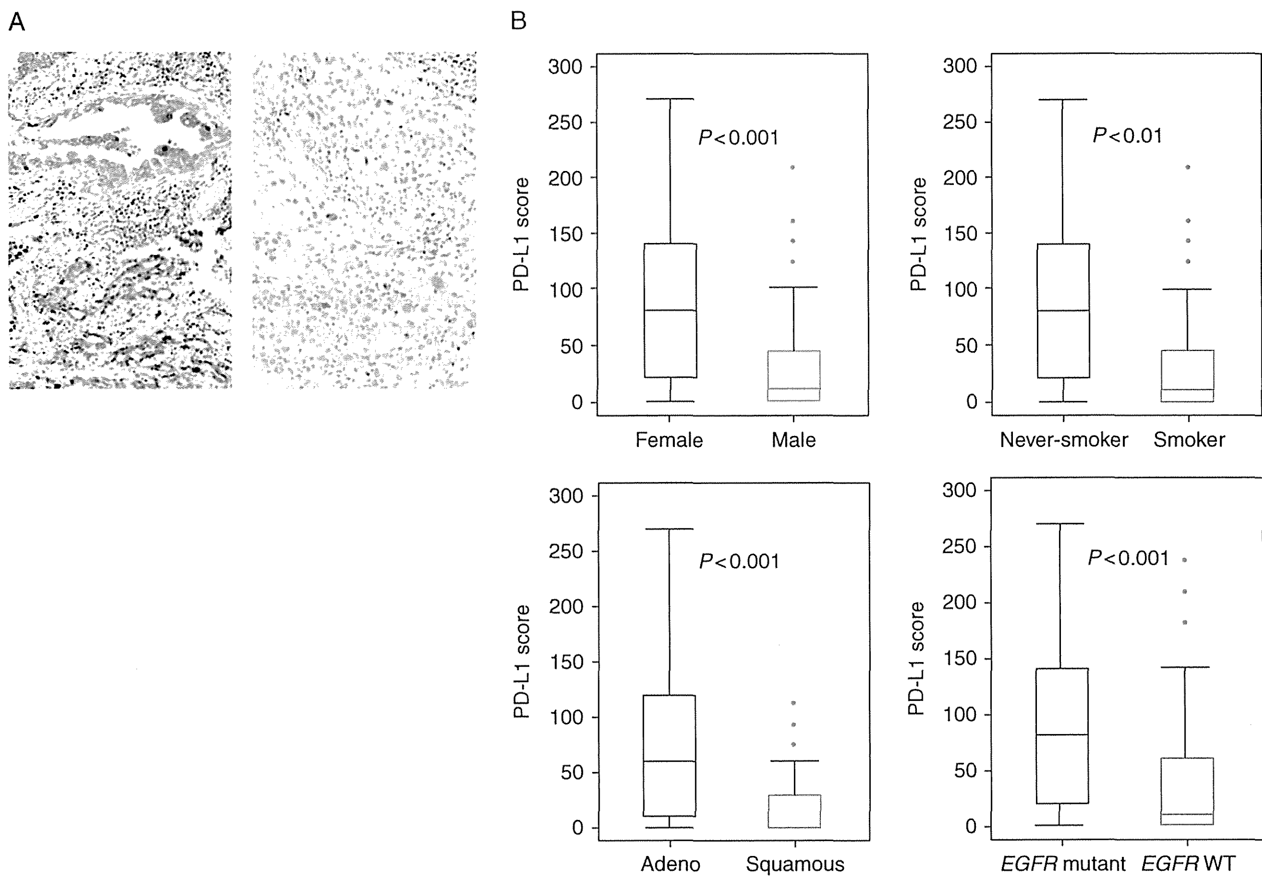

Figure 1. Relation between immunohistochemical staining for PD-L1 in tumor specimens and other patient characteristics. (A) Representative patterns of PD-L1 immunostaining in two NSCLC tumors each with strong (left panels) or weak (right panels) staining intensity. PD-L1 immunoreactivity was detected at the membrane or in the cytoplasm (or both) of tumor cells and stromal lymphocytes. Original magnification, ×400. (B) Significant association of overall PD-L1 staining (*H*) score with sex, smoking status, tumor histology, or *EGFR* mutation status (WT, wild type). Data are presented as box-and-whisker plots, and *P* values were determined with the Wilcoxon rank-sum test.

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detected between expression of PD-L1 and either patient age (≤66 versus >66 years, $P = 0.228$) or tumor p stage (I versus II or III, $P = 0.207$). Given that *EGFR* mutations are more frequently found in women, never smokers, and individuals with adenocarcinoma [2], we carried out multivariate analysis to examine which factors were associated with expression of PD-L1 in the present study. Multivariate analysis revealed that *EGFR* mutation positivity and adenocarcinoma histology were associated with high expression of PD-L1 independently of other patient characteristics ($P = 0.027$ and $P = 0.046$, respectively) (Table 2).

Given the positive association between *EGFR* mutation and high PD-L1 expression in NSCLC tumors, we examined the expression of PD-L1 in NSCLC cell lines whose *EGFR* mutation status had been previously determined. Three (HCC827, PC9, and H1975) of the 14 cell lines examined harbor *EGFR* mutations, whereas the other 11 cell lines are wild type for *EGFR*. Both HCC827 and PC9 harbor an activating in-frame deletion in exon 19 of *EGFR*, whereas H1975 harbors an activating L858R mutation in exon 21 as well as a secondary mutation (T790M) in exon 20 that contributes to the development of resistance to EGFR-TKIs such as gefitinib and erlotinib. Flow cytometric analysis revealed that the level of PD-L1 expression at the cell surface was significantly higher for cell lines with *EGFR* mutations than for those with wild-type *EGFR* ($P = 0.023$) (Figure 2A). To examine the role of oncogenic EGFR signaling in the regulation of PD-L1 expression, we determined the effect of the EGFR-TKI erlotinib on PD-L1 abundance in NSCLC cell lines. Consistent with our previous observations, immunoblot analysis revealed that EGFR phosphorylation was undetectable in *EGFR* mutation-negative A549 cells [9], whereas *EGFR* mutation-positive PC9, HCC827, and H1975 cells manifested a high basal level of EGFR phosphorylation (Figure 2B). Treatment of PC9 and HCC827 cells with erlotinib resulted in marked downregulation of PD-L1 expression at the cell surface (Figure 2C) as well as inhibition of EGFR phosphorylation (Figure 2B), whereas erlotinib did not affect PD-L1 expression or EGFR phosphorylation in A549 cells. The T790M mutation impedes the binding of gefitinib and erlotinib to the ATP binding pocket of the catalytic domain of EGFR. Importantly, erlotinib had no effect not

Characteristic		Coefficient (95% CI)	*P* value
p Stage	I versus II/III	2.0 (−18.4 to 22.4)	0.846
Age (years)	≤66 versus >66	5.3 (−13.8 to 24.4)	0.584
Sex	Female versus male	14.5 (−29.0 to 58.1)	0.511
Smoking status	Smoker versus never smoker	−18.6 (−64.6 to 27.4)	0.426
Histology	Adenocarcinoma versus SCC	25.1 (0.5 to 49.8)	0.046
EGFR status	Mutant versus wild type	25.4 (2.9 to 47.9)	0.027

SCC, squamous cell carcinoma.

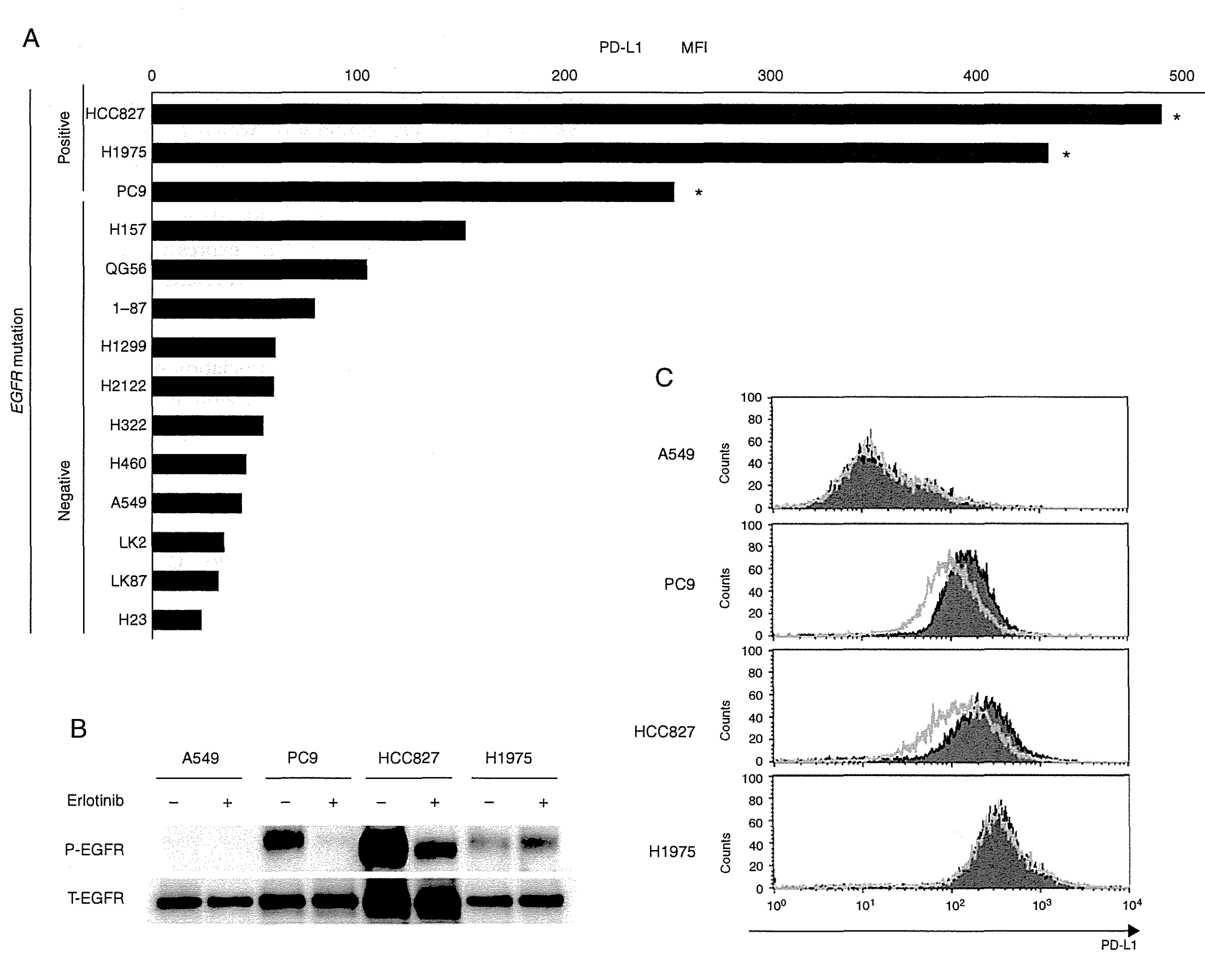

Figure 2. Relation between PD-L1 expression and *EGFR* mutation in NSCLC cell lines. (A) Flow cytometric analysis of PD-L1 expression at the surface of NSCLC cell lines. The mean fluorescence intensity (MFI) for PD-L1 was significantly higher in *EGFR* mutation-positive cell lines (HCC827, H1975, and PC9) than in cell lines with wild-type *EGFR* (*$P < 0.05$, Mann–Whitney *U*-test). (B and C) Immunoblot analysis of phosphorylated (P) and total (T) forms of EGFR (B) as well as flow cytometric analysis of surface PD-L1 expression (C) in the indicated cell lines after incubation for 48 h in the absence [shaded trace in (C)] or presence [green trace in (C)] of 100 nM erlotinib. H1975 harbors a secondary mutation (T790M) of *EGFR* that contributes to the development of resistance to EGFR-TKIs such as gefitinib and erlotinib.

only on EGFR phosphorylation but also on PD-L1 expression in H1975 cells, which harbor both L858R and T790M mutations (Figure 2B and C). Together, these data thus suggested that the expression of PD-L1 is upregulated by EGFR signaling in *EGFR* mutation-positive NSCLC cells.

The median follow-up time for all 164 patients was 55.6 months (range, 0.8–168.4 months). NSCLC patients with a high expression score for PD-L1 had a significantly shorter OS compared with those with a low expression score (median of 55.9 versus

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