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Activity of sputum p38 MAPK is correlated with airway inflammation and reduced FEV₁ in COPD patients

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Data Interpretation D
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Background: Inflammation and remodeling of the small airways are major determinants of the progression and severity of COPD. The present study explored the correlation between sputum p38 mitogen-activated protein kinase (MAPK) activity and airway inflammation and reduction of lung function in the patients with chronic obstructive pulmonary disease (COPD).

Material/Methods: Sputum samples were collected from 48 COPD patients and 12 healthy persons. Sputum p38 MAPK activity was measured by Western blotting and sputum levels of CXCL8 and neutrophil, and lung function was measured. The correlation between p38MAPK activity and airway inflammation and reduction of lung function was analyzed.

Result: Our results showed the significantly increased expression of phospho-p38 MAPK and CXCL8 in the sputum samples of the COPD patients. The p38 MAPK activity was remarkably correlated with the CXCL8 level and neutrophils infiltration in the airway, and the decline of lung function in the COPD patients.

Conclusions: These findings suggest the pivotal role of p38 MAPK in the airway inflammation of COPD patients. We propose p38 MAPK as a potential target for the treatment of COPD.

Key words: **p38 mitogen-activated protein kinase • chronic obstructive pulmonary disease • CXCL8**

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Background

Chronic obstructive pulmonary disease (COPD), characterized by airflow limitation that is not fully reversible, affects approximately 210 million people worldwide and is currently a leading cause of morbidity and mortality worldwide [1–4]. Inflammation and remodeling of the small airways are major determinants for the progression and severity of COPD, as defined by the decline in FEV₁ [5]. The airway inflammation in COPD is generally described as neutrophilic. Several immune cells, proinflammatory chemokines, and cytokines extensively participate in the induction and maintenance of the inflammatory response in the airway [6]. The chemokine CXCL8 (also referred to as interleukin-8), which is secreted from the leukocytic cells (monocytes, T cells, neutrophils, and natural killer cells) and other cells such as epithelial cells and fibroblasts [7], has a well documented role in the pathogenesis and maintenance of airway inflammation in COPD.

In humans and rodents, there exist 2 major forms of CXCL8-77- and 72-amino acid proteins – with a minor 69-amino acid protein [8,9]. CXCL8 production is not constitutive, but is inducible by proinflammatory cytokines such as IL-1 and tumor necrosis factor (TNF)- α [8], bacteria and virus and their products [7,10], and several environmental factors such as hypoxic conditions [11]. Two distinct G-protein-coupled receptors – CXCR1 and CXCR2 – exist for CXCL8 in humans and rodents [12,13]. The primary function of CXCL8 is the induction of chemotaxis in its target cells, especially neutrophils and lymphocytes [10], which involves G $\beta\gamma$ subunit-activated phosphatidylinositol 3-kinase- γ and phosphatidylinositol 3,4,5-trisphosphate (PIP₃) signaling [14,15]. CXCL8 also induces upsurge of cytosolic calcium, which is essential for exocytosis (e.g. mediator release), and respiratory burst [7,10]. Numerous previous studies have suggested the critical role of CXCL8 in the induction and maintenance of airway inflammation in the rodent model and in clinical patients with COPD.

Mitogen-activated protein kinases (MAPKs) play a key role in chronic inflammation, and several complex enzyme cascades have been defined. One of these, the p38 MAPK pathway, is activated by cellular stress and regulates the expression of inflammatory cytokines, including CXCL8, TNF- α and MMPs [16]. Four isoforms of p38 MAPK family (α , β , γ , and δ) exist. Usually, p38 MAPKs are activated by phosphorylation on Thr180 and Tyr182 in the Thr-Gly-Tyr motif of the activation loop by upstream MAPK kinases MKK3 and MKK6, which are in turn activated by the MAPK kinase kinases (MEKK). Once activated, p38 MAPKs effectively act on several downstream kinases, such as MAPK-activated protein kinase (MAPKAPK2/3), and eventually influence the function of some transcription factors, cytoskeletal proteins and translational components, and other enzymes [17].

The role of p38 MAPK has been extensively reported in the induction and maintenance of airway inflammation in COPD [17]. p38 MAPK (measured by phosphorylated p38 MAPK) is activated in alveolar macrophages of COPD lungs [18]. Suppression the activity of p38- α isoform with inhibitor SD-282 effectively decreased TNF- α release from human lung macrophages *in vitro* [19] and attenuated inflammation in a smoking model of COPD in mice [20]. Previously, it was reported that p38 α is involved in the migration of neutrophils and other inflammatory cells [17]. Hence, p38 MAPK is critically involved in the induction of airway inflammation *in vitro* and in the rodent model of COPD. In the present study, we investigated the expression of phospho-p38MAPK in the sputum, and its correlation with airway inflammation and lung function in COPD patients.

Material and Methods

Subjects

Patients with COPD, from the Department of Respiratory Medicine of Hubei University of Science and Technology, were recruited and followed up between October 2007 and May 2010, with approval by the Institutional Ethics Committee. Totally, 48 patients (29 males and 19 females), with average age 66.1 \pm 8.5 years, were included in this study. Patients were excluded if they had a history of asthma, bronchiectasis, tuberculosis, or other confounding diseases, such as severe congestive heart failure (Stage III–IV New York Heart Association), cancer, diabetes, thyrotoxicosis, and autoimmune diseases. Lung function test was performed in all participants with the inhalation of salbutamol sulfate. COPD was defined according to international guidelines (post-bronchodilator FEV₁/FVC ratio <70%), and the severity of COPD was classed according to current Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria [4]. These patients are classified into COPD grade I (13 patients), Grade II (13), grade III (12), and grade IV (10). The control group included the 12 outpatients for routine health check, with matched age and without any known respiratory diseases. The institutional human research review board approved the present study and all patients signed an informed consent.

Collection of sputum samples

Spontaneous or induced sputum samples were collected at admission and before antibiotic administration, according to the previous report with minor modification [21]. Sputum induction was performed with a 3% saline nebulizer and respiratory physiotherapy when required. Only good quality samples (<10 squamous epithelial cells and >25 leukocytes per field) were accepted for processing. Sputum samples were processed microbiologically for semiquantitative culture following accepted

laboratory methods. The supernate of sputum samples were collected and frozen at -70°C for further cytokine measurement.

Protein extraction and western blotting analyzing the phospho-p38 MAPK level

Protein extraction in the sputum cells was performed as described previously [22,23]. The cleared sputum cells were incubated at 37°C for 2 h and then 1×10^5 cells were collected for the protein extraction. The cells were homogenized in a lysis buffer (0.1% Triton X-100, 150 mm NaCl, 25 mm KCl, and 10 mm Tris-HCl, pH 7.4, with protease inhibitors and phosphatase inhibitors). After tissue homogenization and lysis, samples were centrifuged and the supernatant was used for SDS-PAGE. The amount of protein loaded in each lane was 15 μg . Proteins were separated and electrotransferred onto nitrocellulose membrane. Samples were incubated overnight at 4°C with a primary polyclonal antibody against phospho-p38 MAPK (1:400, Santa Cruz Biotechnology, CA) and monoclonal antibody against β -actin (1:1000, Santa Cruz Biotechnology, CA). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10 000, GE Healthcare) for 1 h at room temperature. Immunoreactive proteins were detected by the enhanced chemiluminescence advance kit (GE Healthcare). The intensity of bands was digitally captured and quantitatively analyzed with Image J software. The immunoreactivity of phospho-p38MAPK was normalized to that of β -actin.

Retrospect and quantitative PCR to analyze the mRNA level of CXCL8

The cleared sputum cells were incubated at 37°C for 1 h, and then 1×10^5 cells were collected for the PCR. Total RNA was prepared by using an RNAqueous-4 PCR kit (Ambion, Austin, TX) and reverse transcribed by using a SYBR Green reverse transcription (RT)-PCR Reagents kit (Applied Biosystems, Foster City, CA). The sequences of primers were commercially designed and synthesized by Sangon Biotech (Shanghai, China) included the following: CXCL8: 5'-ATG ACT TCC AAG CTG GCC GTG GCT -3', and 5'- TCT CAG CCC TCT TCA AAA ACT TCT C -3'; β -actin: 5'-GAT GGT GGG TAT GGG TCA GAA GGA-3', and 5'-GCT CAT TGC CGA TAG TGA TGA CCT-3'. Primer concentrations were optimized. Quantitative PCR was performed, and the level of CXCL8 mRNA was normalized to that of β -actin.

Measuring CXCL8 level in the supernate of the sputum samples by ELISA

CXCL8 in the supernate of sputum samples was detected with ELISA kit as described previously [24]. We used 96-well colorimetric "sandwich" ELISA plates (CXCL8 human ELISA Kit, Abcam, Cambridge, MA) to determine CXCL8 content and

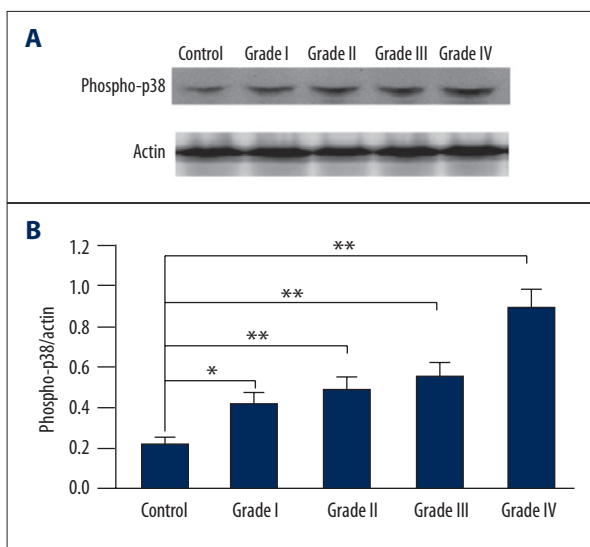


Figure 1. Increased expression of phospho-p38 MAPK in the sputum samples of the COPD patients. (A) representative bands to show the sputum level of phospho-p38 MAPK in COPD patients and healthy individuals; (B) Summarized results to plot the phospho-p38 MAPK level in all groups. * $P < 0.05$; ** $P < 0.01$.

50 μl of human recombinant CXCL8 standards and supernatant samples were run in duplicate following the manufacturer's instructions. The optical density of each well was determined at a wavelength of 450 nm. Samples were considered CXCL8-positive when the signal was higher than the background signal (modified Krebs' solution) and within the range of the standard curve.

Statistical analysis

All data are presented as mean \pm S.E.M. The difference among all groups was analyzed with ANOVA, and the correlations between 2 variances were analyzed with linear correlation regression. $P < 0.05$ was considered statistically significant.

Results

Increased sputum phospho-p38MAPK expression in patients with COPD

The level of phospho-p38 MAPK, the active form of the kinase, was examined in the sputum samples and was compared among the control and the patients with COPD in different stages. The level of phospho-p38 MAPK was significantly increased in the sputum samples of the patients with COPD ($P < 0.05$) when compared to that from the control group (Figure 1). Notably, the level of phosphorylated p38 MAPK significantly increased, paralleling the severity of disease (Figure 1).

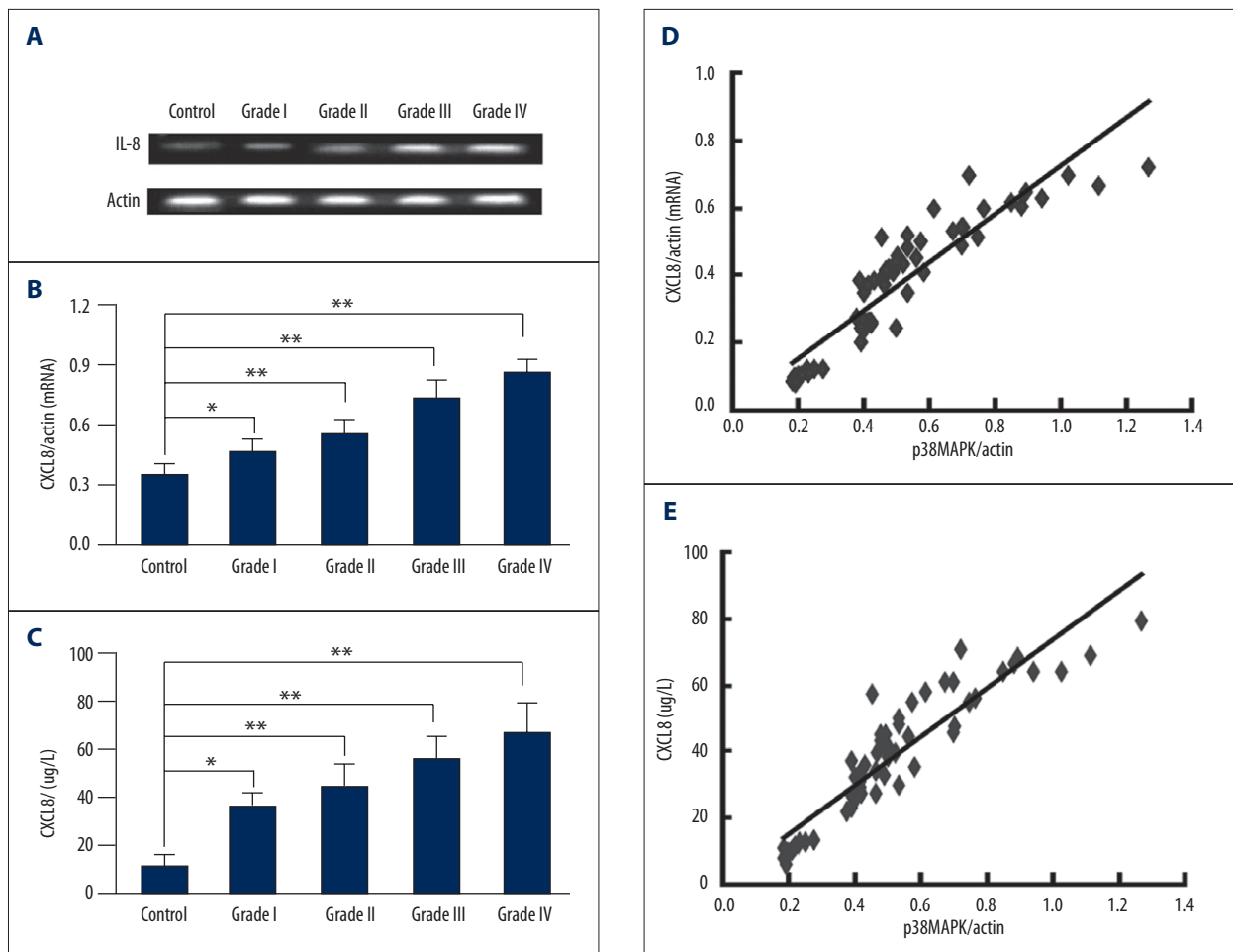


Figure 2. Increased CXCL8 level in the sputum sample of COPD patients, and its correlation with p38 MAPK activity. (A) representative bands to show the mRNA level of CXCL8 in sputum sample of COPD patients and healthy individuals; (B) summarized results to plot the sputum CXCL8 mRNA level in all groups; (C) summarized results to plot the CXCL8 protein level in the sputum samples in all groups; (D, E) phospho-p38 MAPK level was correlated with the sputum CXCL8 mRNA and protein level in the COPD patients. * $P < 0.05$; ** $P < 0.01$

Increased sputum CXCL8 expression and its correlation with phospho-p38 MAPK level in patients with COPD

Provided that CXCL8 was the key chemokine for the induction and development of airway inflammation [7], retroscribe quantitative PCR was performed to examine the mRNA level in the sputum cells in all groups. Compared to the control group, CXCL8 mRNA was significantly increased in the sputum sample of patients with COPD ($P < 0.05$) (Figure 2A, 2B). Consistently, ELISA analysis revealed an increased sputum CXCL8 protein in the patients with COPD (Figure 2C). Both mRNA and protein level of CXCL8 significantly increased, paralleling to the severity of disease (Figure 2A–2C). Further correlation analysis revealed that the mRNA (Figure 2D, $r = 0.571$, $n = 60$, $P < 0.01$) and protein (Figure 2E, $r = 0.531$, $n = 60$, $P < 0.01$) level of CXCL8 in sputum samples was positively correlated with the sputum phospho-p38MAPK level in patients with COPD (Figure 2).

Increased airway inflammation and its correlation with the phospho-p38MAPK in patients with COPD

The primary function of CXCL8 is to induce chemotaxis of inflammatory cells [10], while accumulation of inflammatory immune cells, mainly the neutrophils, in the airway is one of the major factors for the pathogenesis and development of COPD [25]. We examined the number of the immune cells and its correlation with the expression of phosphorylated p38 MAPK in the sputum samples of patients with COPD. Compared to the control group, the number of total immune cells and the percentage of neutrophils were significantly increased in the sputum samples from patients with COPD, paralleling the severity of disease (Figure 3A). Further correlation analysis revealed a significant positive correlation between the neutrophil percentage and the level of sputum phospho-p38 MAPK (Figure 3B, $r = 0.664$, $n = 60$, $P < 0.01$), confirming the critical role

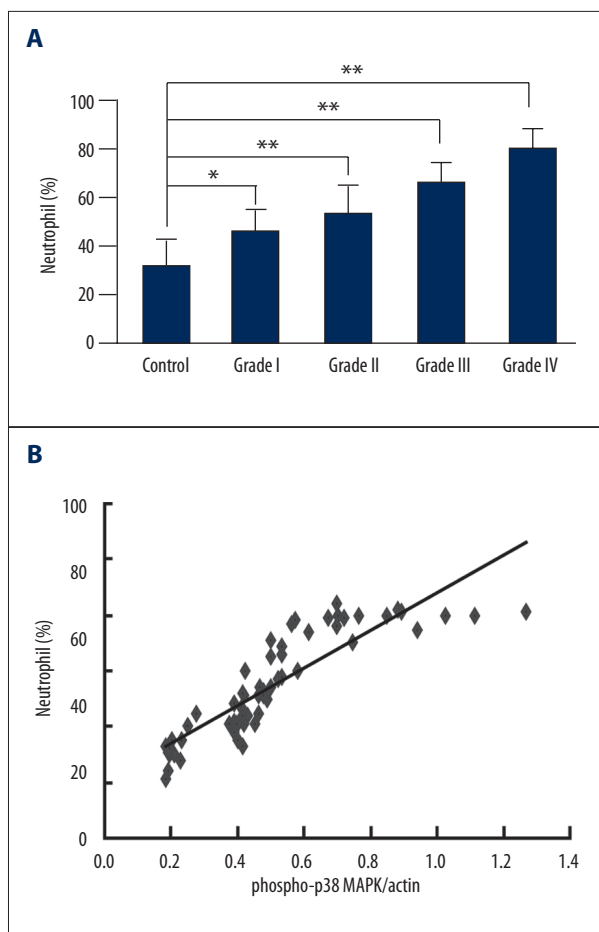


Figure 3. Increased neutrophil in the sputum sample of COPD patients, and its correlation with p38 MAPK activity. (A) summarized results to show the sputum neutrophils in all groups; (B) phospho-p38 MAPK was correlated with the sputum neutrophils in the COPD patients. * $P < 0.05$; ** $P < 0.01$

of phospho-p38MAPK in neutrophil migration and accumulation in the airway of patients with COPD.

Decreased lung function and its correlation with phospho-p38 MAPK in patients with COPD

COPD is characterized as the chronic obstruction of airway flow as demonstrated by the significant reduction of FEV_1 in clinical patients [25]. Here, we examined the correlation between the $FEV_1\%$ and the level of phospho-p38MAPK in COPD patients. COPD patients had significantly decreased $FEV_1\%$ in the lung function test, and the FEV_1 was significantly negatively correlated with the level of phospho-p38 MAPK in the sputum sample of patients with COPD ($r = -0.468$, $n = 60$, $P < 0.05$) (Figure 4). These results suggest that activated p38 MAPK participates in the pathogenic process of the reduction of FEV_1 in patients with COPD.

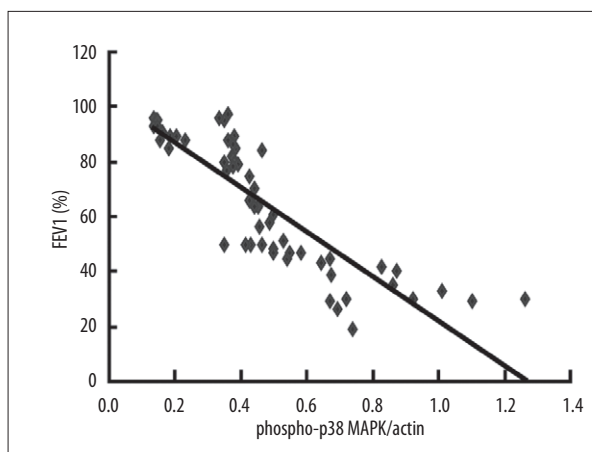


Figure 4. Sputum p38 MAPK activity was inversely correlated with the $FEV_1\%$ in COPD patients ($r = -0.468$, $n = 60$, $P < 0.05$).

Discussion

Increased airway p38 MAPK activity in COPD

The increased activity of p38 MAPK has been extensively documented in several lung diseases in rodent models and in clinical patients. Exposure to cigarette smoke extracts significantly induced p38 MAPK together with nuclear factor- κ B (NF- κ B) activation in airway epithelial cells and macrophages, thereafter inducing the expression of many proinflammatory chemokines and cytokines [26]. Indeed, p38 MAPK activation in alveolar macrophages of the lungs of patients with COPD has been reported [18]. Consistently, in the present study, a significant enhanced expression of the p38 MAPK was observed in the sputum of COPD patients. Notably, the level of sputum p38 MAPK was remarkably correlated with the severity of disease, confirming the critical role of p38 MAPK in the pathogenesis and progression of airway inflammation in this disease. It is likely that the α isoform of p38 MAPK is the most important in the inflammatory response of COPD in the lungs [17].

p38 MAPK was correlated with the CXCL8 in COPD

Extensive evidence indicates the critical involvement of CXCL8 in the pathogenesis and development of several lung diseases [7]. In the lung diseases caused by bacterial or viral infection, exogenous microbes and their production induced CXCL8 protein production in the lung tissue [7], recruiting neutrophils to infection sites to eradicate invasive microbes [27,28]. In bronchioalveolar lavage fluid (BALF) of patients with acute respiratory distress syndrome, significantly increased CXCL8 was observed and correlated with increased neutrophil numbers in BALF [7,29]. In the rodent model and in patients with asthma, CXCL8 directly provokes bronchoconstriction [30] and induces neutrophil infiltration and activation in the airway, preceding

late exacerbation of acute asthmatic attack [31,32]. Similarly, CXCL8 levels, as an indicator of disease activity, significantly increased in both serum and BALF of patients with idiopathic pulmonary fibrosis [33].

Consistently, significantly increased CXCL8 expression, as evidenced by the upregulated mRNA and protein level, was observed in the sputum of COPD patients in the present study. Sputum CXCL8 level was correlated with levels of neutrophil activation markers such as neutrophil myeloperoxidase and elastase, and was inversely correlated with forced expiratory volume and FEV₁% in COPD patients [34–36], indicating that CXCL8 may critically contribute to airway remodeling in COPD patients.

Furthermore, the present study revealed a remarkable correlation between p38 MAPK activity and sputum CXCL8 level in COPD patients, indicating the role of p38 MAPK in the induction of CXCL8 expression and airway inflammation. It was previously reported that infectious bronchitis virus activated the p38 mitogen-activated protein kinase (MAPK) pathway and induced the expression of IL-6 and CXCL8 in cultured human and animal cells [37]. Suppression of p38 MAPK activity inhibited the expression of CXCL8 at the transcription and secretion level in human bronchial epithelial cells [38]. These results suggest that p38 MAPK is a critical factor in modulation of the production of proinflammatory factor (e.g. CXCL8) in airway inflammation in COPD.

p38 MAPK was correlated with airway inflammation in COPD

Diffuse persistent inflammation in the airway is one of the key pathologic features of COPD. Together with proteases and oxidant stimuli, which directly affect lung structures, inflammatory cells actively participate in the pathogenesis of the disease and promote biochemical reactions that result in progressive alteration and remodeling of the lower airways. Typically, neutrophil recruitment to the airways is induced by chemotactic factors like CXCL8 [39] and leukotriene B₄ [40] in COPD patients.

In the present study, sputum level of phospho-p38 MAPK was significantly correlated with the amount of neutrophils,

indicating the possible role of p38 MAPK in the initiation and maintenance of airway inflammation. It was previously reported that p38 α critically participates in the migration of neutrophils, mast cells, and eosinophils induced by several proinflammatory factors [17,41]. Inhibiting activities of p38 MAPK α and β isoform with selective inhibitor SD282 significantly suppressed subchronic tobacco smoke-induced macrophages and neutrophils and cytokine synthesis in murine lung [20]. Transient or long-term administration of the p38 α inhibitor SB681323 significantly decreased the levels of p-HSP27 and TNF- α in whole blood and sputum neutrophils, indicating a remarkable reduction of airway inflammation [17,42–44] in patients with COPD. These studies suggest the pivotal role of p38 MAPK in the induction of airway inflammation in COPD patients, probably through CXCL8 signaling [6,45,46].

p38 MAPK was correlated with the reduced FEV₁% in COPD

In the present study, significantly increased expression of sputum phospho-p38 MAPK was inversely associated with lung function, indicated as the FEV₁%, in patients with COPD. Presence of airflow obstruction, defined as the decreased FEV₁%, is essential in diagnosing COPD in clinical patients [47]. An immunostaining study revealed an inverse correlation between phospho-p38 MAPK-positive alveolar macrophages and FEV₁% in COPD patients [18]. Inhibition of p38 activity for 4 weeks significantly improved the lung function in COPD patients [17,48]. These findings suggest that p38 MAPK is a critical factor in devastation of lung function through the induction of airway inflammation via CXCL8 signaling in patients with COPD.

Conclusions

In conclusion, the present study demonstrated increased p38 MAPK activity in the sputum cells of COPD patients, and p38 MAPK activity was significantly correlated with the progression of airway inflammation and the reduction of lung function in the COPD patients. The present study revealed the pivotal role of p38 MAPK in the development of airway inflammation in COPD patients, and provides significant novel insights into the pathogenesis and treatment of COPD in clinical patients.

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