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Association between Pepsin in Bronchoalveolar Lavage Fluid and Prognosis of Chronic Fibrosing Interstitial Lung Disease

Youlim Kim,[1,2] Yeon Joo Lee,[2,3] Young-Jae Cho,[2,3] Ho Il Yoon,[2,3] Jae Ho Lee,[2,3] Choon-Taek Lee[2,3] and Jong Sun Park[2,3]

[1]Division of Pulmonary, Allergy and Critical Care Medicine, Department of Internal Medicine, Hallym University Chuncheon Sacred Heart Hospital, Chuncheon, Republic of Korea
[2]Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Seoul National University Bundang Hospital, Seongnam, Republic of Korea
[3]Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Republic of Korea

Chronic fibrosing interstitial lung disease (ILD)s are characterized by chronic progressive fibrosis of lung which include idiopathic pulmonary fibrosis (IPF), nonspecific interstitial pneumonia (NSIP), and connective tissue disease-associated interstitial lung disease (CTD-ILD). IPF is an irreversible fibrotic lung disease which results in respiratory failure. Although NSIP and CTD-ILD can be improved or stable by treatment with corticosteroid or immunosuppressant, some of them progress to fibrotic lung diseases. Aspiration of gastric contents is suggested as an aggravating factor of ILDs. We measured pepsin, a marker of gastric aspiration, in bronchoalveolar lavage (BAL) fluid of chronic fibrosing ILD patients to evaluate the association between BAL fluid pepsin and prognosis of chronic fibrosing ILDs. Patients with chronic fibrosing ILDs, who underwent bronchoscopy between December 2010 and April 2015 were prospectively enrolled. Pepsin levels were measured using a commercial ELISA kit. Clinical characteristics, lung function data, and mortality were analyzed. Fifty-one patients with chronic fibrosing ILDs were enrolled (26 with IPF, 15 with NSIP, and 10 with CTD-ILD). Pepsin levels in BAL fluid were 69.87 ± 74.16 ng/mL in IPF, 110.68 ± 94.93 ng/mL in NSIP, and 101.87 ± 88.44 ng/mL in CTD-ILDs. There were no statistically significant differences in BAL fluid pepsin levels among patients with the different chronic fibrosing ILDs. In multivariate regression analysis, higher BAL pepsin levels were associated with higher mortality (adjusted odds ratio [aOR] = 1.021, p = 0.025). BAL fluid pepsin may be used as a prognostic marker for predicting mortality in chronic fibrosing ILD patients.

Keywords: aspiration; bronchoalveolar lavage fluid; interstitial lung disease; pepsin; pulmonary fibrosis
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Introduction

Chronic fibrosing interstitial lung disease (ILD)s are characterized by inflammation and/or fibrosis of the pulmonary parenchyma and include idiopathic pulmonary fibrosis (IPF), nonspecific interstitial lung disease (NSIP), and connective tissue disease-associated ILD (CTD-ILD) (Antoniou et al. 2014). IPF is an irreversible fibrotic lung disease which results in respiratory failure. Although NSIP and CTD-ILD can be improved or remaining stable by treatment with corticosteroid or immunosuppressant, some of them progress to fibrotic lung diseases. Chronic fibrosis of lung can result in progressive deterioration in lung function, worsening respiratory symptoms, deteriorating quality of life, and potential early death (Travis et al. 2013).

Several researches suggested that acid refluxes developed repeatably in IPF patients and acid exposure had an effect on the progression and/or exacerbation of fibrosis (Raghu et al. 2006; Allaix et al. 2014). They thought that aspiration of gastric contents would induce lung injury and inflammatory reactions (Mays et al. 1976; Lee et al. 2010). The acidity of gastric fluid and its constituent enzymes, such as pepsin, are considered to be the essential factors in lung damage (Bathoorn et al. 2011). In fact, abnormal gastro-esophageal reflux is more common (87% to 94%) in individuals with idiopathic pulmonary fibrosis (IPF) (Tobin et al. 1998; Raghu et al. 2006; Savarino et al. 2013), compared with the age- and sex-matched general population (10% to 19%).

Pepsin is stored in the gastric mucosa in its inactive form—pepsinogen—and is activated by acidic environments (Bathoorn et al. 2011). In previous research, pepsin has been reported to be a marker of gastric-to-pulmonary aspiration, and has demonstrated an association with gastro-

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Correspondence: Jong Sun Park, M.D, Ph.D., Seoul National University Bundang Hospital, 82, Gumi-ro 173 Beon-gil, Bundang-gu, Seongnam, Gyeonggi-do 13620, Republic of Korea.
e-mail: jspark.im@gmail.com

esophageal reflux disease and rejection after lung transplantation (Farrell et al. 2006; Stovold et al. 2007). Furthermore, Lee et al. (2010) proposed that recurrent microaspiration may lead to repeated micro-injury of the lung and may cause pulmonary fibrosis. In that study, pepsin levels in bronchoalveolar lavage (BAL) fluid were associated with acute exacerbation of idiopathic pulmonary fibrosis (IPF). However, there are no studies that evaluated pepsin levels in ILDs other than IPF. Moreover, no studied have evaluated whether pepsin levels are associated with the prognosis of chronic fibrosing ILDs.

In this study, we measured pepsin levels in BAL fluid samples from patients with various chronic fibrosing ILDs and evaluated whether they were associated with prognosis in ILDs.

Materials and Methods

Study population and design

The study was conducted prospectively from December 2010 to April 2015 at Seoul National University Bundang Hospital. Patients with chronic fibrosing ILDs (IPF, NSIP, CTD-ILD) were enrolled and underwent bronchoscopy for evaluating the disease. Patients with combined pneumonia, pulmonary tuberculosis, lung malignancy, or other pulmonary diseases were excluded. Pepsin levels in BAL fluid were measured and demographic and clinical data were analyzed. Patients were followed up for longer than 1 year.

This study was conducted in accordance with the amended Declaration of Helsinki. All study participants provided informed written consent, and the Institutional Review Board of Seoul National University Bundang Hospital approved the protocol (no. B-1011-115-001).

Data collection and outcomes

All enrolled patients were reviewed for baseline demographics, history of smoking and medication, clinical data including pulmonary function tests (forced vital capacity, diffusion capacity for carbon monoxide) at baseline. CTD-ILDs were treated by attending physician using corticosteroids or immunosuppressants if CTD-ILDs were aggravated or progressive. To know the effect of proton pump inhibitor (PPI) on pepsin level, use of H2 blockers and/or PPIs were investigated.

The participants underwent BAL via flexible bronchoscopy at the time of admission, and pepsin levels were measured from BAL fluid samples. BAL is a procedure that involves instillation of sterile saline into a sub-segment of the lung, followed by suction and collection of the instillation for analysis. It was performed in a single subsegment of the affected lobe, with at least 100 mL of sterile saline instilled. The BAL fluid was kept on ice and processed within 1 h of collection, then frozen at –80°C. BAL pepsin levels were examined using a commercially available ELISA kit (USCN Life Science, Wuhan, China) (Lee et al. 2012). Using BAL fluid, neutrophil, lymphocyte, eosinophil, and other microbiological culture parameters were analyzed.

Acute exacerbation was defined as the worsening of respiratory symptoms, new-onset ground-glass opacity on high-resolution chest tomography, and no evidence of other causes such as pneumonia, pneumothorax, or heart failure (Raghu et al. 2011). The survival status of the study participants was confirmed from hospital electronic medical record. Information on the additional deaths of patients who were lost to follow-up was supplemented from the Korea National Statistical Office.

Statistical analysis

Data were analyzed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft Corporation, Redmond, USA). Continuous variables are presented as mean and standard deviation, and categorical variables are presented as number and percentage of the total group. Differences in pepsin levels, with and without anti-acid therapy, were assessed using independent t-test analysis, as were differences between survival and non-survival. Chi-square tests were used to compare categorical variables. Risk factors regarding mortality were assessed using multivariate regression analysis. For survival, Kaplan-Meier analysis was performed; $p < 0.05$ was considered as statistically significant.

Results

Baseline characteristics of the study patients

The baseline characteristics of the study groups are shown in Table 1. The mean patient age was 66.7 ± 8.9 years, and more than half of the population was male (n = 30 [58.5%]). Approximately 40% of the patients had a history of smoking, and 4 patients (7.8%) had a history of antacid medication. Patients have prescribed H2 blockers and/or PPIs due to gastroesophageal reflux disease or typical reflux symptoms such as epigastric soreness. IPF was the

Table 1. Baseline characteristics of study groups.

Variables	N = 51
Age (yrs.)	66.7 ± 8.9
Sex (Male, N (%))	30 (58.8)
Smoking Status	
Never-smoker	27 (52.9)
Ex-smoker	20 (39.2)
Unknown	4 (7.8)
Respiratory symptoms	
Cough	30 (58.8)
Dyspnea	39 (76.5)
Anti-acid medication	4 (7.8)
Pulmonary function	
FVC % predicted	70.2 ± 17.8
DLCO % predicted	63.4 ± 21.5
Diagnosis	
Idiopathic pulmonary fibrosis	26 (51.0)
Nonspecific interstitial pneumonia	15 (29.4)
Connective tissue disease-associated ILD	10 (19.6)

Values expressed as mean ± SD, N (%).
BAL, bronchoalveolar lavage; FVC, forced vital capacity; DLCO, diffusion capacity for carbon monoxide.

most common disease, followed by NSIP.

BAL fluid pepsin levels in chronic fibrosing ILDs

The mean pepsin level in patients with IPF was 69.87 ± 74.16 ng/mL, and those of NSIP and CTD-ILD were 110.68 ± 94.93 ng/mL and 101.87 ± 88.44 ng/mL, respectively (Table 2, Fig. 1). BAL fluid pepsin level was not different among various chronic fibrosing ILDs (p = 0.280). The anti-acid therapy did not affect the level of BAL fluid pepsin (Table 3). BAL fluid pepsin levels had a tendency to increase in patients with exacerbation (Table 4).

Comparison of survivor and non-survivor in chronic fibrosing ILDs

The mean follow-up duration was 24.8 ± 23.1 months. Patients who did not survive were much older than those who survived (p = 0.057). Mortality of CTD-ILD was higher than that of other ILDs, because half of them were exacerbation state at the time of enrollment (Tables 4 and 5). The mean pepsin level was higher in the non-survival group (117.8 ± 90.3 ng/mL) compared with the survival group (69.0 ± 74.9 ng/mL) (p = 0.041) (Table 5). The neutrophil count in BAL fluid was also higher in the non-sur-

Table 2. Characteristics and pepsin levels in BAL fluid according to the type of chronic fibrosing interstitial lung disease.

Variables	IPF	NSIP	CTD-ILD	p value
N = 51	26	15	10	
Age, years	67.7 ± 8.8	64.8 ± 10.2	66.7 ± 8.9	0.613
FVC % predicted	71.8 ± 19.1	72.8 ± 16.2	62.7 ± 16.6	0.328
DLCO % predicted	62.3 ± 22.3	71.5 ± 19.6	53.1 ± 19.3	0.127
BAL fluid				
Instilled saline, ml	140.0 ± 20.5	125.0 ± 26.1	135.0 ± 24.1	0.217
Recovered saline, ml	40.3 ± 15.5	40.6 ± 10.4	41.3 ± 18.9	0.986
Pepsin, ng/mL	69.87 ± 74.16	110.68 ± 94.93	101.87 ± 88.44	0.280

Values expressed as mean ± SD.
BAL, bronchoalveolar lavage; FVC, forced vital capacity; DLCO, diffusion capacity for carbon monoxide; IPF, idiopathic pulmonary fibrosis; NSIP, nonspecific interstitial pneumonia; CTD-ILD, connective tissue disease-associated interstitial lung disease.

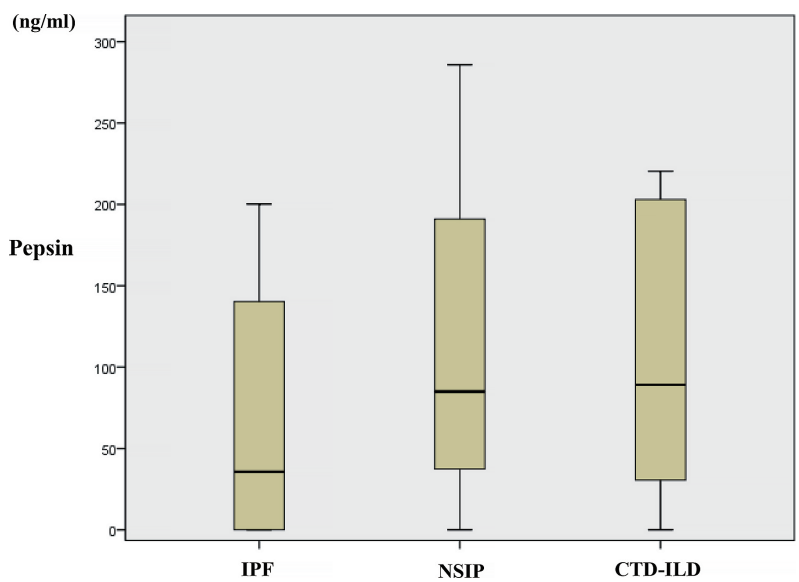

Fig. 1. Pepsin levels in the study patients.
IPF, idiopathic pulmonary fibrosis; NSIP, nonspecific interstitial pneumonia; CTD-ILD, connective tissue disease-associated interstitial lung disease.

Table 3. Bronchoalveolar lavage fluid pepsin level according to the anti-acid therapy.

Variables	No anti-acid treatment*	Anti-acid treatment†	*p* value
N = 51	47	4	
Pepsin, ng/mL	85.77 ± 86.38	116. 09 ± 43.19	0.276

Values expressed as mean ± SD.
*No anti-acid treatment group included 25 patients with IPF, 15 with NSIP, and 7 with CTDILD.
†Anti-acid treatment group included 1 patient with IPF and 3 with CTD-ILD. Abbreviations: IPF, idiopathic pulmonary fibrosis; NSIP, nonspecific interstitial pneumonia; CTD-ILD, connective tissue disease-associated interstitial lung disease.

Table 4. Comparison of patients according to the exacerbation status.

Variables	Stable*	Exacerbation†	*p* value
N = 51	36	15	
Age	65.3 ± 9.3	70.0 ± 7.2	0.057
FVC % predicted	73.5 ± 16.9	60.8 ± 17.7	**0.033**
DLCO % predicted	69.9 ± 19.9	45.9 ± 15.4	**0.001**
Pepsin	73.3 ± 80.4	123.7 ± 84.2	**0.050**
Neutrophil in BAL, %	15.8 ± 20.8	34.2 ± 30.2	**0.044**
Lymphocyte in BAL, %	21.9 ± 23.1	11.1 ± 14.9	0.101

Values expressed as mean ± SD, N (%). Values with statistical significance are in boldface.
*The stable group included 18 patients with IPF, 13 with NSIP, and 5 with CTD-ILD.
†The exacerbation group included 8 patients with IPF, 2 with NSIP, and 5 with CTD-ILD.
FVC, forced vital capacity; DLCO, diffusion capacity for carbon monoxide; IPF, idiopathic pulmonary fibrosis; NSIP, nonspecific interstitial pneumonia; CTD-ILD, connective tissue disease-associated interstitial lung disease.

vival group. It is clear from Fig. 2 that individuals with high levels of pepsin (> 58.4 ng/mL) demonstrated a lower survival rate than those with low pepsin levels ($p = 0.048$).

Risk factors for mortality

Multivariate regression analysis was performed to assess risk factors for mortality in chronic fibrosing ILD patients. The level of pepsin (adjusted odds ratio [aOR] = 1.021; $p = 0.025$) and neutrophil count (aOR = 1.019; $p = 0.050$) in BAL fluid were associated with increased mortality (Table 6).

Discussion

In this study, we obtained BAL fluid samples from patients with chronic fibrosing ILDs and assessed whether there was an association between pepsin levels in BAL fluid and prognosis of ILDs. Our study had two major findings. First, pepsin levels were elevated in BAL fluid obtained from patients with chronic fibrosing ILDs when compared to those reported in a previous study performed in healthy subjects (median concentration of 0.0 ng/ml [Interquartile range (IQR), 0.0-9.4 ng/ml]) (Bohman et al. 2013). BAL fluid pepsin levels were not different among patients with chronic fibrosing ILDs according to the type of ILD. Second, high pepsin level in BAL fluid was associated with mortality in patients with chronic fibrosing ILDs.

The association between pulmonary fibrosis and aspiration has been a long-standing concept (Mays et al. 1976). In previous studies, many researchers have proposed that micro-aspiration of gastric contents is related to microinjury and fibrosis of the lung (Tobin et al. 1998; Raghu et al. 2006). In a group of IPF patients, Tobin et al. (1998) observed acid exposure in the proximal and distal esophagus, and proposed that acid reflux is involved in the pathogenesis of pulmonary fibrosis. The researchers used various tools and molecular markers to monitor acid levels, one of which was pepsin. Pepsin in BAL fluid is a promising marker of aspiration. It is acid-dependent and is converted

Table 5. Comparison between the survived and non-survived.

Variables	Survived	Non-survived*	*p* value
Total group (N = 51)	31	20	
Age (yrs.)	64.8 ± 9.0	69.7 ± 8.2	0.057
FVC % predicted	71.3 ± 17.9	68.2 ± 18.0	0.566
DLCO % predicted	67.3 ± 22.0	56.5 ± 19.5	0.110
Pepsin, ng/mL	69.0 ± 74.9	117.8 ± 90.3	**0.041**
Neutrophil in BAL, /㎕	23.5 ± 50.2	147.9 ± 230.4	**0.027**
Lymphocyte in BAL, /㎕	52.5 ± 105.2	32.9 ± 47.2	0.438

Values expressed as mean ± SD, N (%). Values with statistical significance are in boldface.
BAL, bronchoalveolar lavage; FVC, forced vital capacity; DLCO, diffusion capacity for carbon monoxide; IPF, idiopathic pulmonary fibrosis; NSIP, nonspecific interstitial pneumonia; CTD-ILD, connective tissue disease-associated interstitial lung disease.
*Of the 20 patients who did not survive, 10 had IPF, 2 had NSIP, and 8 had CTD-ILD.

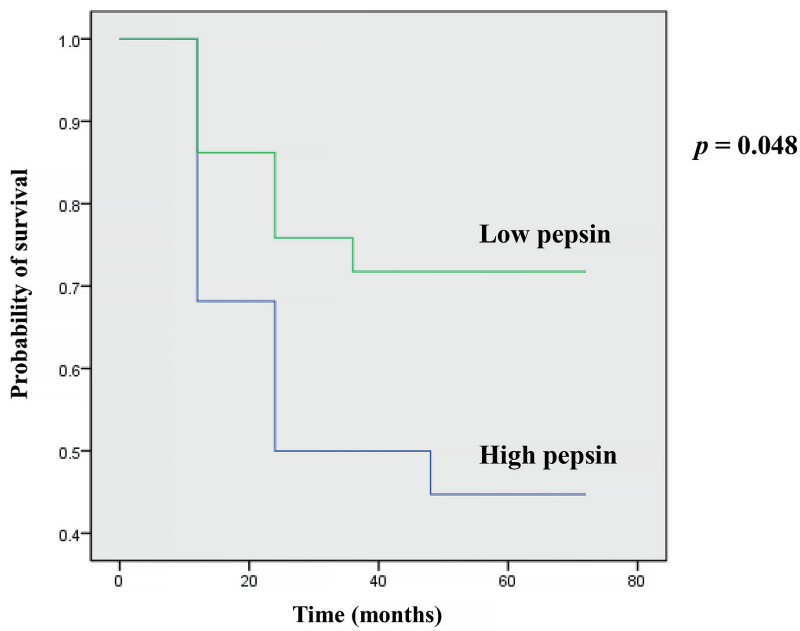

Fig. 2. Kaplan-Meier analysis of survival according to pepsin levels in bronchoalveolar lavage fluid samples.
*A high pepsin level was defined as that more than 58.4 ng/ml. Patients with high pepsin levels were shown to have worse survival rates in Kaplan-Meier analysis ($p = 0.048$).

from its inactive form, pepsinogen, in acidic environments (Bathoorn et al. 2011). Detecting pepsin in BAL fluid was believed to be evidence of micro-aspiration without the occurrence of definite reflux events (Farrell et al. 2006).

Lee et al. (2012) reported that pepsin levels in BAL fluid were increased in patients experiencing acute exacerbation of IPF, suggesting that occult aspiration plays a role in some cases of acute exacerbation of IPF. However, BAL pepsin level was not predictive of survival. In our study, pepsin levels in BAL fluid were measured in patients with chronic fibrosing ILDs, including IPF, and high levels were associated with poor prognosis. Similarly, BAL fluid pepsin levels tended to increase in patients with exacerbation status.

Anti-acid therapy could be a confounding factor affecting pepsin levels. Accordingly, we evaluated the use of PPIs or H_2 blockers at the time of bronchoscopy and determined that BAL fluid pepsin levels did not differ according

Table 6. Regression analysis of risk factors for mortality.

Variables	Adjusted OR	*p* value
Total group		
Age (yrs.)	1.157	0.088
Smoking history (yes)	9.902	0.064
Antacid medication (yes)	0.753	0.846
FVC % predicted	1.052	0.257
DLCO % predicted	0.941	0.137
Pepsin, ng/mL	1.021	**0.025**
Neutrophil count in BAL fluid, /㎕	1.019	**0.050**

Values expressed as mean ± SD, N (%). Values with statistical significance are in boldface.
BAL, bronchoalveolar lavage; FVC, forced vital capacity; DLCO, diffusion capacity for carbon monoxide.

to the use of anti-acid medications.

Previous studies have suggested a relationship between reflux or micro-aspiration and IPF pathogenesis, and the possibility that PPI treatment could improve prognosis in IPF patients (Lee et al. 2011, 2013). However, a recent study reported that PPI treatment did not alter the clinical course of IPF patients (Kreuter et al. 2016). The clinical significance of BAL fluid pepsin levels and the effects of PPI treatment warrant further study.

The most noteworthy aspect of our study was that it was the first to prospectively measure pepsin levels in BAL fluid obtained from patients with various ILDs other than IPF. Moreover, we assessed the association between pepsin level and survival in patients with various ILDs. In the non-survival group, BAL fluid pepsin levels were higher compared with the survival group. This tendency was similar in the fibrotic ILD sub-group.

Limitations to the present study should be considered. First, we did not examine inflammatory cytokines in BAL fluid or blood samples; therefore, we cannot definitively conclude that that high pepsin levels in BAL fluid directly caused inflammation in patients with ILDs. Although, high pepsin levels in BAL fluid was an independent predictor for mortality, it may only represent a simple association and does not confirm a cause-and-effect relationship. Therefore, further studies investigating possible cause-and-effect relationships between BAL fluid pepsin levels and prognosis of ILDs are needed. Second, we did not have normal controls. A recent study reported that airway pepsin levels can be detected in healthy populations that have no risk for aspiration. This study suggested that results of a non-specific pepsin assay to reveal biomarkers for aspiration should be interpreted with caution (Bohman et al. 2013). Third, BAL fluid pepsin levels were examined only one time (at the time of initial evaluation); therefore, changes in BAL pepsin levels according to treatment were not evaluated. Fourth, the sample size of our study was small. Therefore, it was difficult to obtain statistically significant results in some sub-group analyses.

In conclusion, pepsin concentration in BAL fluid was associated with mortality in patients with ILDs, and may be used as a prognostic marker for predicting mortality in chronic fibrosing ILDs.

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Author Contributions

Y.K. contributed to the conception of the study, data collection, data analysis and interpretation, and drafted the manuscript. Y.J.L, and Y.-J.C. contributed to data collection and interpretation and revised the manuscript critically for important intellectual content. H.I.Y, J.H.L, and C.-T.L. contributed to data analysis, interpretation and revised the manuscript critically for important intellectual content. J.S.P. contributed to the conception of the study, data collection, data analysis and interpretation, and drafted the manuscript. All authors approved the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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