

# Serum Levels of TGF- $\beta$ 1 and MCP-1 as Biomarkers for Progressive Coal Workers' Pneumoconiosis in Retired Coal Workers: A Three-year Follow-up Study

Jong Seong LEE<sup>1\*</sup>, Jae Hoon SHIN<sup>1</sup>, Kyung Myung LEE<sup>2</sup>, Ju-Hwan HWANG<sup>1</sup>,  
Jin Ee BAEK<sup>1</sup>, Ji Hong KIM<sup>3</sup> and Byung-Soon CHOI<sup>1</sup>

<sup>1</sup>Occupational Lung Diseases Institute, Korea Workers' Compensation and Welfare Service, Republic of Korea

<sup>2</sup>Forensic DNA Center, NFS, Republic of Korea

<sup>3</sup>Ansan WCI Hospital, Korea Workers' Compensation and Welfare Service, Republic of Korea

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**Abstract:** Various cytokines activated by the inhalation of coal dust may mediate inflammation and lead to tissue damage. Objective of this study was to examine the relationships between coal workers' pneumoconiosis (CWP) progression over a 3 yr period and the serum levels of cytokines in 85 retired coal workers. To investigate the relevance of serum cytokines in CWP, serum levels of interleukin-8 (IL-8), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), and monocyte chemoattractant protein-1 (MCP-1) as progressive CWP biomarkers were studied in relation to the progression of pneumoconiosis over a 3 yr period in 85 patients with CWP. CWP progression was evaluated through paired comparisons of chest radiographs. Median levels of TGF- $\beta$ 1 and MCP-1 were significantly higher in subjects with progressive CWP than in those without CWP progression. The area under the ROC curve for TGF- $\beta$ 1 (0.693) and MCP-1 (0.653) indicated that these cytokines could serve as biomarkers for the progression of CWP. Serum TGF- $\beta$ 1 levels were related to the progression of CWP ( $\beta=0.247$ ,  $p=0.016$ ). The results suggest that high serum levels of TGF- $\beta$ 1 and MCP-1 are associated with the progression of CWP.

**Key words:** Interleukin-8, Monocyte chemoattractant factor-1, Progressive coal workers' pneumoconiosis, Transforming growth factor- $\beta$ 1, Tumor necrosis factor- $\alpha$

## Introduction

Of the various occupational lung diseases, those induced by the inhalation of dusts such as asbestos, crystalline silica, and coal are most prevalent. Dust inhalation can cause a variety of lung diseases such as coal workers'

pneumoconiosis (CWP), progressive massive fibrosis, chronic alveolitis, and emphysema<sup>1</sup>). Notably, crystalline silica has been classified as a class I carcinogen by the International Agency for Research on Cancer<sup>2</sup>). Once a silica threshold has been exceeded, silica-induced pulmonary disease may progress even without further exposure to silica. CWP is a lung disease caused by the inhalation of coal dust. The clinical detection of CWP, however, is currently dependent on radiological and lung function abnormalities, both of which are detectable only in the late

\*To whom correspondence should be addressed.

E-mail address: ljs5075@hanmail.net

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stages of the disease. The identification of accurate and reliable biomarkers would enable earlier detection before irreversible radiological changes occur in the lung<sup>3, 4</sup>.

Cytokines influence various biological events such as inflammation, metabolism, cell growth and proliferation, morphogenesis, fibrosis, and homeostasis. The major sources of cytokines in the lung are epithelial cells, endothelial cells, fibroblasts, and inflammatory cells<sup>5</sup>. Serum cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>6, 7</sup>, Interleukin-8 (IL-8)<sup>6, 8</sup>, transforming growth factor- $\beta$  1 (TGF- $\beta$ 1)<sup>9, 10</sup>, and monocyte chemoattractant protein-1 (MCP-1)<sup>11</sup> have been considered as biomarkers for development or progression of CWP. Although evidence obtained from a various studies on the increased production of these cytokines following exposure crystalline silica or coal mine dust in macrophage and fibroblasts, only a limited number of human validation studies were reported in the literature<sup>1, 6</sup>. These reports suggest the importance of serum cytokines in the CWP, however there are not lots of studied reports about the *in vivo* relevance of predictive discrimination between the levels of cytokines and progression of CWP. To determine the significance of serum cytokines regarding the progression of CWP, a longitudinal design is necessary.

In the present study, we examined the relationship between serum levels of IL-8, TNF- $\alpha$ , TGF- $\beta$ 1, and MCP-1 and CWP findings in retired coal workers with previous cross sectional findings, and examined the relationship between initial levels of same cytokines and 3 yr progressive changes of CWP.

## Subjects and Methods

### *Study design*

A group of 110 male subjects were recruited retired coal workers who were examined for pneumoconiosis for two months at an affiliated hospital of Korea Workers' Compensation & Welfare Service (KCOMWEL) in 2007. We excluded 25 subjects who showed greater than reference serum levels of indices of liver or kidney dysfunction, including aspartate aminotransferase, alanine aminotransferase, or gamma-glutamyl transpeptidase, blood urea nitrogen or creatinine. All of excluded subjects were pneumoconiosis patients (20 cases of 1/0, 4 cases of 1/2, and 1 case of 2/2) and a case showed progressive change (1/0 to 1/1). The progression of CWP was evaluated through paired comparison of chest radiographs made in 2007 and 2010. Ultimately, chest radiographs in 2010 were measured only in 85 subjects, and 85 subjects previously

screened for serum cytokines participated in our follow-up. These subjects were divided into two groups according to the progressive changes identified on chest radiographs (no progression in 67 subjects, progression in 18 subjects).

Pulmonary function was measured in 2007 by using spirometry. The data collected included the forced vital capacity (FVC) and the forced expiratory volume in one second (FEV<sub>1</sub>). The pulmonary function test (PFT) results and personal information, including age, body mass index (BMI), and demographic information (e.g., job and smoking status), were obtained using a questionnaire. All subjects provided their informed consent. The study was approved by the Research Ethics Committee at our institute.

### *Serum cytokine level measurement*

Serum was separated from whole blood samples and stored at  $-80^{\circ}\text{C}$  until the assay. Serum cytokine levels were measured using a bead laser analyzer (Bio-Plex 200, Bio-Rad Laboratories, USA) and sandwich enzyme immunoassays.

### *PFT*

PFT was performed in accordance with the guidelines recommended by the ATS/ERS Task Force<sup>12</sup>) by using a Vmax22 spirometer (SensorMedics, San Diego, CA, USA). The parameters measured included FVC (the volume delivered during an expiration made as forcefully and completely as possible starting from full inspiration), FEV<sub>1</sub> (the volume delivered during the first second of an FVC maneuver), and the FEV<sub>1</sub>/FVC (%FEV<sub>1</sub>/FVC) ratio. The predicted FVC and FEV<sub>1</sub> volumes were calculated using a previously reported equation<sup>13</sup>). Tests of pulmonary function were performed with the patient in the sitting position via closed-circuit method, measuring inhaled and exhaled air during the same test cycle. Tests were carried out until 3 adequate data points had been obtained. The threshold levels of %FVC predicted, %FEV<sub>1</sub> predicted, and the %FEV<sub>1</sub>/FVC ratio were 80%, 80%, and 70%, respectively.

### *Chest radiography*

Chest radiographs were obtained and scored according to the classification rules used by the International Labor Office (ILO 2002)<sup>14</sup>). Classifications of radiographs were decided by the guidelines of the pneumoconiosis review committee of KCOMWEL in Korea. Briefly, radiographs were identified after consensus of classification was acquired between two experienced radiologists.

**Table 1. Patient characteristics**

	NP (n=67) <sup>†</sup>	P (n=18) <sup>c</sup>	<i>p</i> -values
Age, yr	64.8 (41.9–84.0)	63.5 (44.0–78.0)	0.767 <sup>a</sup>
BMI, kg/m <sup>2</sup>	22.7 (17.2–31.6)	23.7 (16.1–26.9)	0.519 <sup>a</sup>
Exposure period, yr	18.2 (7.0–37.0)	16.5 (5.0–35.0)	0.198 <sup>a</sup>
Ex-exposure period, yr	18.0 (0.5–42.0)	18.8 (2.0–31.0)	0.214 <sup>a</sup>
%FVC predicted	91.5 (61.3–125.6)	89.7 (62.3–112.2)	0.250 <sup>a</sup>
%FEV <sub>1</sub> predicted	86.8 (41.9–129.2)	83.6 (50.6–110.4)	0.268 <sup>a</sup>
%FEV <sub>1</sub> /FVC ratio	71.3 (65.2–76.1)	66.7 (43.0–81.8)	0.464 <sup>a</sup>
Smoking, N (%)			
Never	7 (10.4)	4 (22.2)	0.405 <sup>b</sup>
Past	32 (47.8)	8 (44.4)	
Current	28 (41.8)	6 (33.3)	

<sup>†</sup> NP, CWP subjects without progression; P, CWP subjects with progression. <sup>a</sup> Calculated by Mann-Whitney *U* test, median (range). <sup>b</sup> Calculated by  $\chi^2$ -test, number of cases (%). <sup>c</sup> Three-year progression was evaluated by paired comparison of chest radiographs. Category 0 (3 subjects): 0/0 (2) to 1/0, 0/1 (1) to 1/1. Category I (8 subjects): 1/0 (2) to 1/1, 1/1 (3) to 1/2, 1/1 (2) to 2/1, 1/2 (1) to 2/1. Category II (7 subjects): 2/1 (3) to 2/2, 2/2 (3) to 4A, 2/3 (1) to 3/2.

**Table 2. Median levels of serum cytokines as related to CWP progression**

	NP (n=67) <sup>†</sup>	P (n=18) <sup>c</sup>	<i>p</i> -values	
			Univariate <sup>a</sup>	Multivariate <sup>b</sup>
IL-8, pg/ml	14.7 (6.8–38.4)	16.8 (10.5–26.4)	0.056	0.054
TNF- $\alpha$ , pg/ml	2.03 (0.47–18.98)	1.91 (0.73–9.19)	0.817	0.926
TGF- $\beta$ 1, ng/ml	74.4 (30.4–119.8)	90.7 (49.3–122.9)	0.012	0.006
MCP-1, pg/ml	30.6 (10.5–238.7)	37.2 (16.3–64.0)	0.048	0.354

<sup>a</sup> Calculated by Mann-Whitney *U* test, median (range). <sup>b</sup> Calculated using a logistic regression model to adjust for age, BMI, exposure period, smoking status (never/past/current), and pulmonary function (%FVC predicted, %FEV<sub>1</sub> predicted, and %FEV<sub>1</sub>/FVC ratio).

### Statistical analyses

Levels of serum IL-8, TNF- $\alpha$  and MCP-1 were log-normally distributed. The values were log-transformed for parametric statistical tests such as regression analysis. The Mann-Whitney *U* test was used to identify differences between the study groups. A logistic regression model was constructed to compare non-progressive CWP patients with progression cases, then adjusted for age, BMI, exposure period, ex-exposure period, smoking status, and pulmonary function. Receiver operating characteristic (ROC) curve analysis was used to assess the biomarker potential of each cytokine for the discrimination of CWP progression. Statistical “cutoff” values were calculated by minimizing the distance between the point with specificity=1 and sensitivity=1 and various points on the ROC curve. For ROC analysis, an area under the curve (AUC) of 1.0 indicates perfect discrimination, whereas an area of 0.5 indicates that the test discriminates no better than chance. Values of *p*<0.05 were considered statistically sig-

nificant. All statistical evaluations were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA).

## Results

### Study subjects

The general characteristics of the study subjects are shown in Table 1. Among the CWP patients, 67 and 18 subjects were classified as having non-progressive and progressive CWP, respectively. General characteristics, including median age, BMI, exposure period, pulmonary function (%FVC predicted, %FEV<sub>1</sub> predicted, %FEV<sub>1</sub>/FVC ratio), and smoking status did not show difference between the two study groups.

### Serum cytokine levels

Serum cytokine levels as related to CWP progression are shown in Table 2. Median level of TGF- $\beta$ 1 was significantly higher in subjects with progressive CWP

**Table 3. Area under the ROC curve for serum cytokines according to CWP progression**

Cytokines	AUROC	SE	95% CI	<i>p</i> -values	Cut off	Sensitivity (%)	Specificity (%)
IL-8	0.65	0.07	0.52–0.78	0.056	-	-	-
TNF- $\alpha$	0.48	0.09	0.31–0.65	0.817	-	-	-
TGF- $\beta$ 1	0.69	0.07	0.55–0.84	0.012	79.2 ng/ml	61.1	61.2
MCP-1	0.65	0.08	0.50–0.81	0.048	34.3 pg/ml	66.7	67.2

AUROC, area under the ROC curve; SE, standard error; CWP, coal workers' pneumoconiosis; 95% CI, 95% confidence interval. In all subjects (n=85).

**Table 4. Stepwise multiple regression analysis for serum cytokines**

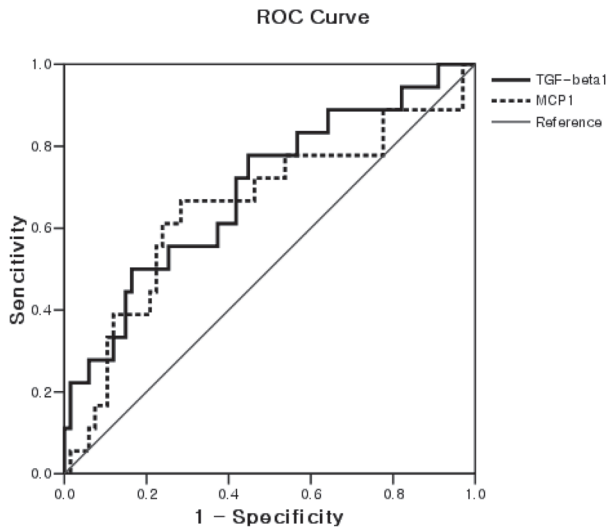
Dependents	Variables		B <sup>†</sup>	SE	$\beta$	<i>p</i> -values
	Independents					
Log TNF- $\alpha$ (pg/ml)	Constant		-0.276	0.205		0.183
	Log IL-8		0.485	0.173	0.295	0.006
	adjusted R <sup>2</sup>		0.076			
	F		7.887 ( <i>p</i> = 0.006)			
Log IL-8 (pg/ml)	Constant		1.105	0.128		0.000
	Log TNF- $\alpha$		0.179	0.058	0.295	0.003
	TGF- $\beta$ 1		0.002	0.001	0.339	0.001
	BMI		-0.011	0.005	-0.240	0.014
	ILO category		0.048	0.024	0.191	0.050
	adjusted R <sup>2</sup>		0.235			
TGF- $\beta$ 1 (ng/ml)	Constant		-129.544	70.618		0.000
	Progression of CWP		12.264	4.961	0.247	0.016
	Log MCP-1		30.060	11.920	0.252	0.014
	%FEV <sub>1</sub> /FVC ratio		2.238	0.969	0.229	0.023
	adjusted R <sup>2</sup>		0.178			
	F		7.069 ( <i>p</i> < 0.001)			
Log MCP-1 (pg/ml)	Constant		1.300	0.069		0.000
	TGF- $\beta$ 1		0.002	0.001	0.259	0.014
	Smoking status		0.084	0.036	0.242	0.022
	adjusted R <sup>2</sup>		0.124			
	F		6.955 ( <i>p</i> = 0.002)			

<sup>†</sup> Subjects: CWP patients with or without progression (n=85). B, regression coefficients; SE, standard error;  $\beta$ , standardized B. Independent variables: age, BMI, exposure period, smoking status (yes/no), ILO category of CWP, progression of CWP (yes/no), %FVC predicted, %FEV<sub>1</sub> predicted, %FEV<sub>1</sub>/FVC ratio, and measured serum cytokines.

(90.7 ng/ml vs. 74.4 ng/ml) (*p*=0.012). This effect held true in the adjusted logistic regression model (*p*=0.006 for all comparisons). Although median level of MCP-1 was significantly higher in subjects with progressive CWP (37.2 pg/ml vs. 30.6 pg/ml) (*p*=0.048), this effect disappeared upon analysis with the adjusted logistic regression model (*p*=0.842). Although median levels of IL-8 and TNF- $\alpha$  were similar in subjects with and without progressive CWP, the median IL-8 level tended to increase in association with the progression of CWP (14.7 pg/ml vs. 16.8 pg/ml, *p*=0.056).

#### ROC analysis of serum cytokine levels

The area under the ROC curve for serum cytokine levels as related to the progression of CWP is shown in Table 3 and Fig. 1. The AUC of the ROC curve for serum TGF- $\beta$ 1 level was 0.69 (95% CI, 0.55–0.84). A serum TGF- $\beta$ 1 level of 79.2 ng/ml was determined as the optimal cutoff value with resulting sensitivity and specificity of 61.1% and 61.2%, respectively. The AUC of the ROC curve for serum MCP-1 levels was 0.65 (95% CI, 0.50–0.81). A serum MCP-1 level of 34.3 pg/ml was determined as the optimal cutoff value with resulting sensitivity and specific-



**Fig. 1. ROC curve for TGF- $\beta$ 1 and MCP-1 according to progression of CWP (n=85). The areas under the ROC curve for TGF- $\beta$ 1 and MCP-1 were 0.69 (95% CI, 0.55–0.84,  $p=0.012$ ) and 0.65 (95% CI, 0.50–0.81,  $p=0.048$ ), respectively. The optimal cutoff values of TGF $\beta$ 1 and MCP1 for the progression of pneumoconiosis were 79.2 ng/ml (sensitivity and specificity of 61.1% and 61.2%) and 34.3 pg/ml (sensitivity and specificity of 66.7% and 67.2%), respectively.**

ity of 66.7% and 67.2%, respectively.

#### *Relationships between serum cytokine levels and associated variables*

Stepwise multiple regression analysis (Table 4) showed that serum TGF- $\beta$ 1 levels were related to the progression of CWP ( $\beta=0.247$ ,  $p=0.016$ ), %FEV1/FVC ratio ( $\beta=0.229$ ,  $p=0.023$ ) and log MCP-1 levels ( $\beta=0.252$ ,  $p=0.014$ ) (adjusted  $R^2=0.178$ ,  $p<0.001$ ). Serum levels of IL-8, TNF- $\alpha$ , and MCP-1 were not related to the progression of CWP.

## Discussion

CWP results from exposure to coal mine dust and is characterized by a progressive fibrotic reaction in the lung that can cause functional damage and irreversible changes<sup>1</sup>. Pulmonary fibrosis is an irreversible accumulation of connective tissue in the interstitium of the lung. Research on animal models and studies of human lung disease suggest that the initiating events may be a combination of pulmonary injury and the recruitment of inflammatory cells<sup>15</sup>.

The identification of biomarkers that are accurate and reliable in the prediction and early detection of CWP is imperative for the implementation of timely intervention strategies<sup>3</sup>. The present study was performed using prospective findings collected over a 3 yr period (2007–2010)

from retired coal workers who were examined for pneumoconiosis. Serum levels of inflammatory cytokines may be elevated in these status<sup>16, 17</sup>. In the present study, we excluded subjects who showed greater than reference serum levels of indices of liver-kidney dysfunction.

IL-8 is a chemokine secreted by a variety of cells, including fibroblasts, in response to IL-1 and TNF- $\alpha$  stimulation and is an important activator and chemoattractant for neutrophils. The accumulation of inflammatory leukocytes in the lung is hallmark of either acute or chronic pulmonary inflammation<sup>18</sup>. IL-8 is one of the important mediators in the lung inflammation induced by crystalline silica<sup>19, 20</sup>. In present study, we found that levels of IL-8 levels were related to those of serum TNF- $\alpha$  ( $\beta=0.295$ ,  $p=0.003$ ). Levels of IL-8 were reportedly elevated in the supernatants of spontaneous or dust-stimulated monocytes isolated from peripheral blood and sera from non-progressing CWP patients<sup>8</sup>. We previously found that serum levels of IL-8 were related to the presence of CWP, but were not related to the progression of CWP<sup>6</sup>. In the present study, although no difference between study groups was identified, serum IL-8 levels tended to increase in association with CWP progression ( $p=0.056$ ) and ILO category ( $\beta=0.191$ ,  $p=0.050$ ). Future studies will be required to ascertain the role of IL-8 for the progression of CWP using more longitudinal follow-up. TNF- $\alpha$  is a proinflammatory cytokine, important in the early onset of inflammation, development, and progression of several diseases, including pulmonary fibrosis<sup>1, 3</sup>. Workers who showed abnormally high dust-stimulated levels of TNF- $\alpha$  had an increased risk of CWP progression. Serum TNF- $\alpha$  levels were reported to be a powerful tool for the estimation of disease progression in patients with coal dust-induced pneumoconiosis, even after any occupational exposure had ended<sup>19</sup>. In our previous study, high levels of serum TNF- $\alpha$  are associated with the progression of CWP in 1 yr follow-up. In the present study, however, serum TNF- $\alpha$  levels were not related to progression of CWP. Future studies will be required to ascertain the role of TNF- $\alpha$  for the progression of CWP using more longitudinal follow-up.

The major sources of TGF- $\beta$  proteins are blood platelets, alveolar macrophages, monocytes, and neutrophils<sup>21</sup>. TGF- $\beta$  is expressed in injured lung tissue and produced by inflammatory cells removed from the lung. TGF- $\beta$  production is increased prior to collagen synthesis. The cytokine is mainly produced by alveolar macrophages and can be deposited in epithelial cells in areas of lung regeneration and remodeling. Borm and Schins<sup>22</sup> proposed that



TGF- $\beta$ 1 release from silica-stimulated monocytes was significantly lower than baseline levels of release. These previous studies have demonstrated that TGF- $\beta$  has anti-fibrotic properties and that its pro/anti-fibrotic action may be concentration dependent<sup>7)</sup>. Vanhee *et al.*<sup>10)</sup> reported that TGF- $\beta$  levels were significantly higher in the BAL fluid of patients with simple pneumoconiosis in comparison with those in control subjects and patients with progressive massive fibrosis. However, TGF- $\beta$ 1 continues to be regarded as the most important of the growth factors involved in pulmonary fibrogenesis<sup>9)</sup>. Notably, aberrant tissue repair may lead directly to progressive fibrosis<sup>15)</sup>. Gosset *et al.*<sup>23)</sup> reported that coal and silica dust-stimulated TGF release was significantly higher in workers than in non-exposed individuals. Jagirdar *et al.*<sup>24)</sup> reported that scar tissue in the progressive massive fibrosis (PMF) lesions contained TGF- $\beta$ . These data suggest a major role for TGF- $\beta$  in silicosis, particularly in the development of PMF. In fetal monkey study<sup>25)</sup>, over expression of TGF- $\beta$ 1 within lung results in severe and progressive fibrosis in lung parenchyma and pleural membrane. In the present study, serum TGF- $\beta$ 1 level in subjects with progressive CWP were higher than those in patients without progressive CWP (90.7 ng/ml vs. 74.4 ng/ml,  $p=0.012$ ). The ROC curve analyses identified TGF- $\beta$ 1 (AUC=0.69) levels as predictive of CWP progression. The associated threshold was 79.2 ng/ml (sensitivity, 61.1%; specificity, 61.2%). Applying stepwise multiple regression analysis, serum TGF- $\beta$ 1 levels were correlated with the progression of CWP ( $\beta=0.247$ ,  $p=0.016$ ) and the %FEV1/FVC ratio ( $\beta=0.229$ ,  $p=0.023$ ). These results suggest that levels of serum TGF- $\beta$ 1 might serve as biomarkers for the progression of CWP and the decreasing of lung function.

MCP-1 is a potent chemotactic factor for blood monocytes<sup>26)</sup> and produced by various inflammatory cells<sup>27)</sup>. MCP-1 may play an important role in the development of pulmonary fibrosis, but not in the initiation of acute lung inflammation<sup>28, 29)</sup>. MCP-1 levels are reported to increase in the bronchoalveolar lavage fluid of CWP patients<sup>11)</sup>. MCP-1 may also be involved in the development of chronic macrophagic inflammation in CWP patients. This process stems from the pulmonary overproduction of MCP-1. Fibroblasts and type II cells might also mediate the enhanced production of MCP-1 in CWP patients. However, no human study has been performed in patients with progressive CWP. In the present study, the median serum MCP-1 level in subjects with progressive CWP was higher than that in patients without progressive CWP (37.2 pg/ml vs. 30.6 pg/ml,  $p=0.048$ ). The AUC values for the

predictive value of MCP-1 (0.65) identified the cytokine as a reasonable biomarker. The cutoff MCP-1 value for the progression of pneumoconiosis was 34.3 pg/ml (sensitivity, 66.7%; specificity, 67.2%). Stepwise multiple regression analysis showed that serum MCP-1 levels correlated with serum TGF- $\beta$ 1 levels ( $\beta=0.259$ ,  $p=0.014$ ). These results suggest that levels of serum MCP-1 might serve as a biomarker for the progression of CWP.

The study has several limitations. One is that the follow-up period of 3 yr was insufficient to draw conclusions about the long-term progression of CWP. Another limitation concerns the lack of data on co-factors such as serum leptin (for TNF- $\alpha$ )<sup>30, 31)</sup> and neurotrophic factor (for IL-8)<sup>32)</sup>. Serum cytokines levels can be measured by various techniques, the enzyme-linked immunosorbent assay, flow cytometry, and a biochip array<sup>33)</sup>. The optimal cutoff values may, therefore, differ depending on the method of analysis. In the current study, serum levels of TGF- $\beta$ 1 and MCP-1 tended to increase in association with CWP progression. The ROC analysis identified TGF- $\beta$ 1 and MCP-1 levels as potential biomarkers.

In conclusion, high serum levels of TGF- $\beta$ 1 and MCP-1 are associated with the progression of CWP. Further longitudinal follow-up studies are needed to investigate the potential of serum cytokines for use as CWP biomarkers. Future studies will be required to ascertain the cytokine profiles of bronchoalveolar lavage fluid, exhaled breath condensate, and lung tissue.

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