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ORIGINAL RESEARCH

Serum Osteopontin, KL-6, and Syndecan-4 as Potential Biomarkers in the Diagnosis of Coal Workers' Pneumoconiosis: A Case–Control Study

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Background: Coal worker's pneumoconiosis (CWP) is a chronic occupational disease mainly caused by coal dust inhalation in miners. This study aimed to investigate the clinical value of Osteopontin (OPN), KL-6, Syndecan-4 and Gremlin-1 as serum biomarkers in CWP.

Patients and Methods: We integrated reported lung tissues transcriptome data in pneumoconiosis patients with silica-exposed alveolar macrophage microarray data to identify four CWP-associated serum biomarkers. The serum concentrations of Osteopontin, Krebs von den Lungen-6 (KL-6), Syndecan-4 and Gremlin-1 were measured in 100 healthy controls (HCs), 100 dust-exposed workers (DEWs) and 200 patients of CWP. Receiver operating characteristic (ROC) curve analysis was used to determine the sensitivity, specificity, cut-off value and area under the curve (AUC) value of biomarkers.

Results: The pulmonary function parameters decreased sequentially, and the serum OPN, KL-6, Syndecan-4 and Gremlin-1 concentrations were increased sequentially among the HC, DEW and CWP groups. Among all participants, multivariable analysis revealed that these four biomarkers were negatively correlated with the pulmonary function parameters (all $p<0.05$). Compared with HCs, patients with higher OPN, KL-6, Syndecan-4 and Gremlin-1 had higher risk for CWP. The combination of OPN, KL-6, and Syndecan-4 can improve the diagnostic sensitivity and specificity of CWP patients differentiated from HCs or DEWs.

Conclusion: OPN, KL-6 and Syndecan-4 are novel biomarkers that can be used for CWP auxiliary diagnosis. The combination of three biomarkers can improve the diagnostic values of CWP.

Keywords: coal workers' pneumoconiosis, biomarker, osteopontin, Krebs von den Lungen-6, Syndecan-4, Gremlin-1

Introduction

Coal worker's pneumoconiosis (CWP) is an incurable but preventable chronic lung disease caused by long-term inhalation of coal dust.[1] It is a kind of fibrous lung disease characterized by chronic inflammatory response caused by activation of macrophages and endothelial cells in the lung.[2] Despite decades of prevention efforts, CWP remains a public health problem worldwide.[3] China is a country with a high incidence of occupational diseases. By the end of 2018, there were more than 975,000 cases of occupational diseases, of which 873,000 were pneumoconiosis, accounting for about 90% of the total reported occupational diseases.[4] The new cases diagnosed pneumoconiosis were 19,468 in 2018.[5] Due to the low performance of occupational health monitoring and relatively strict diagnostic criteria for

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pneumoconiosis, the actual number of cases is likely to be much higher than the reported.[6] At present, the clinical diagnosis of CWP mainly depends on occupational exposure history and radiological abnormalities. Because the disease is progressive and incurable, early diagnosis or early prevention is especially important to control this disease. Previous studies showed elevated levels of serum markers such as IL-6, IL-8, propylparaben, TGF-β1 in patients with pneumoconiosis[7–9] and indicated the potential role of serum biomarkers for the early diagnosis. However, although these biomarkers show promise, they have not yet been applied in clinical settings. Therefore, further exploration of novel biomarkers is necessary to establish a foundation for future clinical applications.

As previously reported,[10–12] after integrating analysis of published lung tissue transcriptome data in pneumoconiosis patients and silica-exposed alveolar macrophage microarray data, we found that four serum biomarkers (Osteopontin [OPN], KL-6, Syndecan-4 and Gremlin-1) were potentially associated with CWP. Study revealed that patients with pneumoconiosis exhibited elevated expression of OPN and Gremlin-1 in their lung tissue.[10] Previous study suggested that Syndecan-4 highly expressed in cultured alveolar macrophages with silica exposure.[11] A previous study showed that serum KL-6 concentrations were elevated in patients with asbestosis.[12] Therefore, these biomarkers are all associated with pneumoconiosis.

OPN is a phosphorylated acidic glycoprotein that can be secreted by lots of cells, including immune cells, osteoblasts, inflammatory cells, fibroblasts, and cancer cells.[13] It is also considered to be a key cytokine that regulates various physiological and pathophysiological processes, such as inflammatory infiltration, tissue reconstruction, and tissue fibrosis.[14] Studies have shown that OPN is highly induced in the lungs of mice exposed to carbon nanotubes, and plays a key role in TGF-β1 signaling activation and myofibroblast differentiation, and promotes the formation of pulmonary fibrosis.[14]

Krebs von den Lungen-6 (KL-6) is a mucin-like glycoprotein mainly expressed in damaged or regenerating bronchial epithelial cells and alveolar type II pneumocytes.[15] KL-6 also acts as a chemokine for human fibroblasts, and high concentrations of KL-6 were found in epithelial lining fluid to trigger intra-alveolar fibrosis.[16] KL-6, as a marker, has potential value in the clinical evaluation of ILD.[15,17]

Syndecan-4, a member of the transmembrane heparin sulfate proteoglycan family, binds to extracellular effector molecules and regulates cellular processes such as tissue homeostasis, inflammation, fibrosis, and tumor metastasis.[18–20] Syndecan-4 has been shown to express on numerous cells, such as alveolar macrophages, fibroblasts, endothelial cells, and epithelial cells.[21–23] Interestingly, conflicting results have been reported. One study has shown that Syndecan-4 can inhibit the development of pulmonary fibrosis in mice by attenuating TGF-β signaling pathway,[23] while another study revealed that Syndecan-4 can promote TGF-β-induced epithelial mesenchymal transition (EMT) in A549 cells.[24]

Gremlin-1 is one of the bone morphogenetic protein (BMP) antagonist and contains a cysteine knot, which is a member of the cysteine knot superfamily.[25] Gremlin-1 is considered to be a multifunctional protein that may have a variety of environmental and cell-related functions. It was demonstrated that there were increased mRNA levels of Gremlin-1 in lung tissues of IPF patients and bleomycin-induced mouse fibrosis models,[26] and that Gremlin-1 played an important role in the pathogenesis of idiopathic pulmonary fibrosis (IPF).[27] In addition, Gremlin-1 mRNA levels were upregulated in the lungs of mice exposed to asbestos[28] and patients with pneumoconiosis.[10]

Although the relationship between OPN, KL-6, Syndecan-4, Gremlin-1 and pulmonary fibrosis has been reported, the role of these biomarkers in the diagnosis and staging of pulmonary fibrosis and CWP has not been studied except limited evidence from KL-6. Therefore, the purpose of this study is to explore the relationship between four biomarkers and CWP.

Materials and Methods

Study Population

From February 2019 to July 2021, we enrolled a total of 400 subjects, of which 100 healthy controls (HCs) were from the health monitoring center of Sinopharm Tongmei general hospital, and 100 dust-exposed workers (DEWs) and 200 coal workers' pneumoconiosis patients (CWPs) were recruited from Sinopharm Tongmei General Hospital. All CWP patients were diagnosed according to China National Diagnostic Criteria for pneumoconiosis (GBZ70-2015), which was

consistent with the judgment of opacity profusion according to the 1980 International Labour Organization (ILO) Classification of Pneumoconiosis, and all the enrolled patients completed chest CT, X-ray and physical examination. According to the size, number and distribution of the opacity profusion on high kilovolt chest X-ray, CWP patients were divided into stage I, stage II and stage III. The chest X-rays were evaluated by three independent physicians with national certification and agreement by at least two independent physicians.

Diagnostic criteria for stage I of CWP: The overall profusion of small opacities is category 1, and the distribution of range reaches at least two zones of lungs. Diagnostic criteria for stage II of CWP: (1) The overall profusion of small opacities is category 2, and the distribution of range reaches more than four zones of lungs; (2) Or the overall profusion of small opacities is category 3, and the distribution of range reaches four zones of lungs. Diagnostic criteria for stage III of CWP: (1) Large opacities appear, with the long diameter not less than 20mm and the short diameter greater than 10mm; (2) Or the overall profusion of small opacities is category 3, and the distribution of range reaches more than four zones of lungs and small opacities aggregation appear; (3) Or the overall profusion of small opacities is category 3, and the distribution of range reaches more than four zones of lungs and large opacities appear.

The inclusion criteria of CWPs include: (1) male miners with a history of coal dust exposure for 10 years or more and diagnosed with CWP; (2) Over than 40 years old. The exclusion criteria of CWPs include: (1) other major respiratory diseases, such as other interstitial lung disease, lung cancer, and tuberculosis. (2) Comorbidity such as congestive heart failure, rheumatic system-related diseases, liver fibrosis, renal fibrosis and other related fibrosis diseases. (3) Previous history of other systemic malignancies or chemotherapy or radiotherapy. The inclusion criteria of DEWs included: (1) male miners with a history of coal dust exposure for 10 years or more and did not diagnosed with CWP. (2) Over than 40 years old. The exclusion criteria are consistent with CWPs. The inclusion criteria of HCs included: (1) Healthy male without a history of coal dust exposure. (2) Over than 40 years old. The exclusion criteria are consistent with CWPs.

All the study population lived and worked in Datong, and they were matched in age, gender, BMI among the 3 groups, and duration of coal dust exposure between DEWs and CWPs. This study was approved by the Institutional Ethics Committee, and written informed consent was obtained from all subjects in accordance with Helsinki declaration (NO. 2017–25-1; NO. 201902).

Data Collection

Experienced interviewers used structured questionnaires to interview the participants. The contents of the questionnaire include personal general characteristics (age, height, weight, family history, disease history), occupational history (dust exposure time, type of work, personal protection), occupational diseases (CWP stage, date of first diagnosis, disease progression), and lifestyle (smoking history, number of cigarettes, quit smoking or not). Approximately 10 mL of fasting venous blood was collected from each participant during the hospitalization. Serum was obtained by centrifugation at 2000 rpm for 10 min and stored at −80 °C until assayed.

The pulmonary function test was conducted according to the guidelines of the physiological experimental group of the hospital pulmonary function room, which is consistent with the guidelines recommended by the ATS/ERS task force.[29] The measured parameters include FEV1 (the maximal volume of air exhaled in the first second of a forced expiration from a position of full inspiration), FVC (the maximal volume of air exhaled with maximally forced effort from a maximal inspiration), the percent of predicted FEV1 (FEV1% predicted), the percent of predicted FVC (FVC % predicted), and the FEV_1 / FVC ratio (% FEV_1 / FVC).

Screening of Target Biomarkers

Our team previously published transcriptome data about lung tissues of 10 silicosis patients obtained when they received lung transplantation and lung tissues of 7 non-diseased donors from a Chinese Han population.[10] We compared the top 20 differentially up-regulated genes between the two groups, analyzed and tested these 20 differentially up-regulated genes on UniProt (https://www.uniprot.org), and found that 8 of them could exist in the form of extracellular region or secreted. Finally, by searching the functions of these 8 genes, the top 2 genes involved in inflammation and fibrosis are OPN and Gremlin-1.

Li et al published microarray data about the genetic profile in cultured alveolar macrophages with silica exposure.[11] We compared the top 20 differentially up-regulated genes between the silica exposure and vehicle control groups, analyzed these 20 differentially up-regulated genes on UniProt (https://www.uniprot.org), and found that 4 of them could exist in the form of extracellular region or secreted. Finally, by searching the functions of these 4 genes, the top 1 gene involved in inflammation and fibrosis is Syndecan-4.

Because a previous study reported that the concentration of KL-6 in the serum of asbestosis was increased,[12] our experiment used it as a control and verified its results in CWP. Therefore, this study focuses on the potential value of four biomarkers in the diagnosis of CWP.

Measurements of OPN, KL-6, Syndecan-4 and Gremlin-1 Concentrations

The serum OPN, KL-6, Syndecan-4 and Gremlin-1 concentrations were measured by commercially available ELISA kits (OPN kit lot: RX105857H; KL-6: RX106444H; Syndecan-4: RX105979H; Gremlin-1: RX101950H; Ruixinbio, Quanzhou, China). Absorbance at 450 nm was measured using the Rayto RT-6100 (Rebio, Los Angeles, USA). All assays were conducted in strict accordance with the manual procedures provided by the kits.

Statistical Analysis

All data were analyzed using SPSS 26.0 software (IBM, New York, USA) and GraphPad Prism 8 software (University of California San Diego, California, USA). The differences among the three groups were tested by Kruskal–Wallis test or one-way ANOVA. Differences in demographic characteristics and selected variables between two groups were calculated by Student's *t*-test. Pearson χ^2 test was used for categorical variables. Logistic analysis was used to analyze the association between biomarkers and the risk of CWP, while linear regression was applied to analyze the association between biomarkers and pulmonary function parameters. Receiver operating characteristic (ROC) curve was used to analyze the serum biomarker levels and determine the cut-off value that resulted in obtaining the optimal diagnostic accuracy of each biomarker between CWP group and DEW group or HC group. A p value of <0.05 was considered statistically significant.

Results

Basic Characteristics of the Study Population

The basic characteristics for all study population are shown in Table 1. There was a significant difference in smoking status (p<0.001), smoking index (p=0.012), and pulmonary function parameters (p<0.001) among HCs, DEWs and CWP

Table 1 Basic Characteristics of the Study Population

Characteristics	Health Controls	DEWs	CWPs	*p*-value
Age, years	60.45±9.07	61.56±9.09	62.71±8.86	0.115
BMI	23.60±2.95	23.75±3.39	22.88±3.25	0.062
Smoking status (N, %)				<0.001
Non-smokers	30 (30.00)	17 (17.00)	19 (8.00)	
Smokers	70 (70.00)	83 (83.00)	181 (92.00)	
Smoking index	21.55±19.19	26.28±24.55	29.45±23.20*	0.012
Duration of exposure, years	NA	22.64±8.53	23.61±7.83	0.222
Pulmonary function				
FVC observed (L)	3.72±0.65	3.48±0.85	2.82±0.84*#	<0.001
FVC% predicted	95.15±13.30	91.32±19.27	79.30±20.42*#	<0.001
FEV1 observed (L)	2.84±0.54	2.55±0.75*	1.80±0.73*#	<0.001
FEV1% predicted	91.66±13.30	84.50±21.83*	64.84±23.70*#	<0.001
FEV1/FVC (%)	76.20±4.17	72.51±9.15*	62.34±13.55*#	<0.001

Notes: *p* value was computed by Kruskal–Wallis test among HCs, DEWs, and CWPs; *p* value was calculated by Mann–Whitney test between two groups. *Compared with Health controls, p < 0.05; #Compared with DEWs, p < 0.05. Data are presented as mean ±SD.
Abbreviations: BMI, body mass index; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; DEWs, dust-exposed workers; CWPs, coal workers' pneumoconiosis; N, number.

patients. The proportion of smokers and smoking index increased sequentially among the three groups and were highest in CWP patients. The pulmonary function decreased sequentially among the three groups. As expected, the CWP patients had the lowest FEV1 observed, FVC observed, FEV1% predicted, FVC % predicted, and FEV1/FVC ratio, which was significantly different from DEWs or HCs.

Serum Concentrations of OPN, KL-6, Syndecan-4 and Gremlin-1

As shown in Figure 1 and Table S1, the serum OPN, KL-6, Syndecan-4 and Gremlin-1 concentrations increased sequentially among the three groups, and were higher in the DEW group, highest in the CWP group. These four biomarkers' concentrations in CWP patients showed remarkable difference from those in HCs ($p<0.001$). When comparing with DEW group, level of OPN, KL-6, Syndecan-4 were significantly higher in CWP group ($p<0.001$). The serum concentration of Gremlin-1 was higher in CWP group, although it did not reach statistical significance ($p=0.158$). No significant differences were found in the serum OPN, KL-6, Syndecan-4 and Gremlin-1 concentrations among the different stages of CWP. As shown in Figure 2A, serum OPN, KL-6, Syndecan-4 and Gremlin-1 were positively associated with each other.

In addition, we divided CWP patients into three group according to their types of pulmonary ventilation dysfunction, which are obstructive pattern of impairment (OPI), restrictive pattern of impairment (RPI), and mixed pattern of impairment (MPI). We analyzed the serum biomarker levels of patients with different types of pulmonary ventilation dysfunction, and found that there was no significant difference between different groups (Figure S1). The level of Syndecan-4 and KL-6 was a little higher in patients with RPI.

Correlations Between Biomarkers and Pulmonary Function

Interestingly, these four biomarkers were negatively correlated with the pulmonary function (FVC observed, FEV1 observed and FEV1/FVC ratio) among all participants (Figure 2B). After adjusted for age, BMI, duration of exposure and smoking index, these pulmonary function parameters significantly decreased with the increase of biomarkers' concentration (all $p < 0.001$) (Table 2). There was no significant correlation between biomarkers and pulmonary function in CWP group alone or DEW group alone (Tables S2 and S3).

Associations Between Biomarkers (OPN, KL-6, Syndecan-4 and Gremlin-1) and CWPs

As shown in Table 3, compared with HCs, univariate logistic regression analysis showed that all four biomarkers were positively correlated with CWPs, and the correlation was more strongly after adjusted for other confounding factors. The adjusted odds ratio (OR) of OPN, KL-6, Syndecan-4 and Gremlin-1 for CWP were 3.88 (95% CI: 2.34–6.42), 1.01 (1.01–1.02), 4.26 (2.78–6.52) and 1.01 (1.00–1.01), respectively. Compared with DEWs, univariate logistic regression analysis showed that only Gremlin-1 was not associated with CWP risk, which was consistent with multivariate logistic regression analysis. The adjusted OR (95% CI) of OPN, KL-6 and Syndecan-4 for CWP were 1.53 (1.34–1.75), 1.01 (1.00–1.01) and 1.40 (1.16–1.69), respectively.

ROC Curve Analysis for Identification of CWP

ROC curve analysis was used to evaluate the discriminatory power of the serum biomarkers to differentiate CWP patients from HCs and DEWs. Compared with HCs, the AUC for OPN, KL-6, Syndecan-4 and Gremlin-1 were 0.970, 0.924, 0.917, 0.868, respectively. The cut-off values for OPN, KL-6, Syndecan-4 and Gremlin-1 were 10.58ng/mL, 674.83 U/mL, 5.76 ng/mL, and 777.3 ng/mL, respectively. The diagnostic sensitivity and specificity were 85.5% and 93.0% for OPN, 82.0% and 94.0% for KL-6, 77.5% and 93.0% for Syndecan-4, 89.5% and 67.0% for Gremlin-1, respectively (Table 4 and Figure 3A). The AUC for OPN was larger than that for other three biomarkers, indicating that the order of diagnostic accuracy by the ROC curve was OPN, KL-6, Syndecan-4 and Gremlin-1 when distinguishing CWP patients from HCs (Figures 3A and S2).

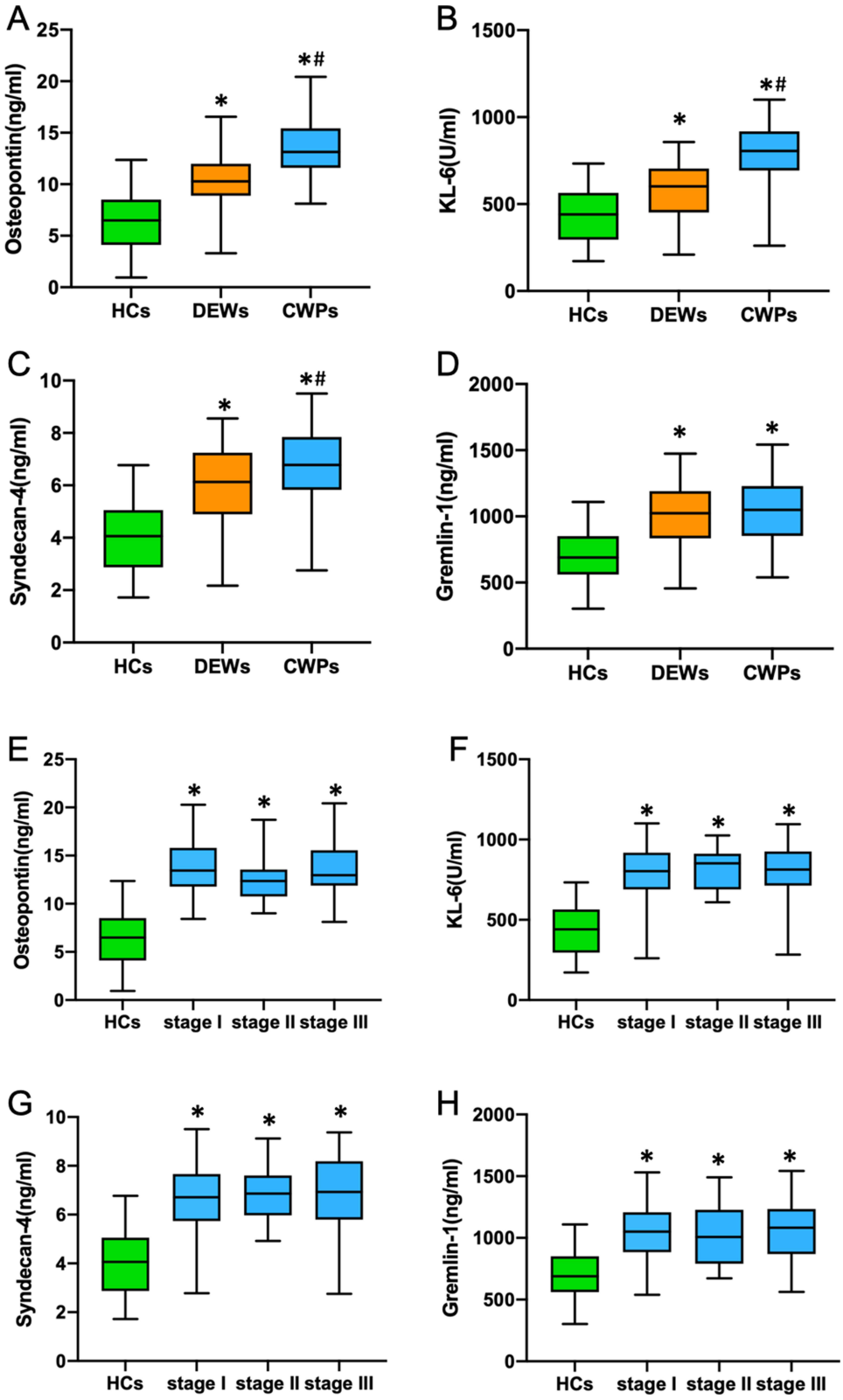

Figure 1 Comparison of serum concentrations of biomarkers among the HCs, DEWs and CWPs groups. (**A**) Osteopontin; (**B**) KL-6; (**C**) Syndecan-4; (**D**) Gremlin-1; (**E**) Osteopontin; (**F**) KL-6; (**G**) Syndecan-4; (**H**) Gremlin-1. *Compared with HCs, $p < 0.05$; #Compared with DEWs, $p < 0.05$. *p* value was computed by Kruskal–Wallis test among different stage of CWPs in Osteopontin, KL-6, Syndecan-4 and Gremlin-1. *p* >0.05. Bars represent median with interquartile 25–75, min-maximum.

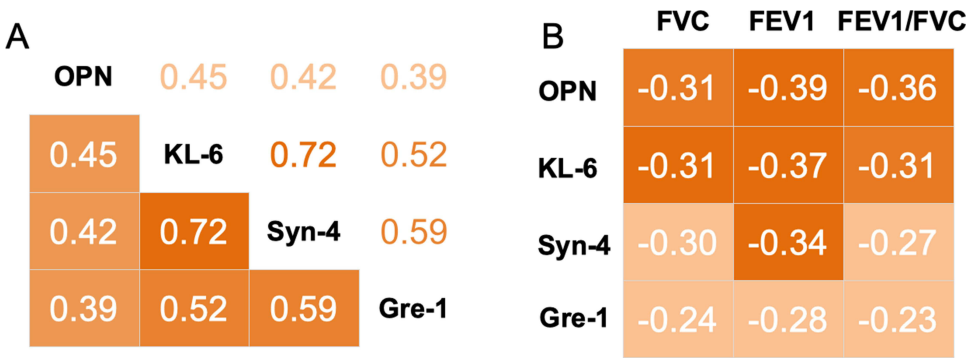

Figure 2 (**A**) Correlations among Osteopontin, KL-6, Gremlin-1 and Syndecan-4 in all participants. (**B**) Correlations between Osteopontin, KL-6, Gremlin-1, and Syndecan-4 and pulmonary function parameters in all participants. The numbers represent the value of Pearson coefficient.

Compared with DEWs, we found that the AUC for OPN, KL-6, and Syndecan-4 were 0.788, 0.823, and 0.643, respectively. The cut-off values were 12.16 ng/mL, 745.63 U/mL, and 6.06 ng/mL, respectively. The diagnostic sensitivity and specificity were 70.0% and 79.0% for OPN, 64.5% and 88.0% for KL-6, and 72.5% and 48.0% for Syndecan-4, respectively (Table 4 and Figure 3B). The AUC for KL-6 was larger than that for OPN and Syndecan-4, indicating that the order of diagnostic accuracy by the ROC curve was KL-6, OPN, Syndecan-4 when identifying CWP patients from DEWs (Figures 3B and S3).

Combined Serum Biomarkers for Identification of CWP

As shown in Table 5, compared with HCs, the combination of three biomarkers (OPN, KL-6, syndecan-4) had the greatest efficacy in diagnosing CWP (Figure 3C), with a sensitivity and specificity of 96.5% and 98.0%, respectively. When compared with DEWs, the combination of two biomarkers (OPN, KL-6) and the combination of three biomarkers (OPN, KL-6, syndecan-4) were consistent in diagnosing CWP (Figure 3D). The sensitivity and specificity of combining two biomarkers were 76.0% and 85.0%, respectively. The sensitivity and specificity of combining three biomarkers were 73.0% and 89.0%, respectively.

Table 2 Correlations Between Biomarkers and Pulmonary Function Among All Participants

Characteristics	Univariate Analysis			Multivariable Analysis		
	β	95% CI	P value	β	95% CI	P value
FEV1/FVC						
Osteopontin	−1.12	-(1.41–0.84)	<0.001	−0.87	-(1.21–0.54)	<0.001
KL-6	−16.79	-(21.86–11.72)	<0.001	−12.38	-(17.97–6.80)	<0.001
Syndecan-4	−1.84	-(2.48–1.20)	<0.001	−1.21	-(1.91–0.50)	0.001
Gremlin-1	−10.70	-(15.15–0.62)	<0.001	−5.54	-(10.33–0.74)	0.024
FEV1						
Osteopontin	−0.08	-(0.09–0.06)	<0.001	−0.05	-(0.07–0.03)	<0.001
KL-6	−1.32	-(1.65–0.99)	<0.001	−0.90	-(1.25–0.55)	<0.001
Syndecan-4	−0.15	-(0.19–0.11)	<0.001	−0.09	-(0.13–0.04)	<0.001
Gremlin-1	−0.87	-(1.16–0.57)	<0.001	−0.44	-(0.74–0.13)	0.005
FVC						
Osteopontin	−0.06	-(0.08–0.04)	<0.001	−0.03	-(0.06–0.01)	0.002
KL-6	−1.18	-(1.55–0.82)	<0.001	−0.77	-(1.15–0.39)	<0.001
Syndecan-4	−0.14	-(0.18–0.09)	<0.001	−0.08	-(0.12–0.03)	0.001
Gremlin-1	−0.80	-(1.12–0.48)	<0.001	−0.41	-(0.73–0.08)	0.014

Notes: Univariate analysis: single factor logistic regression; Multivariable analysis: adjusted for age, BMI, duration of exposure and smoking index.

Table 3 The Association Between Serum Biomarkers and CWP by Univariate and Multivariate Analysis

Characteristics	Model 1		Model 2		Model 3	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
CWP vs HC						
Osteopontin	3.09 (2.28–4.19)	<0.001	3.15 (2.29–4.32)	<0.001	3.88 (2.34–6.42)	<0.001
KL-6	1.01 (1.00–1.01)	<0.001	1.01 (1.00–1.01)	<0.001	1.01 (1.01–1.02)	<0.001
Syndecan-4	3.51 (2.66–4.64)	<0.001	3.66 (2.73–4.91)	<0.001	4.26 (2.78–6.52)	<0.001
Gremlin-1	1.01 (1.00–1.01)	<0.001	1.01 (1.00–1.01)	<0.001	1.01 (1.00–1.01)	<0.001
CWP vs DEW						
Osteopontin	1.55 (1.37–1.75)	<0.001	1.55 (1.37–1.75)	<0.001	1.53 (1.34–1.75)	<0.001
KL-6	1.01 (1.00–1.01)	<0.001	1.01 (1.00–1.01)	<0.001	1.01 (1.00–1.01)	<0.001
Syndecan-4	1.43 (1.21–1.67)	<0.001	1.42 (1.20–1.67)	<0.001	1.40 (1.16–1.69)	<0.001
Gremlin-1	1.00 (1.00–1.01)	0.078	1.00 (1.00–1.01)	0.101	1.00 (1.00–1.01)	0.134

Notes: Model 1 was univariate analysis; Model 2 adjusted for age, BMI and smoking index; Model 3 adjusted for age, BMI and smoking index and pulmonary function.

Table 4 Cut-Off Values and the Identifying Ability of Osteopontin, KL-6, Syndecan-4, and Gremlin-1 by ROC Curve Analysis

Variables	Identifying HCs				Identifying DEWs			
	Osteopontin	KL-6	Syndecan-4	Gremlin-1	Osteopontin	KL-6	Syndecan-4	Gremlin-1
AUC	0.970	0.924	0.917	0.868	0.788	0.823	0.643	0.547
95% CI	0.954–0.985	0.895–0.954	0.886–0.947	0.828–0.909	0.734–0.842	0.777–0.869	0.577–0.709	0.479–0.615
Cut-off	10.58 ng/mL	674.83 U/mL	5.76 ng/mL	777.3 ng/mL	12.16 ng/mL	745.63 U/mL	6.06 ng/mL	–
Sensitivity, %	85.5	82.0	77.5	89.5	70.0	64.5	72.5	–
Specificity, %	93.0	94.0	93.0	67.0	79.0	88.0	48.0	–

Abbreviations: HCs, health controls; DEWs, dust-exposed workers; AUC, area under the curve; 95% CI, 95% confidence interval.

Discussion

In our present study, we found that pulmonary function decreased sequentially in HCs, DEWs and CWP groups, while the concentrations of biomarkers OPN, KL-6, Syndecan-4 and Gremlin-1 increased sequentially. These four biomarkers were negatively correlated with the pulmonary function among all participants, and pulmonary function parameters significantly decreased with the increase of biomarkers' concentration. We also found that these four serum biomarkers have high diagnostic value in differentiating CWP patients from HCs. However, only three biomarkers, OPN, KL-6, and Syndecan-4, have high diagnostic value in differentiating CWP patients from DEWs. The order of diagnostic accuracy was OPN, KL-6, Syndecan-4 and Gremlin-1 when distinguishing CWP patients from HCs, while the order of diagnostic accuracy was KL-6, OPN, Syndecan-4 when identifying CWP patients from DEWs. The combination of OPN, KL-6, and Syndecan-4 can improve the diagnostic sensitivity and specificity of CWP.

OPN was first discovered in malignant cells in 1979.[30] It is a glycoprotein containing three isoforms, OPN-A, OPN-b, and OPN-c, which can be secreted or intracellular, and exhibits different post-translational modifications depending on the tissue of expression.[31] The biological functions of OPN in different physiological and pathophysiological states are diverse. It plays an important role in the development of immune-mediated inflammatory diseases and modulates the host response to infection. OPN is a key mediator of the inflammatory response, primarily as a chemotactic agent for immune cells. It mainly induces the secretion of cytokines by triggering different white blood cells to form an immune response. For example, several inflammation-related lung diseases depend on OPN signaling. In chronic obstructive pulmonary disease (COPD), OPN is up-regulated and associated with disease exacerbation, possibly by interfering with the function of antimicrobial proteins and thereby reducing the host's ability to defend against bacterial infection.[32] OPN also can be released by aging pulmonary artery smooth muscle cells and is involved in the pathogenesis of pulmonary hypertension.

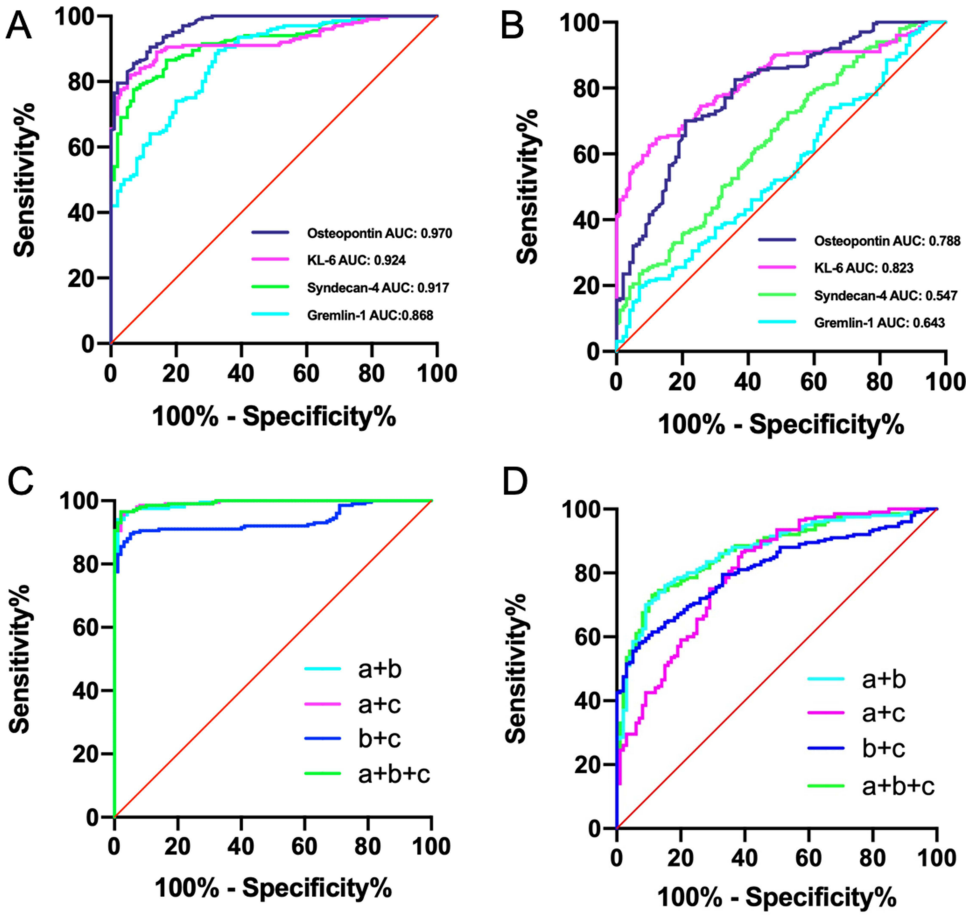

Figure 3 (**A**) ROC curve analysis to differentiate patients with CWP from HCs. (**B**) ROC curve analysis to differentiate patients with CWP from DEWs. (**C**) Combined ROC curve analysis to differentiate patients with CWP from HCs. (**D**) Combined ROC curve analysis to differentiate patients with CWP from DEWs. a+b: combined Osteopontin and KL-6; a+c: combined Osteopontin and Syndecan-4; b+c: combined KL-6 and Syndecan-4; a+b+c: combined Osteopontin, KL-6 and Syndecan-4. a: Osteopontin; b: KL-6; c: Syndecan-4.

It has been reported that OPN plasma concentrations are positively associated with elevated pulmonary artery pressure in COPD patients with ischemic heart disease.[33] The pathogenesis of asthma is mediated by interleukin-33 (IL-33) by enhancing the production of amphiregulin by memory T helper 2 cells. Amphiregulin directly programs eosinophils to an inflammatory state along with epidermal growth factor receptor (EGFR) signaling and stimulates them to produce OPN, which acts as a pro-fibrotic mediator in this process.[34]

OPN is also associated with lung injury of different etiologies. Animal studies have shown that certain forms of carbon nanotubes (CNTs) are potent fibrosis inducers in the lung, causing bronchial, interstitial, and pleural fibrosis, characterized by excessive deposition of collagen fibers and scarring of associated tissues.[35] It has been reported that

Table 5 Cut-Off Values and the Identifying Ability of Combined Osteopontin, KL-6, and Syndecan-4 by ROC Curve Analysis

Variables	Identifying HCs				Identifying DEWs			
	Model 1	Model 2	Model 3	Model 4	Model 1	Model 2	Model 3	Model 4
AUC	0.991	0.993	0.935	0.994	0.867	0.802	0.812	0.867
95% CI	0.984–0.998	0.987–0.999	0.907–0.964	0.988–0.999	0.826–0.908	0.748–0.855	0.765–0.859	0.827–0.908
Sensitivity, %	94.0	96.5	89.5	96.5	76.0	86.5	58.0	73.0
Specificity, %	99.0	97.0	95.0	98.0	85.0	61	93.0	89.0

Notes: Model 1: Osteopontin + KL-6; Model 2: Osteopontin + Syndecan-4; Model 3: KL-6 + Syndecan-4; Model 4: Osteopontin + KL-6 + Syndecan-4.
Abbreviations: HCs, health controls; DEWs, dust-exposed workers; AUC, area under the curve; 95% CI, 95% confidence interval.

OPN is highly and persistently induced by CTNs during both acute and chronic phases of fibrosis in mouse lungs, and that OPN promotes lung fibrosis by activating TGF-β1 signaling and promoting myofibroblast differentiation, and increased fibrous matrix protein production.[14] Lung interstitial fibrosis initiates with an acute inflammatory response, and after the acute inflammatory and fibrotic response, the pathological effect gradually transforms into chronic inflammatory fibrosis, which eventually manifests as mild inflammation, increased extracellular matrix (ECM) protein deposition, and the formation of fibrotic foci.[36] It has been reported that OPN can be used to distinguish IPF from other idiopathic interstitial lung diseases.[37] In this study, our results first revealed that OPN has a high value in the diagnosis of CWP patients, whether in differentiating HCs or DEWs. It is suggested that OPN can be used as a novel biomarker of CWP. However, OPN concentrations did not differ in different stages of CWP, so it may not be used as a biomarker for disease stage. At the same time, there was a negative correlation between OPN and pulmonary function among all participants, and pulmonary function parameters significantly decreased with the increase of OPN concentration.

KL-6, also known as human mucin-1 (MUC1), is a high molecular weight glycoprotein mainly produced by damaged or regenerating alveolar type II pneumocytes and bronchial epithelial cells.[15] KL-6 not only plays a major role in fibroblast stimulation and apoptosis inhibition in normal lung tissue but also promotes the chemotactic activity and anti-apoptotic effect of lung fibroblasts, but the exact mechanism remains unclear.[38] Elevated serum KL-6 levels are known to be a marker of active pulmonary fibrosis. Several recent studies showed that serum KL-6 levels were significantly higher in interstitial pneumonia with autoimmune features (IPAF) patients than in non-fibrotic lung disease (non-FLD) patients and healthy controls.[39,40] However, an interesting observation is that the findings on the association between KL-6 and the FVC % predicted varied from study to study: one study showed that KL-6 was negatively correlated with the FVC % predicted,[40] another study demonstrated that there was no significant correlation between KL-6 and the FVC % predicted.[39] Serum KL-6 concentration is a biomarker for distinguishing IPF from other interstitial lung diseases (ILD) and also serves as a predictor of acute exacerbation of IPF.[15] A previous study showed that serum KL-6 concentrations were significantly elevated in patients with asbestosis, especially compared with DEWs and HCs.[12] Similar results were obtained in our study, where the serum KL-6 concentration was significantly increased in CWP patients compared with DEWs and HCs, and it could be used as a biomarker for the auxiliary diagnosis of CWP disease. To the best of our knowledge, although we are not the first to demonstrate that KL-6 can act as a biomarker for asbestosis, we are the first to identify its particular value in the diagnosis of CWP. Our study also demonstrated a correlation between KL-6 and pulmonary function among all participants. KL-6 levels were not significantly different in different stages of CWP, so they may not be used as a biomarker for evaluating disease stage.

Syndecan-4 is a heparin sulfate proteoglycans (HSPG) that can be expressed in a variety of cells. It consists of a core protein and heparin sulfate glycosaminoglycan (GAG), which binds to numerous cytokines and growth factor side chains to exert biological functions.[20] Syndecan-4 is reported to exist in soluble form when cleaved from the cell surface by disintegrin, matrix metalloproteinase 7, metalloproteinase 9 or metalloproteinase 17.[41] A study showed that serum Syndecan-4 levels were significantly higher in patients with acute pneumonia than in healthy controls and were negatively correlated with pneumonia severity scores.[41] Interestingly, another study showed that Syndecan-4 levels were significantly lower in community-acquired pneumonia (CAP) patients compared to healthy controls, especially in non-survivors.[20] To the best of our knowledge, no studies have explored the relationship between Syndecan-4 and CWP. Our study first revealed that serum Syndecan-4 levels were significantly higher in CWP patients than in DEWs and HCs, indicating that serum Syndecan-4 has a high value in the auxiliary diagnosis of CWP disease, suggesting that it can be used as a novel biomarker for the diagnosis of CWP. At the same time, our experiments did find a correlation between Syndecan-4 and pulmonary function among all participants. Serum Syndecan-4 concentrations were not significantly different in CWP stages I, II, and III, suggesting that it may not be used as a biomarker for assessing disease stage.

The association of Gremlin-1 with pulmonary fibrosis was first investigated in a 2006 study that demonstrated Gremlin-1 expression in IPF. The study also showed that Gremlin-1 levels are higher in IPF and that it is induced by TGF-β.[27] A previous study demonstrated that Gremlin-1 mRNA levels were upregulated in an asbestos-exposed mouse lung model. Simultaneous analysis of cultured human bronchial epithelial cells showed that asbestos-induced Gremlin-1 expression could be prevented by TGF-β receptor inhibitors as well as by inhibitors of the mitogen-activated protein kinase kinase/extracellular signal-regulated protein kinase pathway.[28] A recent study revealed that Gremlin-1

concentration in systemic sclerosis (SSc) patients was significantly higher than health controls. Patients with SSc-associated ILD had significantly elevated serum Gremlin-1 levels compared to SSc patients without ILD.[42] Another study demonstrated that serum gremlin-1 level was obviously elevated in IPF patients, followed by non-IPF ILD patients and healthy controls.[43] An interesting phenomenon emerged from our study. Compared with HCs, serum Gremlin-1 concentrations were significantly increased in CWP patients, but there was no significant difference in serum Gremlin-1 levels between CWP patients and DEWs. This differential result indicates that Gremlin-1 is not a good biomarker for the diagnosis of CWP. Although the screening of Gremlin-1 was carried out through rigorous scientific design and standard research ideas, the results were not satisfactory. One of the possible reasons for this is that we used transcriptomic data of lung tissue from silicosis and non-disease donors (not exposed to dust) for screening. The transcriptome data only revealed transcriptome differences between silicosis and HCs but did not reveal transcriptome differences between silicosis and DEWs. This is also consistent with our experimental results. Another possible explanation is that mRNA needs to undergo sophisticated and complex post-transcriptional modification and then translation to proteins, and the transcriptome data does not have a one-to-one correspondence with the proteomic data. Therefore, proteomics, transcriptomics and genomics among the three groups (CWP, DEW, HC) will help to discover novel CWP biomarkers in the future.

Interestingly, the levels of these three biomarkers were not significantly different in different stages of CWP. The specific reasons for this phenomenon are not yet clear, and perhaps the following analysis can explain this phenomenon. Firstly, the target biomarkers we selected are based on their involvement in the pathogenesis of CWP. These biomarkers may play an important role in lung inflammation and fibrosis, and inflammation and partial fibrosis are involved in all three stages of CWP. Secondly, the staging of CWP is an imaging-based diagnosis rather than a functional diagnosis. This may lead to inconsistent severity and staging of the disease. Biomarkers are more indicative of the severity of the disease.

When compared with HCs, ROC curve analysis showed that OPN had the largest AUC, indicating that it is more effective than KL-6, Syndecan-4 and Gremlin-1 in diagnosing CWP. The values of the first three-biomarker combination (OPN, KL-6, Syndecan-4) were higher than that of any single serum biomarker. However, the AUC of the first three-biomarker combination was only 0.003 higher than the AUC of the two-biomarker combination (OPN, KL-6). When compared with DEWs, ROC curve analysis showed that KL-6 had the largest AUC, followed by OPN and Syndecan-4. The values of the three-biomarker combination and the two-biomarker combination (KL-6 and OPN) were consistent and higher than any single serum biomarker. Considering the practical application of biomarkers in clinical diagnosis, we feel that the combination of OPN and KL-6 may be of great value in the auxiliary diagnosis of CWP. Although the clinical application of these biomarkers still requires large-scale, multicenter validation, it also provides a new direction for CWP diagnosis.

There are several limitations in our study. First, we did not continuously measure biomarkers and therefore cannot assess the dynamic changes in disease progression and prognosis. Secondly, we enrolled 200 patients with CWP, and there was no equal distribution of stage I, stage II and stage III patients. Our initial purpose of the experiment is to study the role of biomarkers in the diagnosis of CWP, rather than in different stages. We can further study the role of biomarkers in different stages by matching the number of people in different stages in the follow-up study. In addition, although we revealed that the biomarkers had a high value in the diagnosis of CWP, the specific mechanism was unclear and further studies were needed to clarify.

Conclusions

This study revealed that OPN, KL-6 and Syndecan-4 are novel biomarkers that can be used for CWP auxiliary diagnosis. The combination of three biomarkers can improve the diagnostic sensitivity and specificity of CWP. Our study also did find a negative correlation between biomarkers and pulmonary function among all participants.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval

This study was approved by the Ethics Committee of the China-Japan Friendship Hospital (NO. 2017-25-1) and the Ethics Committee of the Sinopharm Tongmei General Hospital (NO.201902).

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Disclosure

The authors report no conflicts of interest in this work.

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