

Correlation analysis of surfactant protein A and surfactant protein D with lung function in exhaled breath condensate from lung cancer patients with and without COPD

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Abstract. Pulmonary surfactant protein A (SP-A) and pulmonary surfactant protein D (SP-D) are associated with the pathogenesis of chronic obstructive pulmonary disease (COPD). The aim of the present study was to determine the correlation between SP-A, SP-D and lung function in patients with COPD. A total of 60 patients with lung cancer undergoing unilateral lobectomy were selected and divided into three groups, including a non-COPD group (n=20), a COPD treatment group (n=20) and a COPD control group (n=20). The levels of SP-A and SP-D were detected in the exhaled breath condensate (EBC) using ELISA analysis. Tissue samples were obtained during lobectomy via resection of the adjacent lung tissues, located >5 cm from the nodule. Immunohistochemistry and reverse transcription-quantitative polymerase chain reaction analysis was performed. The proportion of SP-A⁺ alveolar type II (ATII) cells and the mRNA levels of SP-A and SP-D in lung tissue were measured. In addition, the correlation between SP-A and SP-D in EBC, SP-A and SP-D mRNA in lung tissue, forced expiratory volume in 1 sec (FEV1) and the ratio of SP-A⁺ ATII, was evaluated. The expression levels of SP-A and SP-D were significantly increased in patients of the non-COPD group compared with the other two groups (P<0.05). In addition, the expression levels of SP-A were positively correlated with FEV1 and the ratio of SP-A⁺ ATII (P<0.05). The expression levels of SP-D exhibited no correlation with FEV1 and ratio of SP-A⁺ ATII (P>0.05). The results

of the present study indicated that the SP-A and SP-D levels in EBC were correlated with lung function, which contributed to COPD diagnosis. Future studies are required to further investigate the results of the present study.

Introduction

Chronic obstructive pulmonary disease (COPD) is a multifactorial disease characterized by irreversible limitations to airflow. According to the World Health Organization, COPD is expected to be the third leading cause of mortality worldwide by the year 2020, accounting for ~3 million mortalities annually (1). A number of factors have been identified to be risk factors for COPD, including environmental and genetic factors. Although an inherited deficiency of α_1 -antitrypsin accounts for 1-2% of COPD cases, studies have suggested that there may be a strong genetic component (2,3). Air pollution is a major risk factor for and primary cause of COPD, and results in increased COPD morbidity in industrialized countries (4), and whereas only 10-20% of heavy cigarette smokers develop COPD (5).

Pulmonary surfactant and its components, including pulmonary surfactant proteins (SP)-A and -D, have been demonstrated to serve important roles in host defense, bronchiolar stability and the regulation of inflammatory processes within the lung (6,7). Human SP-A, located on chromosome 10 consists of two functional genes (SPA1 and SPA2) exhibiting opposite genetic variability (8). Human SP-D, located proximal to the centromere, is associated with the SP-A locus (9). SP-A has been demonstrated to correlate with the pathogenesis of COPD and was considered to be a potential COPD biomarker (10). In addition, SP-D levels were demonstrated to correlate with alterations in Respiratory Disease Questionnaire scores, which may be a useful biomarker to track the health outcomes of patients with COPD (11). Polymorphism studies have demonstrated an association between tuberculosis, and SP-A and SP-D alleles (12). SP-D and club cell protein16 are two proteins synthesized predominantly in type II pneumocytes and club cells of lungs, and can be used as markers to track the health status and disease progression of patients with COPD (13).

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Exhaled breath condensate (EBC) is an evolving tool for the noninvasive evaluation of lung disease (14), which is widely used in the diagnosis of various respiratory diseases (15). Significant differences have been observed in EBC between patients with COPD patients and healthy individuals (16). Although intensive studies into EBC have been performed (17,18), the correlation between SP-A, SP-D and lung function in EBC requires further investigation.

In the present study, EBC samples from 60 patients with lung cancer were collected. The levels of SP-A and SP-D in EBC and lung tissue were measured. The correlation between SP-A, SP-D and lung function in patients with COPD was analyzed.

Materials and methods

Participants. A total of 60 patients with lung cancer undergoing a unilateral lobectomy were recruited from Tianjin Chest Hospital (Tianjin, China) between October 2012 and April 2013 for the present study. Patient characteristics are presented in Table I. According to their lung function, all individuals were divided into a non-COPD group (n=20) and a COPD group (n=40). According to a random number table, the COPD group was further randomly divided into a COPD control group (n=20) and a COPD treatment group (n=20). The COPD treatment group were given a 5-day lung protection treatment (atomized inhalation of 1,000 μ g ipratropium bromide, 30 μ g ambroxol and 2 mg budesonide 3 times/day; intravenous injection of 150 mg ambroxol hydrochloride dissolved in 100 ml saline and 0.2 g doxofylline dissolved in 100 ml saline once/day) once they were hospitalized. The non-COPD group and COPD control group received no intervention.

The present study was approved by the ethics committee of Tianjin Chest Hospital and written consent was obtained from all patients and their families prior to enrollment. The diagnosis of patients with COPD was performed in accordance with the Guidelines for Diagnosis and Treatment of Chronic Obstructive Pulmonary Disease (2013 revision) (19). The standard method of confirming COPD is the examination of lung function: Following treatment with a bronchial dilation agent, the patients were defined as incomplete reversible airflow limitation with forced expiratory volume in 1 sec (FEV1) <80%, and FEV1/forced vital capacity (FVC) <70%. The exclusion criteria were as follows: i) Patients with a lung infection or COPD acute attack in the short term (one month prior); ii) patients with other chronic diseases (for example, bronchiectasis, asthma, pulmonary interstitial fibrosis or silicosis); iii) patients with chronic diseases of the heart, brain, liver, kidney or other systems; and iv) patients taking medication, including antibiotics, cortical hormone, bronchodilators or expectorants.

Sampling of EBC and lung tissue. EBC was obtained following the 5-day lung protection treatment described above using an RTube condenser (Model Austin TX 78720; RTube starter kit; Respiratory Research Inc., Austin, TX, USA). Patients were required to gargle to clean up oral secretions and wear a nasal splint prior to EBC collection. Patients breathed through a disposable biting mouthpart. A one-way valve in the collecting tube allowed the exhaled air to enter the collecting tube and be

Table I. Clinical data analysis of recruited patients with lung cancer.

Characteristic	Group		
	Non-COPD	COPD control	COPD treatment
No. of females (%)	11 (55%)	10 (50%)	8 (40%)
Age, years	54.65 \pm 5.77	53.75 \pm 8.86	56.55 \pm 7.55
No. of smokers (%)	7 (35%)	9 (45%)	11 (55%)
FEV1 (%)	96.91 \pm 7.09	62.11 \pm 7.01 ^a	60.68 \pm 7.37 ^a

^aP<0.05 vs. non-COPD group. n=20 per group. Age and FEV1% are presented as the mean \pm standard deviation. COPD, chronic obstructive pulmonary disease; FEV1%, the forced expiratory volume for 1 sec expressed as a percentage of the forced vital capacity.

condensed into liquid through a cooling tube. The procedure lasted 10-15 min and 1-3 ml condensate was collected. Patients were required to ingest the saliva during process of collection. Collection was stopped if the patient coughed. The condensate was removed into cryovial and stored at -80°C for subsequent use.

Pneumonectomy was performed to obtain lung tissue specimens under aseptic conditions. Lung tissue samples were obtained during lobectomy through resection of the adjacent lung tissues, located >5 cm from the nodule. Partial samples were fixed in 10% formalin at room temperature for 12 h and cut into 5- μ m paraffin sections, which were used for immunohistochemistry experiments. At the time of sampling, other lung tissues were immediately frozen in liquid nitrogen and stored at -80°C; these were used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

ELISA analysis. Levels of SP-A and SP-D in EBC were measured using ELISA assay kits (cat nos. RGP011R and RGP011R; BioVendor Laboratorni Medicina, a.s., Brno, Czech Republic), according to the manufacturer's protocol. A total of 10 μ l each sample was analyzed on the same ELISA plate, including a negative control. Detection was performed at 450 nm using a multiscan spectrum microplate reader (model ELx808; BioTek Instruments, Inc., Winooski, VT, USA). The concentrations of SP-A and SP-D were calculated from the calibration curve drawn from the data for standard albumin.

RT-qPCR analysis. Total RNA from lung tissue was extracted using aUNIQ-10 Column total RNA purification kit (Sangon Biotech Co., Ltd., Shanghai, China). cDNA was generated following the manufacturer's protocol of a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany). The expression levels of SP-A and SP-D were analyzed using RT-qPCR with FastStart Universal SYBR-Green Master (Roche Diagnostics GmbH) and a Bio-Rad CFX96 qPCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturers' protocol. The primers used were as follows: SP-A forward, 5'-ATGTCA CTGTGTCTTTGGCTTTC-3' and reverse, 5'-TCAAATTC

ACAAACAGCCAGCC-3'; SP-D forward, 5'-AAAGTGTCTGGGAGAAGA-3' and reverse, 5'-TCAGTCATGCTCAGGAAA-3'. qPCR analysis was carried out in a total volume of 20 μ l, containing 9 μ l 2X SYBR-Green qPCR Mix (Takara Bio, Inc., Otsu, Japan), 0.1 μ M each specific primer and 100 ng template cDNA. The reaction mixtures were heated to 94°C for 30 sec, followed by 40 cycles at 94°C for 10 sec, 60°C for 45 sec, and 72°C for 45 sec. The housekeeping gene GAPDH was used as a reference gene, with primers as follows: GAPDH forward, 5'-CGGAGTCAACGGATTGGTCGTAT-3' and reverse, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. The primers for the RT-qPCR experiments were designed using Primer Premier version 5.0 (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Sangon Biotech Co., Ltd. All RT-qPCR analysis was performed in triplicate and quantified using the $2^{-\Delta\Delta C_q}$ method (20).

Immunohistochemical staining. The lung tissue slides were deparaffinized in xylene, hydrated through a graded series of ethanols (anhydrous ethanol, 95% ethanol and 75% ethanol for 5 min, respectively), and rehydrated in deionized water. Inactivation of endogenous peroxidase was performed using 3% H_2O_2 in methanol for 10 min. Antigen retrieval was performed by heating with citric acid solution (pH 6.0) for 30 sec at 125°C and subsequently for 25 min at 94°C, followed by cooling for 15 min. Tissues were blocked with 1% bovine serum albumin (Shenhang Biotechnology Co., Ltd., Shanghai, China) at room temperature for 20 min, and subsequently incubated with diluted primary mouse anti-human SP-A antibody (cat no. sc-7700; 1:200) and anti-thyroid transcription factor-1 (TTF-1) antibody (cat no. sc-12524; 1:200) (both from Abcam) overnight at 4°C. Following washing with PBS, sections were incubated with secondary anti-mouse SP-A antibody (cat no. sc-7701; 1:500) and anti-mouse TTF-1 antibody (cat no. sc-12525; 1:500) (both from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at 37°C for 30 min and visualized with diaminobenzidine and hematoxylin-eosin staining at room temperature for 15 min. The proportions of SP-A⁺ alveolar type II (ATII) cells were evaluated under a light microscope (BX50; Olympus Corporation, Tokyo, Japan) at x400 magnification.

Statistical analysis. All data are expressed as the mean \pm standard deviation. Statistical analysis was performed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). Paired sample t-test was conducted when comparing the expression levels of SP-A and SP-D in EBC in the COPD treatment group prior to and following lung protection treatment. Multiple comparisons between the groups were performed using one-way analysis of variance followed by Student-Newman-Keuls test. Correlation analysis was conducted using Spearman's rank correlation test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinical data analysis. As presented in Table I, the clinical data of patients, including sex, age, smoking history and FEV1, was analyzed in the three groups, including the non-COPD group, COPD control group and COPD treatment group.

No statistically significant difference was observed in sex, age and smoking between patients of the three groups. FEV1 in patients with COPD was significantly decreased compared with patients in the non-COPD group ($P < 0.05$). There was no significant difference in FEV1 between the COPD control group and COPD treatment group ($P > 0.05$).

Expression levels of SP-A and SP-D measurement. The mRNA expression levels of SP-A and SP-D in lung tissue were measured using RT-qPCR (Fig. 1A). Significant differences in SP-A and SP-D expression were observed among the three groups ($P < 0.05$). SP-A in the non-COPD group (3.21 ± 0.52) exhibited a significantly increased mRNA expression compared with the COPD control group (2.25 ± 0.95) and COPD treatment group (2.75 ± 0.15 ; $P < 0.05$). SP-D expression exhibited a similar profile, with the highest levels (2.59 ± 0.44) in the non-COPD group and lowest expression levels (2.00 ± 0.13) in the COPD control group ($P > 0.05$).

In addition, the expression levels of SP-A and SP-D in EBC were measured using ELISA analysis (Fig. 1B). The non-COPD group exhibited the highest SP-A expression of 8.57 ± 0.73 pg/ml and SP-D expression of 4.55 ± 0.99 ng/ml, which were significantly increased compared with the other two groups ($P < 0.05$). The COPD control group exhibited the lowest expression levels of SP-A and SP-D ($P < 0.05$). In the COPD treatment group, lung protection treatment for 5 days promoted SP-A (5.82 ± 0.27 vs. 4.98 ± 0.96 pg/ml; $P < 0.05$) and SP-D expression (4.01 ± 0.8 vs. 3.66 ± 0.79 ng/ml; $P < 0.05$) in EBC.

Histological analysis of lung tissue. As presented in Fig. 2, SP-A and TTF-1 immunostaining was performed to measure the proportion of SP-A⁺ ATII in patient lung tissues. Areas of ATII cells, club cells and alveolar macrophages were observed to be positive for SP-A immunostaining. In addition, ATII cells, bronchial epithelial cells and club cells were positive for TTF-1 immunostaining. The proportions of SP-A⁺ ATII cells in the non-COPD group, COPD control group and COPD treatment group was 62.00 ± 5.86 , 44.00 ± 5.89 and $52.55 \pm 10.46\%$, respectively.

Correlation between expression levels of SP-A, SP-D and lung function. The correlation between the expression levels of SP-A, SP-D and lung function was analyzed in all three patient groups, as exhibited Fig. 3. The results of the present study demonstrated that FEV1 was positively correlated with the expression levels of SP-A in EBC ($r = 0.494$, $P < 0.05$) and SP-D ($r = 0.253$, $P < 0.05$). Additionally, FEV1 in lung tissue was positively correlated with the mRNA expression of SP-A ($r = 0.264$, $P < 0.05$) and SP-D ($r = 0.281$, $P < 0.05$).

The correlation between the proportion of SP-A⁺ ATII and lung function was analyzed. A positive correlation between SP-A⁺ ATII and FEV1 was observed ($r = 0.795$, $P < 0.05$).

Correlation between the expression of SP-A, SP-D and SP-A⁺ ATII. The correlation between the content of SP-A and SP-D in EBC, and the corresponding mRNA in lung tissue, was analyzed (Fig. 4). There was a positive correlation between SP-A in EBC and SP-A mRNA in lung tissue ($r = 0.542$, $P < 0.05$). A positive correlation additionally existed between

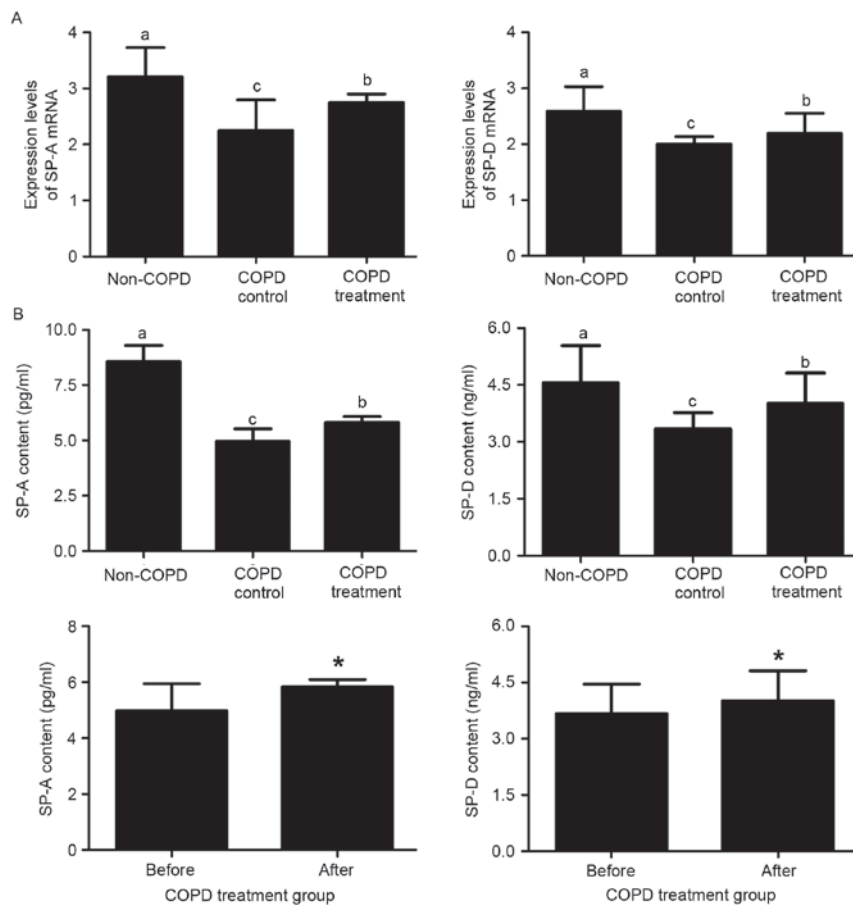


Figure 1. Measurement of the expression levels of SP-A and SP-D. (A) Expression levels of SP-A and SP-D mRNA in lung tissue were measured by using the reverse transcription-quantitative polymerase chain reaction and (B) contents of SP-A and SP-D in EBC were measured using ELISA analysis in samples from the non-COPD group, COPD control group and COPD treatment group following lung protection treatment for 5 days. Different letters above the bars indicate significantly different values ($P<0.05$) calculated using one-way analysis of variance followed by a Student-Newman-Keuls test. * $P<0.05$ vs. before. SP, surfactant protein; EBC, exhaled breath condensate; COPD, chronic obstructive pulmonary disease.

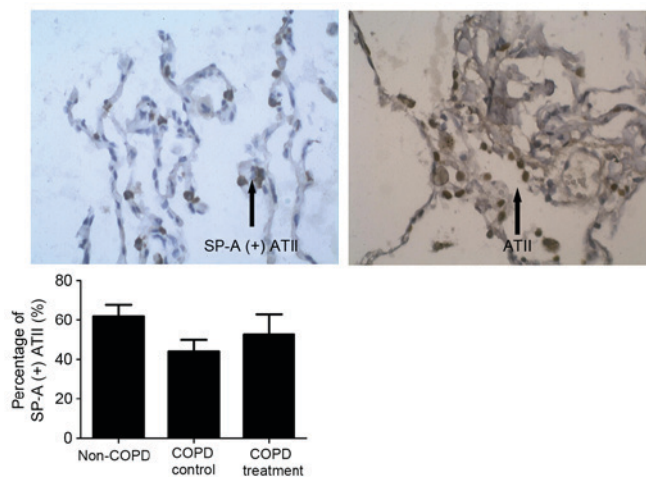


Figure 2. Histological analysis of lung tissue. SP-A (left) and thyroid transcription factor-1 (right) immunostaining was performed on lung tissue from a patient with COPD. The proportions of SP-A⁺ ATII cells in the non-COPD, COPD control and COPD treatment group (5 days) were calculated. Magnification, x400. SP-A, surfactant protein A; COPD, chronic obstructive pulmonary disease; ATII, alveolar type II cell.

the content of SP-D in EBC and the mRNA expression of SP-D in lung tissue ($r=0.478$, $P<0.05$). In addition, the proportion of

SP-A⁺ ATII exhibited a positive correlation with SP-A content in EBC ($r=0.522$, $P<0.05$) and mRNA expression levels of SP-A in lung tissue ($r=0.299$, $P<0.05$). Notably, no correlation was observed between SP-A⁺ ATII and SP-D contents in EBC ($r=0.145$, $P>0.05$). No correlation was observed between SP-A⁺ ATII and the mRNA expression levels of SP-D in lung tissue ($r=0.200$, $P>0.05$).

Discussion

Epidemiological studies have demonstrated that respiratory disease is associated with notable morbidity and that its diagnosis is complex. As described previously, COPD is expected to be the third leading cause of mortality worldwide by the year 2020 (1). Lung function is frequently severely impaired in patients with COPD, which markedly increases the risk of pulmonary complications, particularly following thoracic surgery (21). Traditional methods used in lung function diagnosis include bronchoscopy with bronchoalveolar lavage, sputum collection and sputum induction, which are not suitable for use in ambulant individuals, particularly children (22). EBC is able to successfully compensate for the shortcomings of traditional techniques, and may be used for the analysis of dissolved nonvolatile compounds, including proteins, lipids, oxidants and nucleotides, and their associations with

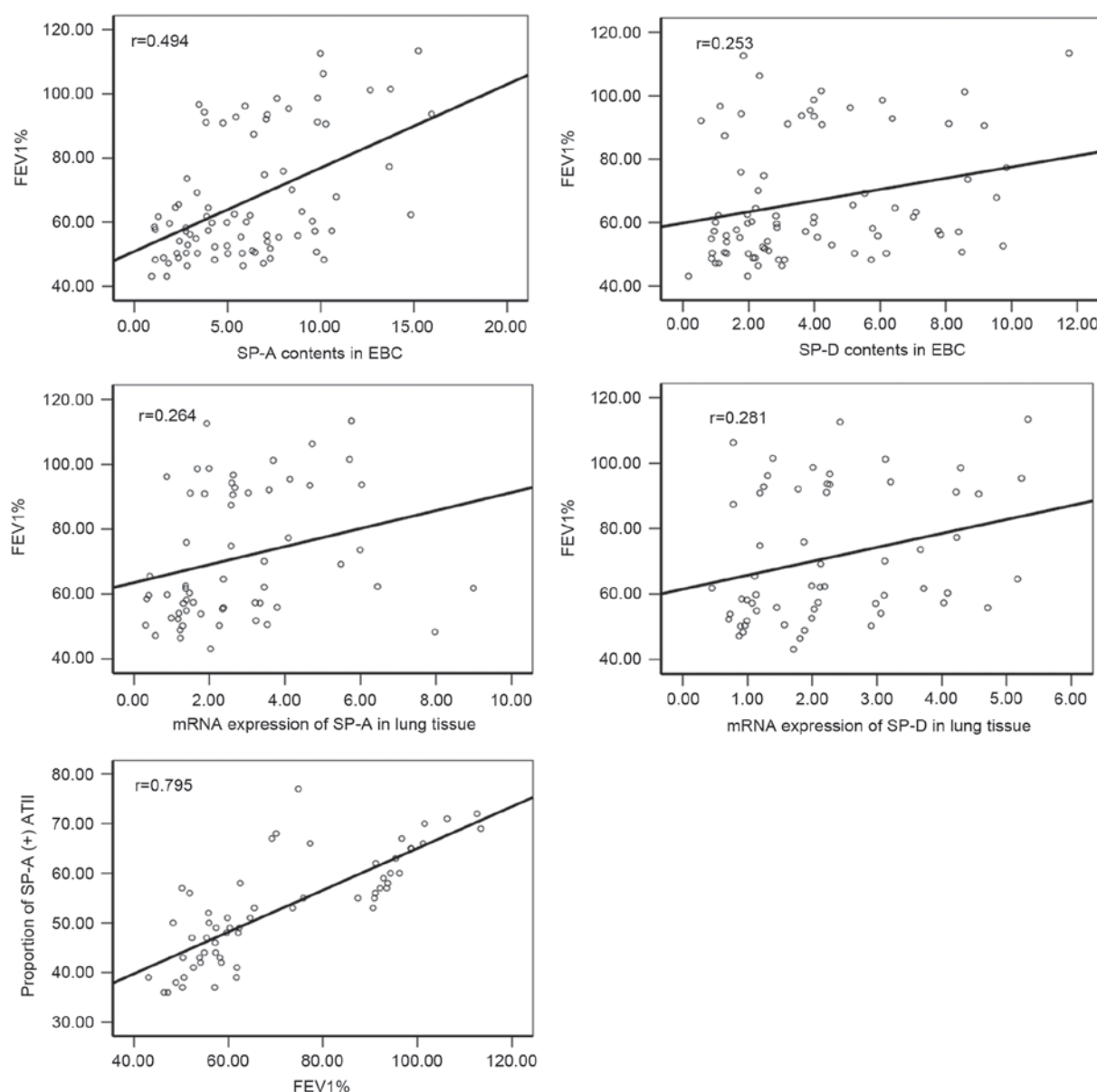


Figure 3. Analysis of the correlation between SP-A and SP-D expression, SP-A⁺ ATII and lung function, among all patient groups. EBC, exhaled breath condensate; SP, surfactant protein; FEV1, forced expiratory volume in 1 sec; ATII, alveolar type II cell.

physiological and pathological processes in the lung. The correct assessment of lung function is of importance for diagnosis.

To date, research into COPD has primarily focused on inflammatory mediators, and studies of alveolar surfactant proteins have predominantly used the serum test (23), which may not reflect real lung function in patients. The structures of surfactant proteins (SP-A and SP-D) are well-characterized. SP-A and SP-D are hydrophilic collagen glycoproteins and belong to the C-type lectin family. SP-A (700 kDa) is composed of six triple helix subunits. SP-D (1 MDa) is composed of 4 tri-polymers (24). SP-A was demonstrated to be highly expressed in type II pneumocytes, accounting for 50% of the total surfactant proteins, and exhibited a similar function to SP-D in reducing inspiratory pulmonary resistance, increasing pulmonary compliance and inhibiting inflammatory responses (25). In the present study, the contents of SP-A and SP-D in EBC, their corresponding mRNA levels and

the proportion of SP-A⁺ ATII in lung tissue were measured. The correlation between lung function (FEV1) and surfactant proteins (SP-A and SP-D) was analyzed.

As described previously, SP-A and SP-D are abundantly secreted in the alveolar space and airway. SP-A and SP-D in EBC or lung tissue were analyzed using ELISA and RT-qPCR experiments in the present study. The contents of SP-A and SP-D in EBC, and the mRNA expression levels in lung tissue of patients with COPD, were significantly decreased compared with the non-COPD group, and were significantly increased following treatment, which was inconsistent with previous analyses of the expression in serum and bronchoalveolar lavage fluid (23,26). This inconsistency may be explained by the specificity of expression of SP-A and SP-D. Previously, there has been controversy over the expression of SP-A and SP-D. Compared with non-smokers with COPD, smokers with COPD were demonstrated to exhibit the highest SP-D concentrations in bronchoalveolar lavage fluid,

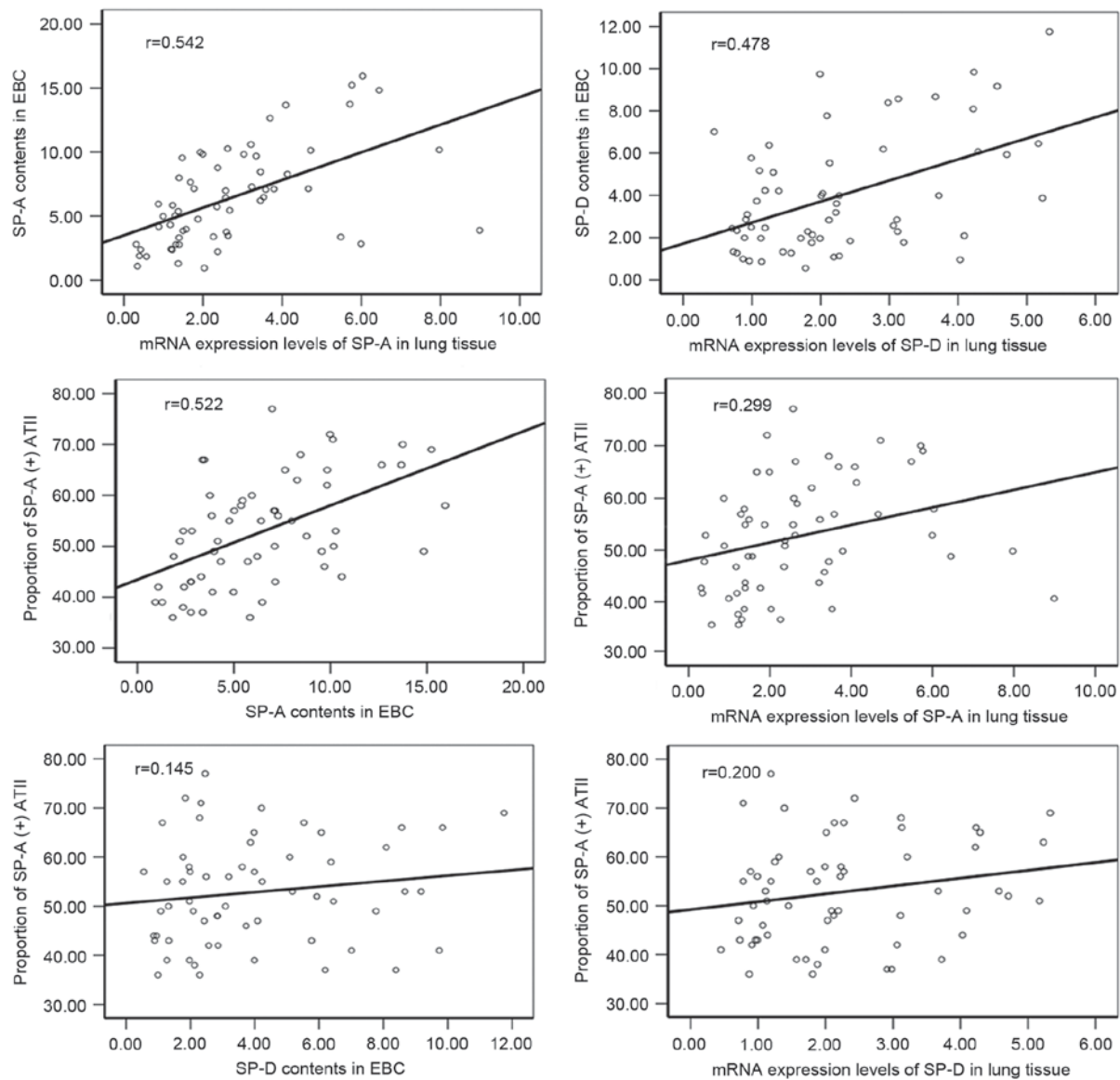


Figure 4. Analysis of the correlation between the expression levels of SP-A and SP-D, and SP-A⁺ ATII. EBC, exhaled breath condensate; SP, surfactant protein; ATII, alveolar type II cell.

and the lowest serum SP-D concentrations, particularly elderly smokers (23). By contrast, Liu *et al* (26) reported that patients with COPD exhibited significantly increased serum SP-D concentrations compared with healthy subjects. Serum SP-D levels higher than the baseline were observed to be associated with a >95% increased risk of exacerbations in patients with COPD (27).

Pulmonary surfactants reduce the surface tension at the air-liquid interface. FEV1 may reflect lung function, which was observed to be damaged in patients with COPD compared with non-COPD subjects in the present study. The results of the present study demonstrated a positive correlation between SP-A and SP-D in EBC, and SP-A and SP-D mRNA in lung tissue, which illustrated that SP-A and SP-D levels in EBC may reflect SP-A and SP-D levels in lung tissue. SP-A and SP-D levels have been reported to correlate with age and smoking history (28). A previous study demonstrated that the serum SP-D level is negatively associated with FEV1 in older patients with COPD (11). A correlation between SP-D levels in

bronchotracheal alveolar lavage fluid, in serum, or the ratio of SP-D in bronchotracheal alveolar lavage fluid and serum, and FEV1/FVC was observed in smokers (23). In the present study, SP-A and SP-D positively correlated with FEV1 in EBC or in lung tissue, which suggested that SP-A and SP-D in EBC and lung tissue may be correlated with lung function.

In the present study, histological analysis illustrated that the proportion of SP-A⁺ ATII cells in the COPD control group was the lowest, which was consistent with a previous study (29). ATII cells have been observed to be involved in lung injury repair and are present at a decreased number in patients with lung injury (30,31). Correlation analysis additionally demonstrated that the proportion of SP-A⁺ ATII was positively correlated with FEV1, and SP-A level in EBC and lung tissue. In patients with COPD, the loss of lung function may reduce the proportion of SP-A⁺ ATII, thereby further affecting the SP-A protein levels. The proportion of SP-A⁺ ATII may reflect the function of ATII cells in secreting alveolar surfactants, which may be responsible for airway obstruction in patients

with COPD. SP-A protein levels may reflect the real lung function of patients with COPD.

In conclusion, the expression levels of SP-A and SP-D in the EBC of non-COPD subjects were increased compared with patients with COPD, which positively correlated with the lung function indicator FEV1, and expression levels of SP-A and SP-D mRNA in lung tissue. Additionally, the expression levels of SP-A and SP-D in EBC positively correlated with SP-A* ATII. The results of the present study demonstrated that SP-A and SP-D levels in EBC may reflect lung function. It may therefore be feasible to diagnose COPD via analysis of SP-A and SP-D expression levels in EBC.

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