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Surfactant protein A expression and distribution in human lung samples from smokers with or without chronic obstructive pulmonary disease in China

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Abstract

Cigarette smoking is considered the main risk factor for chronic obstructive pulmonary disease (COPD), although the mechanism remains unknown. surfactant protein A (SP-A) is thought to protect the lung from smoking-induced damage, but related studies performed in China are scarce. The aim of the study is to assess alterations of SP-A expression and distribution in lung samples from Chinese smokers with or without COPD.

This cross-sectional study assessed 45 men in Wuhan Tongji Hospital after lobectomy for lung cancer in June 2010 to September 2010. Peripheral lung specimens were collected from control nonsmokers without airflow obstruction (nonsmoking group, n = 15), smokers without airflow obstruction (smoking group, n = 15), and patients with COPD (COPD group, n = 15). SP-A expression levels in lung tissue samples and its distribution in lung cells, type II pneumocytes (PNII), and alveolar macrophages (MACR) were determined by immunoblotting and immunohistochemistry.

SP-A levels were significantly decreased in the COPD group (1.00 ± 0.25) compared with the smoking (2.31 ± 0.64) and nonsmoking (8.03 ± 2.80) groups; the smoking group also showed significantly reduced levels compared with the nonsmoking group ($P < .05$). PNII expressing SP-A were less abundant in the COPD group ($39.3\% \pm 7.1\%$) compared with the smoking group ($76.2\% \pm 29.8\%$), whereas SP-A$^+$ MACR were more abundant ($92.4\% \pm 7.1\%$ vs $68.5\% \pm 20.2\%$) (all $P < .05$). Among the 30 smokers, forced expiratory volume in one second (% predicted) was positively correlated with SP-A levels ($r = 0.739$) and the rate of SP-A+ PNII ($r = 0.811$), and negatively correlated with the rate of SP-A+ MACR ($r = -0.758$) (all $P < .05$).

Changes in SP-A expression and distribution in lung tissues may be involved in COPD pathogenesis in smokers.

Abbreviations: COPD = chronic obstructive pulmonary disease, FEV_1 = forced expiratory volume in 1 second, FVC = forced vital capacity, MACR = macrophages, OD = optical density, PNII = type II pneumocytes, PS = pulmonary surfactant, SP-A = surfactant protein A, SPs = surfactant-associated proteins, TTF-1 = thyroid transcription factor-1.

Keywords: alveolar macrophages, chronic obstructive pulmonary disease, smoking, surfactant associated protein A, type II pneumocytes

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1. Introduction

Cigarette smoking is generally considered the most important contributor to chronic obstructive pulmonary disease (COPD),[1] but only 10% to 20% of smokers develop COPD,[2] indicating that other extrinsic or intrinsic factors may be involved in COPD pathogenesis. Prospectively identifying individuals susceptible to COPD among smokers would help initiate more specific and effective interventions to prevent COPD as early as possible. Previous studies have shown that α1-antitrypsin,[3] transforming growth factor-β1,[4] mEPHX17,[5] tumor necrosis factor-α,[6] and surfactant protein A (SP-A)[7] may be associated with COPD, but the mechanism underlying patient susceptibility to COPD among smokers remains unclear.

Pulmonary surfactant (PS) is an essential substance in the lung tissue and consists of lipids and proteins. Surfactant-associated proteins (SPs) can be divided into SP-A, SP-B, SP-C, and SP-D, with SP-A being the most abundant.[8] SP-A is mainly synthesized and secreted by type II pneumocytes (PNII) and belongs to group III of C-type lectin family, whose members have a structure consisting of multiple globular "head" regions linked by triple-helical, collagen-like, strands.[9] This protein group also includes SP-D and the serum proteins mannan-binding protein, conglutinin, and collectin-43, all of which bind to the C1q receptor found on a wide variety of cells, including macrophages, to act as opsonins.[10]

SP-A is currently thought to play an important role in inflammation regulation and host immune defense response against infection.[11] Previous studies have shown that SP-A can protect the lung from smoking-related damage.[12–14] In addition, studies have reported that SP-A gene polymorphism is associated with susceptibility to COPD[7] and the severity of this disease.[15]

Based on the above, we hypothesized that SP-A expression and distribution in the lung tissue may differ between individuals susceptible and nonsusceptible to COPD. Therefore, the present study aimed to explore the expression of SP-A in lung tissue samples from patients with lung cancer to provide further evidence regarding the role of SP-A and smoking in the pathogenesis of COPD. We found that alterations in SP-A expression and distribution in lung tissues may participate in COPD pathogenesis in smokers.

2. Materials and methods

2.1. Participants

A total of 45 male patients hospitalized at Wuhan Tongji Hospital for lobectomy due to lung cancer from June to September 2010 were assessed in this cross-sectional study. They were diagnosed by postoperative pathological examinations. No evidence of distant tumor metastasis was found by head magnetic resonance imaging enhancement, bone emission computed tomography, lymph node ultrasound, or abdominal ultrasound. Patients with tuberculosis, asthma, bronchiectasis, cystic fibrosis, and any other chronic diseases were excluded. According to the Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease 2017 Report[16] and smoking history, the patients were divided into 3 groups, including control nonsmokers without airflow obstruction (nonsmoking group, n = 15), smokers without airflow obstruction (smoking group, n = 15), and patients with COPD (COPD group, n = 15). Preoperative clinical data, including age, body mass index (BMI), and smoking index were collected via the electronic medical record system of the hospital. The study was approved by the ethical committees of the Wuhan Tongji Hospital and informed consent was obtained from the patient

2.2. Pulmonary function test and smoking index

Pulmonary function measurements were performed within 1 week before surgery, using a Jaeger pulmonary function meter (flow spirometer), measuring lung ventilation function parameters such as forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1) and FEV_1/FVC. Pulmonary function data were measured after a bronchodilation test, 20 minutes after inhalation of salbutamol 400 μg. The smoking index was determined as the number of cigarettes consumed per day multiplied by years of smoking, and recorded a week before surgery.

2.3. Sample preparation

Four lung tissue pieces (about 1 cm) per patient were obtained at least 5 cm away from the lung tumor; 1 was stored at −80°C for Western blot and 1 was fixed with 4% paraformaldehyde for >24 hours for immunohistochemistry, respectively.

2.4. Western blot

Total SP-A protein expression levels in the lung tissue were assessed, with β-actin as an internal reference. Lung tissue lysates were obtained and equal amounts total protein (50 μg) were separated by 12.5% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophorosis under reducing conditions. The protein bands were then transferred onto Polyvinylidene fluoride membranes. Detection was performed with mouse anti-human-SP-A (Abcam, ab51891, 1:2000 dilution) and rabbit anti-human-actin (Santa Cruz, sc-1616-R; 1:1500 dilution) antibodies. After film exposure, the bands were quantified with the Image Pro Plus 6.0 software, based on integrated optical density (IOD) values.

2.5. Immunohistochemistry

Immunochemistry was performed by 2 investigators in a blinded manner. SP-A expression in situ and distribution were assessed in different lung cells, including PNII and alveolar macrophages (MACR). Thyroid transcription factor-1 (TTF-1) is mainly found in the thyroid, lung, and brain tissues in humans,[17,18] and mostly located in nuclei of PNII in the lung. A study has tagged TTF-1 to count type II alveolar epithelial cells.[19] SP-A and TTF-1 in formalin-fixed, paraffin-embedded lung tissue sections were detected by the SP method. Primary mouse anti-human-TTF-1 (Santa Cruz, sc-53136, 1:200 dilution) and mouse anti-human-SP-A (Abcam, ab51891, 1:1000 dilution) antibodies were used to assess total PNII and SP-A+ PNII, respectively. Bovine serum albumin was used as negative control. Sections were counterstained with Mayer hematoxylin. SP-A optical density (OD) per square millimeter semiquantitatively reflected total SP-A expression in situ.[20] The rates of SP-A+ PNII and MACR reflected SP-A distribution in the lung tissue. SP-A expression was determined in 20 randomly selected high power fields.

2.6. Statistical analysis

SPSS 18.0 (SPSS Inc, Chicago, IL) was used for data analysis. Data are mean ± standard deviation (SD). Group comparisons were performed by 1-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. Associations were assessed by Pearson correlation analysis. Two -ided $P < .05$ was considered statistically significant.

3. Results

3.1. Patient baseline characteristics

The clinical data of the subjects are shown in Tables 1 and 2. Age and BMI were similar among the 3 groups, and smoking index

Table 1

General characteristics and pulmonary function of the participants.

Variables	COPD	Smoking	Nonsmoking
Cases (males)	15	15	15
Age, y	57.3 ± 10.2	55.7 ± 10.4	56.3 ± 11.2
BMI, kg/m^2	22.1 ± 3.0	22.5 ± 3.4	21.9 ± 2.4
SI	865 ± 270	766 ± 228	—
FEV_1/FVC (%)	55.8 ± 10.4	82.4 ± 10.0[*]	86.9 ± 11.6[*]
FEV_1 (%pred)	61.5 ± 10.2	79.5 ± 6.1[*]	79.2 ± 5.9[*]

BMI = body mass index, COPD = chronic obstructive pulmonary disease, FEV_1 = forced expiratory volume in 1 second, FVC = forced vital capacity, pred = predicted, SI = smoking index.

[*] $P < .05$ compared with the COPD group.

Table 2

Characteristics and SP-A levels of the participants.

	No	Age, y	SI	BMI, kg/m²	FEV₁/FVC (%)	FEV₁ (% pred)	SP-A IOD in WB	SP-A-positive of MACR (%)	SP-A-positive of PNII (%)
Nonsmoking group	1	55	0	20.8	85	97	9.65	33.3	78.6
	2	60	0	19.9	76	71	3.73	67.4	67.8
	3	43	0	26.3	88	112	9.74	41.3	93.6
	4	48	0	22.1	75	76	4.88	45.5	79.5
	5	68	0	18.4	86	92	11.18	28.8	93.6
	6	48	0	21.9	78	82	8.69	67.5	85.4
	7	65	0	24.5	78	86	6.84	42.8	87.1
	8	39	0	20.7	75	88	8.70	40.0	92.6
	9	35	0	23.2	79	89	9.73	31.8	89.8
	10	58	0	22.3	76	79	5.28	42.6	97.5
	11	67	0	19.7	84	95	9.65	38.1	94.6
	12	70	0	19.0	83	95	10.53	35.7	85.0
	13	67	0	25.3	73	70	4.82	89.7	89.6
	14	62	0	24.0	86	97	12.58	26.3	87.2
	15	59	0	20.1	75	75	4.37	55.6	89.4
Smoking group	1	71	1040	25.1	76	70	1.82	93.6	33.6
	2	49	1015	25.8	86	100	2.81	29.4	97.5
	3	39	380	24.6	78	73	1.33	85.8	56.7
	4	65	1075	21.5	87	101	3.42	50.1	89.0
	5	45	625	17.2	75	77	3.03	76.3	53.3
	6	40	800	27.9	77	78	1.72	56.6	69.0
	7	59	975	26.4	79	79	1.79	77.6	76.6
	8	48	520	26.4	80	79	3.28	64.4	87.9
	9	68	720	18.6	83	86	2.27	54.7	61.4
	10	58	660	21.3	86	81	1.82	69.2	82.7
	11	65	880	21.0	87	83	2.49	64.2	95.4
	12	62	600	23.2	77	81	2.72	74.4	79.9
	13	47	650	18.3	84	100	2.57	39.5	96.9
	14	64	1050	19.8	70	76	1.89	95.2	45.7
	15	55	495	20.4	76	73	1.70	96.8	45.7
COPD group	1	67	650	19.6	68	69	1.28	77.3	28.2
	2	72	1300	18.8	61	41	0.51	100.0	32.9
	3	46	560	22.3	68	68	1.17	84.2	45.6
	4	45	750	20.6	47	46	0.68	97.3	34.7
	5	65	864	26.1	53	47	0.78	98.7	39.1
	6	59	1100	18.4	55	54	1.00	94.5	36.3
	7	59	1290	20.3	63	67	1.17	89.3	59.0
	8	66	875	23.5	58	57	1.17	94.3	39.6
	9	67	855	22.7	60	60	1.06	89.6	34.7
	10	43	525	25.1	69	61	1.02	92.6	44.9
	11	66	1080	20.1	65	61	1.30	90.9	42.6
	12	53	900	19.1	68	57	1.08	97.2	37.5
	13	58	1140	21.6	38	43	0.86	99.5	38.8
	14	54	520	25.5	69	68	1.24	80.9	35.0
	15	39	570	27.6	66	39	0.66	100.0	41.0

BMI = body mass index, COPD = chronic obstructive pulmonary disease, FEV₁ = forced expiratory volume, FVC = forced vital capacity, IOD = integrated optical density, MACR = alveolar macrophages, PNII = type II pneumocytes, pred = predicted, SI = smoking index, SP-A = surfactant protein A, WB = western blot.

did not significantly differ between the smoking and COPD groups (all $P > .05$). Meanwhile, FEV₁/FVC and FEV₁ (% predicted) were significantly lower in the COPD group compared with the smoking and nonsmoking groups ($P < .05$); the latter 2 groups showed comparable values.

3.2. Total SP-A protein expression levels by western blot

Standardized IOD values for SP-A bands were 1.00 ± 0.25, 2.31 ± 0.64, and 8.03 ± 2.80 in the COPD, smoking, and nonsmoking groups, respectively, indicating statistically significant differ-

ences ($P < .05$). Both the COPD and smoking groups had lower values compared with the nonsmoking group (Fig. 1 and Table 2).

3.3. Total SP-A expression levels detected by immunohistochemistry

Positive signals for SP-A were found in the cytoplasm of PNII and MACR (Fig. 2A). IOD values were $47,732 \pm 13,799/mm^2$, $103,112 \pm 47,386/mm^2$, and $153,130 \pm 7467/mm^2$ in the COPD, smoking, and nonsmoking groups, respectively, indicating
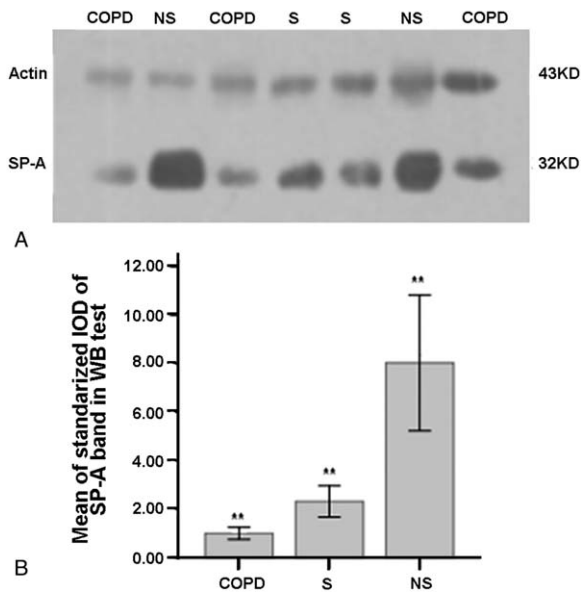
Figure 1. SP-A protein levels by Western blot SP-A protein amounts in the 3 groups were assessed by Western blot and immunoreactive bands are shown in (A). Mean ± standard deviation of standardized IOD for SP-A is shown in (B). **$P < .05$ (n = 15). SP-A = surfactant protein A.

statistically significant differences ($P < .05$) (Fig. 2B and Table 2), as shown above by immunoblotting.

3.4. Distribution of SP-A in different lung cells

Specimens from 30 smokers (smoking and COPD groups) were assessed by immunohistochemistry to evaluate the distribution of SP-A in different cells. The total amounts of PNII in the COPD and smoking groups were $520 \pm 206/mm^2$ and $327 \pm 135/mm^2$,

respectively, whereas the rates of SP-A positive cells were 39.3% $\pm 7.1\%$ and 76.2% $\pm 29.8\%$, respectively (n = 15, $P < .05$). The total number of PNII in the COPD group was higher than that of the smoking group, but SP-A-positive PNII were less abundant in the COPD group compared with the smoking group. The total amounts of MACR in the COPD and smoking groups were $482 \pm 188/mm^2$ and $159 \pm 73/mm^2$, respectively, whereas the rates of SP-A+ MACR were 92.4% $\pm 7.1\%$ and 68.5% $\pm 20.2\%$ in the COPD and smoking groups, respectively (n = 15, $P < .05$). The COPD group had larger amounts of MACR and a higher rate of SP-A-positive cells compared with the smoking group (Figs. 3 and 4, and Table 2).

3.5. Associations of SP-A expression and distribution with various parameters

Correlation analysis was performed with data of the 30 smokers (smoking and COPD groups). Standard SP-A's IOD obtained by immunoblotting was positively correlated with FEV_1 (% predicted) ($r = 0.739$, $P < .05$). SP-A's IOD per square millimeter of lung tissue obtained by immunohistochemistry was also positively correlated with FEV_1 (% predicted) ($r = 0.677$, $P < .05$). In addition, there was a positive correlation between the rate of SP-A positive PNII and FEV_1 (% predicted) ($r = 0.811$, $P < .05$). The rate of SP-A-positive alveolar macrophages was negatively correlated with FEV_1 (% predicted) ($r = -0.758$, $P < .05$). There were no significant associations of lung tissue SP-A expression or distribution in different cells with age, BMI, and smoking index (all $P > .05$).

4. Discussion

In this study, SP-A levels were significantly decreased in COPD patients compared with the remaining individuals. At the cellular level, less PNII and more MACR expressing SP-A were found in the COPD group compared with the smoking group. Finally, in smokers, FEV_1 (% predicted) was positively correlated with SP-A
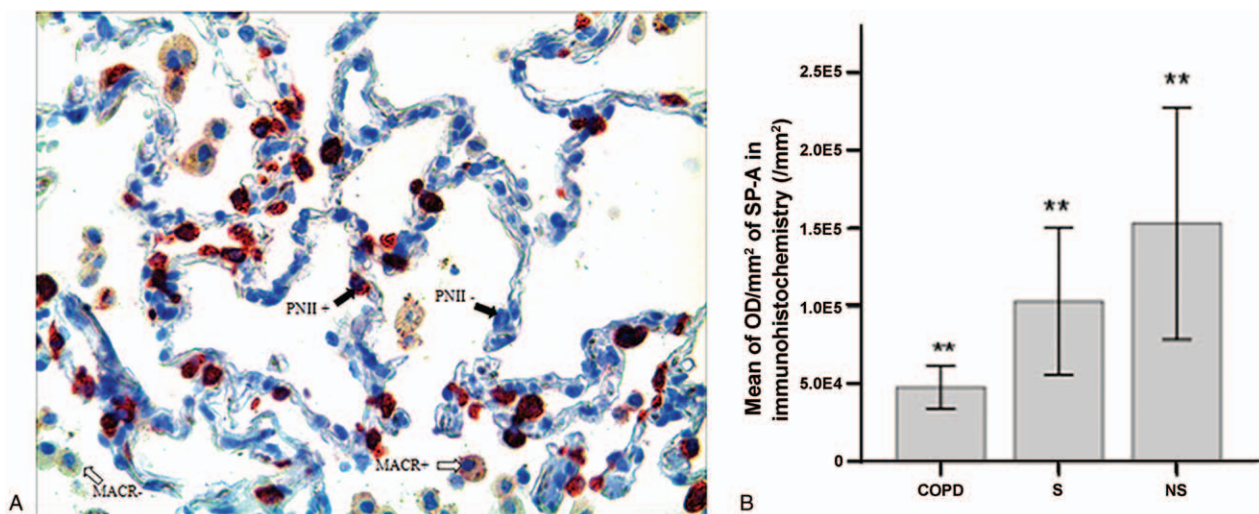

Figure 2. Total SP-A expression levels assessed by immunohistochemistry. (A) SP-A signals (dark red) were mostly distributed in the cytoplasm of PNII and MACR, with limited amounts in the alveolar and interstitial spaces (200×). (B) Quantitation of (A), expressed as IOD per square millimeter of 4-μm-thick lung tissue sections, that is, semi-quantitative measurement of total lung SP-A expression levels in situ. **$P < .05$ (n = 15). MACR = alveolar macrophages, PNII = type II pneumocytes, SP-A = surfactant protein A.

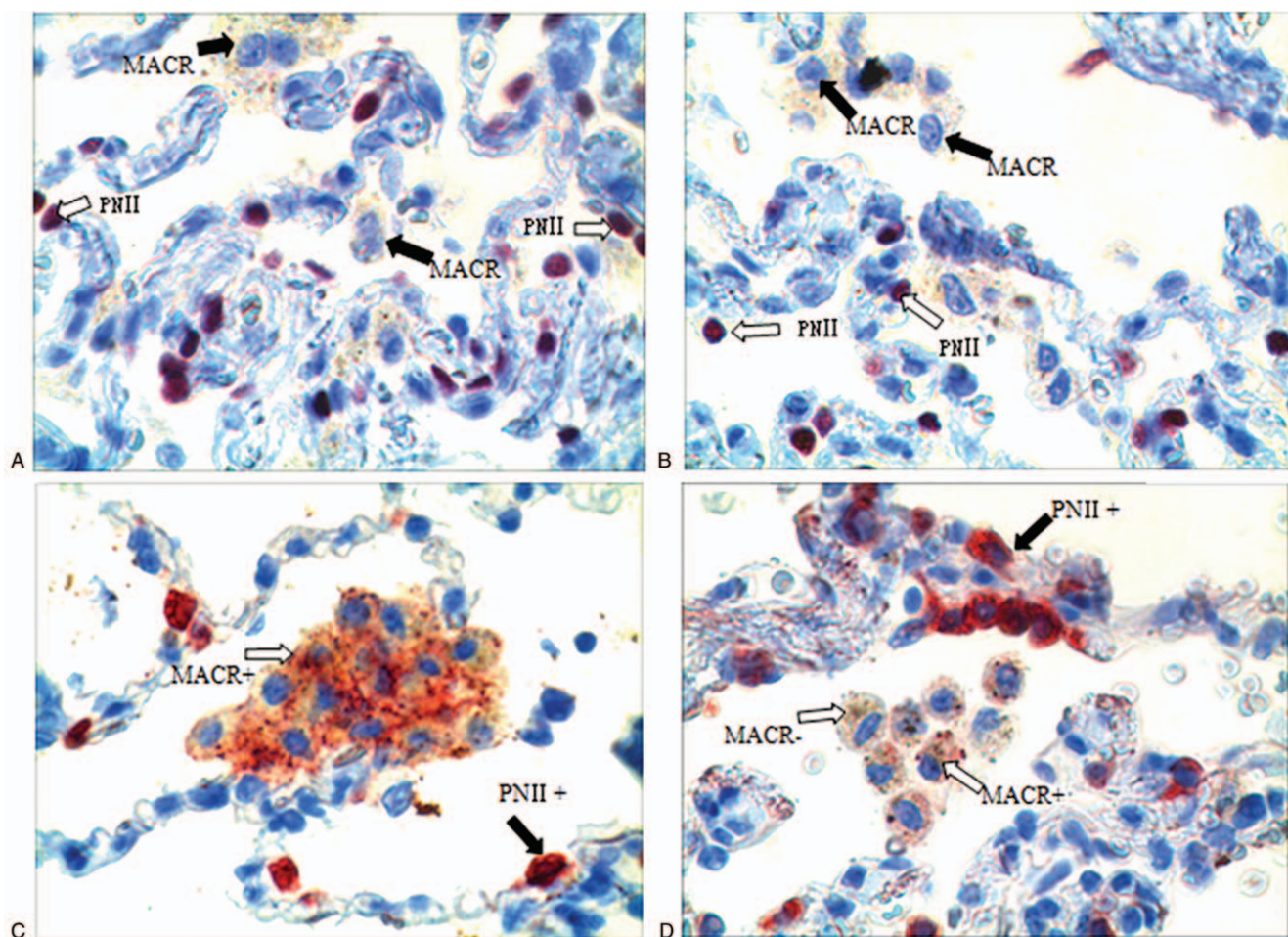**Figure 3.** Distribution of SP-A in different lung cells. (A) and (B) show TTF-1 stained samples (400×). PNII were displayed as cubic or roundish purple cells with large nuclei and limited cytoplasm. MACR, located at the alveolar space or close to alveolar wall, had an irregular shape with multiple blue-stained nuclei, foamed cytoplasm and foreign granules. (A), a representative sample of the COPD group; (B), a representative sample of the smoking group. (C) and (D) show SP-A antibody stained samples (400×). SP-A+ PNII were displayed as cubic cells on the alveolar wall, with blue roundish nuclei and red cytoplasm. SP-A+ MACR, located at the alveolar space or close to the alveolar wall, had an irregular shape with multiple blue-stained nuclei, foamed cytoplasm and foreign granules. (C), a representative sample of the COPD group; (D), a representative sample of the smoking group. COPD=chronic obstructive pulmonary disease, MACR=alveolar macrophages, PNII=type II pneumocytes, SP-A=surfactant protein A, TTF-1=Thyroid Transcription Factor-1.

levels and the rate of SP-A+ PNII, and negatively correlated with the rate of SP-A+ MACR.

As shown above, smokers had reduced SP-A amounts, indicating that smoking could reduce SP-A levels in the lung tissue. SP-A is considered to protect the lung tissue from oxidative stress, inflammation, and infection, also helping clear apoptotic cells from the lung.[11,13] Long-term smokers with reduced SP-A amounts in the BALF would be susceptible to respiratory infections, and the inability to eliminate apoptotic cells would result in abnormal lung tissue regeneration.[21] Two recent independent studies suggested that long-term smoking rats have decreased SP-A levels in the lung tissue and BALF, confirming that smoking interferes with SP-A metabolism and associated protective effects in the lung.[22,23]

Compared with smokers without COPD (smoking group), smokers with COPD (COPD group) showed reduced SP-A amounts, more PNII but lower amounts of SP-A+ PNII, and more total and SP-A+ MACR. Increased PNII amounts in the lung tissue of smokers with COPD could be explained by the Milic-Emili's tissue repair theory.[24] After lung tissue damage in COPD

patients, the differentiation and proliferation of cells are induced to compensate the missing cells; in lung damage and chronic inflammation, PNII would proliferate and differentiate into PNI.[25] Besides, proliferating PNII produce some substances, including SP-A, for the prevention against harmful factors.[26] Compared with smokers without COPD, those with COPD have more MACR, as reported previously.[27] Due to damage induced by smoking, SP-A expression and metabolism are altered. The abnormal SP-A in the alveoli is taken up by MACR, leading to increased amounts of SP-A-positive MACR. This alteration is closely correlated to the extent of airway obstruction. It was pointed out that SP-A could stimulate MACR and increase their ability to uptake viruses, bacteria, allergens, and apoptotic cells.[28] As an opsonin, SP-A might stimulate MACR to take up abnormal SP-A from the alveoli and distribute more SP-A.

Compared with smokers not suffering from COPD, the COPD group had a lower rate of SP-A-positive PNII; this is also related to the extent of airway obstruction, which may be caused by decreased PNII function, in which cells secrete less SP-A. These changes became more obvious with disease aggravation. Under
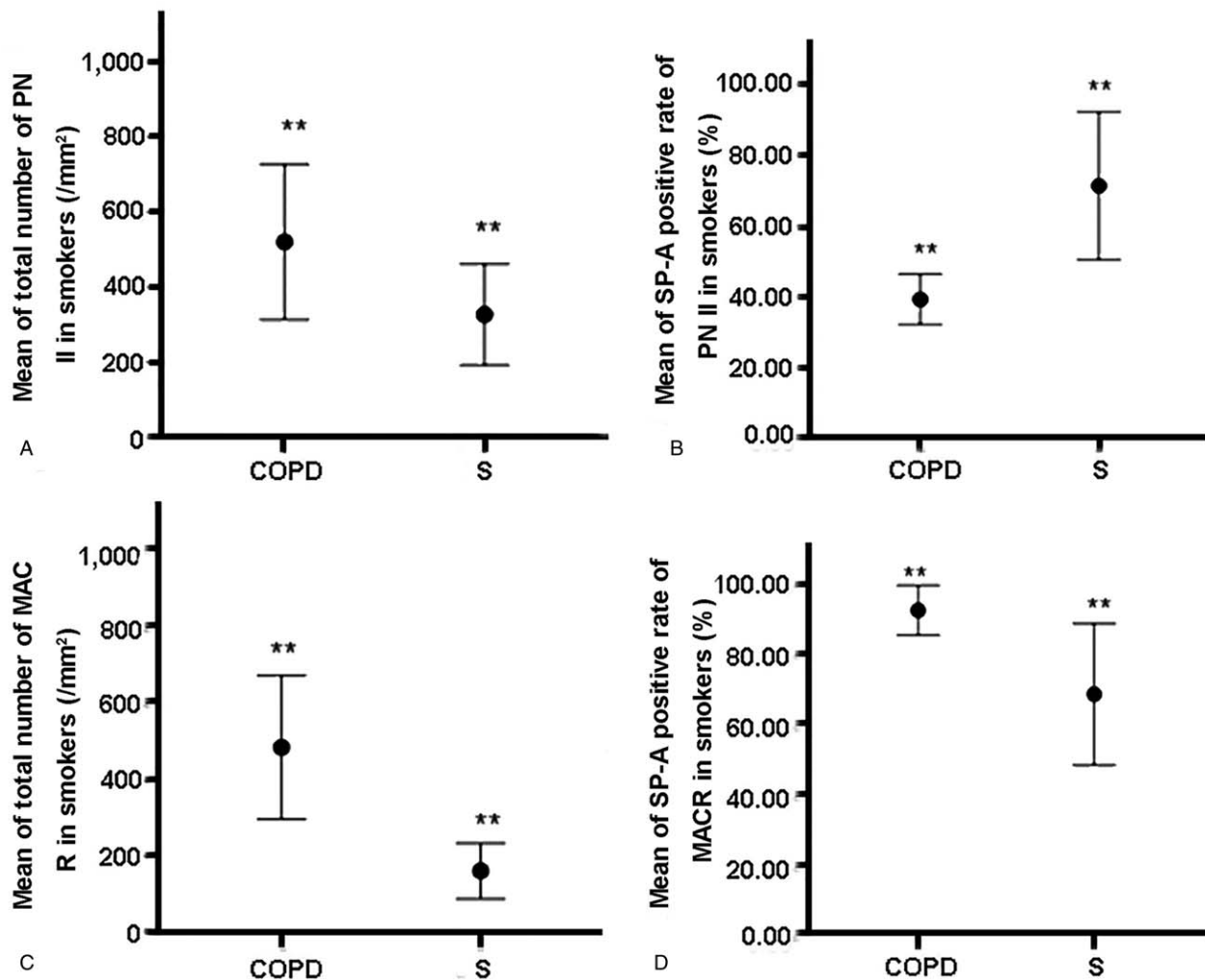
Figure 4. Quantitation of immunohistochemistry data. (A) The COPD group had larger amounts of PNII compared with the smoking group. (B) The COPD group had a lower rate of SP-A+ PNII compared with the smoking group. (C) The COPD group had larger amounts of MACR compared with the smoking group. (D) The COPD group had a higher rate of SP-A+ MACR compared with the smoking group. **$P < .05$ (n = 15). COPD = chronic obstructive pulmonary disease, MACR = alveolar macrophages, PNII = type II pneumocytes, SP-A = surfactant protein A.

continuous stimulation of cigarettes, the alveolar epithelial barrier is repeatedly damaged by oxidative stress; the COPD-susceptible DNA would undergo oxidative damage, leading to DNA mutation[29–31] and PNII secreting less SP-A. In agreement, it was shown that genetic variants of surfactant proteins, for examples, single-nucleotide polymorphisms and haplotypes, are associated with lung disease throughout life in several populations.[32] As shown above, FEV_1 (% predicted) was associated with alterations of SP-A expression, which might be closely related to the extent of airflow limitation.

Based on the above findings, we speculate that in smokers susceptible to COPD, the quality and amounts of SP-A produced by PNII are reduced; in individuals not susceptible to COPD, additional factors may protect PNII cells, allowing the latter to produce more SP-A of good quality and prevent COPD induced by smoking. Further investigation is required to determine such factors.

The limitations of this study should be mentioned. First, we did not demonstrate the causality between SP-A expression and distribution and COPD, and only provided observational findings regarding susceptibility to COPD. Meanwhile, both methods used for SP-A, including Western blot and immunohistochemistry, are merely semiquantitative; however, immunohistochemistry also helps assess SP-A distribution in cells. Therefore, additional studies are required to confirm the current findings and comprehensively determine the role of SP-A in COPD.

5. Conclusions

Overall, the above findings suggested that changes of SP-A expression and distribution in the lung tissue play an important role in the pathogenesis of COPD in smokers. Such alterations of SP-A may constitute a potential maker for identifying smokers more likely to develop COPD. Further studies are required to comprehensively determine the role of SP-A in COPD.

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Author contributions

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