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SERPINA1 Methylation Levels are Associated with Lung Cancer Development in Male Patients with Chronic Obstructive Pulmonary Disease

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Purpose: The mechanism of lung cancer (LC) in male patients with chronic obstructive pulmonary disease (COPD) has not been well understood, and the early diagnosis is currently challenging. The study aimed to explore the association of DNA methylation levels with LC development in male COPD patients.

Patients and Methods: A total of 147 male participants were divided into four groups, ie, COPD+LC group, COPD group, LC group, and control (CON) group. The methylation levels of human serine protease inhibitor A1 (*SERPINA1*) and the serum levels of inflammatory biomarkers were compared among groups. Multivariate logistic regression was performed to explore the correlation of inflammatory biomarkers and gene methylation with lung cancer combining COPD.

Results: *SERPINA1* methylation levels were significantly higher in the COPD+LC group than that in the COPD group and LC group, respectively (all $p < 0.05$). The serum levels of interleukin (IL)-1β, IL-17, and transforming growth factor (TGF)-β1 were significantly higher in the COPD+LC group than in the LC group (all $p < 0.05$). The *SERPINA1* methylation levels were positively correlated with the IL-1β levels (r = 0.5188, $p = 0.0012$). The AUC (area under curve) of *SERPINA1* methylation for the diagnosis of LC in COPD was 0.677 (sensitivity of 52.2% and specificity of 78.2%).

Conclusion: The methylation of *SERPINA1* is linked to LC in patients with COPD. The *SERPINA1* methylation levels were positively correlated with the IL-1β levels. These findings may be of diagnostic value.

Keywords: *SERPINA1* methylation, lung squamous cell carcinoma, chronic obstructive pulmonary disease, male

Introduction

Chronic obstructive pulmonary disease (COPD) is associated with a large public health burden as its high disability and mortality. Lung cancer (LC) is the second most common malignant tumor in the world, accounting for 11.4% of all cancers.[1] Smoking is a common risk factor for COPD and LC, while COPD itself is a high-risk factor for LC.[2] The development of tumor-associated inflammation has received increasing attention. Studies have explored the genetic susceptibility to LC, epigenetic changes, and DNA damage repair in patients with COPD who develop LC.[3] It has been revealed potential links between epigenomics including DNA methylation, non-coding, and microRNA expression, and post-translational modifications of histones and COPD and LC.[4]

The human serine protease inhibitor A1 (*SERPINA1*) gene encoding alpha1-antitrypsin (AAT) is located at chromosome 14q32.13.[5,6] *SERPINA1* is involved in the pathophysiology of emphysema and it is a biomarker for COPD in individuals exposed to smoke or biofuels.[7–12] Two family-based cohorts showed that *SERPINA1* methylation was linearly correlated with the severity of lung function in COPD.[7] It was shown that the *SERPINA1* gene significantly interacted with smoking status, associated with

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a higher risk of COPD in Chinese.[13] In addition, *SERPINA1* seems to be indirectly involved in the distant metastasis of various cancers, including lung, ovarian, cervical, colorectal, and breast tumors.[14,15] As for lung cancer, a study explored the change of *SERPINA1* was associated with worse survival rates in non-small cell lung cancer (NSCLC).[8] However, the methylation levels of *SERPINA1* in the male patients with COPD comorbid with LC have not been well studied in detail.

Therefore, the study aimed to explore the association of DNA methylation levels with LC development in male COPD patients.

Materials and Methods

Study Population

In total, 147 participants (male, 40–90 years old) from the Biological Resource Database of Ruijin Hospital (January 2019 to December 2020) were enrolled in the study. The participants in our study were recruited randomly. They were divided into four groups: COPD+LC (n=23), COPD (n=56), LC (n=40), and CON (n=28, healthy subjects without a history of COPD or LC). A postbronchodilator FEV1/FVC (the ratio of forced expiratory volume in 1s of forced vital capacity) less than 0.70 is required for a diagnosis of COPD, which was diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease Guidelines.[16] Post-bronchodilator spirometry, body plethysmography, and diffusion capacity were measured using the Jaeger MasterScreen^{TM} (CareFusion, Germany). Additionally, the diagnosis of LC was based on the histological findings and TNM stage, as delineated in the 8th edition of American Joint Committee on Cancer.[17] The study was approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, and written informed consent was obtained from all participants. Patients were excluded if they had a history of pneumonia, acute exacerbation of COPD, antibiotic use in the past 3 months, or secondary LC. Exclusion criteria also included a history of asthma, poorly managed cardiovascular disease, arrhythmia, any neurological, endocrinal, immunological, mental, gastrointestinal, hepatic, or hematological abnormalities, and malignancy in any other organ system.

Research Protocol

Clinical data including age, smoking history, lung function parameters including postbronchodilator ratio of forced expiratory volume in 1 second (FEV_1) to forced vital capacity (FVC) (FEV_1/FVC%) and FEV_1 in percent predicted (FEV_1% pred), residual volume to total lung capacity (RV/TLC%), diffusion capacity (transfer factor) for carbon monoxide (DLCO), the pathological type of LC (LUSC [lung squamous cell carcinoma], LUAD [lung adenocarcinoma], SCLC [small cell lung cancer]), and TNM stage (I–II/III–IV) were collected. Tumor biomarkers such as carcinoembryonic antigen (CEA), neuron specific enolase (NSE), squamous cell carcinoma antigen (SCC), cytokeratin 19 fragment antigen 21-1 (CY211), and cancer antigen 125 (CA125) were measured along with the NLR (neutrophil to lymphocyte ratio) using a Cobas E 601 system (Roche Diagnostics, Penzberg, Germany). The serum levels of interleukin (IL)-1β, IL-17, and transforming growth factor (TGF)-β1 in the peripheral blood were determined using ELISA kits (Shanghai Fuhan Biotechnology Co., Ltd, Shanghai, China).

To detect the methylation levels of *SERPINA1*, 5mL of venous blood was collected from patients after fasting and added to a sodium citrate-containing anticoagulant tube. For separation, the blood was centrifuged at 2000 rpm for 20 min and stored at 4°C for further examination. Genomic DNA (500ng) was extracted from the peripheral blood using the Zymo-Research Kit. After bisulfite transformation, DNA purification and PCR amplification were performed. Pyrosequencing was used to detect CpG sites. PyroMark software (Qiagen, Hilden, Germany) was used to design specific primers for five CpG sites based on *SERPINA1* sequences (Table 1). Subsequently, PyroMark Q48 (Qiagen, Hilden, Germany) was used to sequence the PCR products. The methylation levels of the target genes were quantified using PyroQ-CpG software (Qiagen, Hilden, Germany).

Statistical Analysis

All Data were analyzed using SPSS, v 21.0 (SPSS Inc., Chicago, IL, USA). Categorical variables were presented as percentages. Continuous variables showing normal distribution were expressed as mean ± standard deviation and those showing a skewed distribution were expressed as the median (interquartile ranges). Shapiro–Wilk test was used to determine whether the data obeyed homogenous distribution. For homogenous distribution, differences were compared using the *t*-test

Table 1 Primer Sequence Information

Gene	Primer Name	Primer Sequence
SERPINA1	1. *SERPINA1*-F	GGTGGTGTTTGAAGTTGAGG
	1. *SERPINA1*-Rbio	CAAATCCCAACCAATAAACTTAACC
	1. *SERPINA1*-S	GGAGATAGGGTTTTGT

and one-way ANOVA. Meanwhile, for non-normal distribution, comparisons were performed using the rank-sum test and Kruskal–Wallis rank-sum test. Multiple linear regression was performed to explore the correlation of tumor, inflammatory biomarkers, and gene methylation with LC comorbid with COPD. The methylation levels of the *SERPINA1* were visualized using the ggplot2 3.3.5 package in R 4.0.3 software. The comparisons between tumor biomarkers, Inflammatory biomarkers, and *SERPINA1* methylation levels were visualized using GraphPad Prism v.8 (San Diego, CA, USA). Receiver operating characteristic (ROC) curve analysis was performed to evaluate the sensitivity and specificity of *SERPINA1* methylation levels in predicting patients with COPD comorbid with LC. P <0.05 was considered statistically significant.

Results

Clinical Characteristics

All of the groups contained a high proportion of smokers and ex-smokers, and the smoking index (pack*year, average pack number per day multiplied by years of smoking) in the COPD group was significantly higher than that in the LC group (p =0.030) and CON group ($p < 0.001$). Most tumors were detected in the upper lobe of the lung and for adenocarcinoma is the most common type of tumor. Most patients were in the advanced stage III–IV at the time of enrollment without treatments. The levels of FEV_1/FVC, FEV_1% pred, and DLCO% in the COPD+LC group and COPD group were significantly lower than those in the LC group and CON group ($p < 0.001$, respectively). There was no significant difference in RV/TLC% and age among the four groups (Table 2).

Furthermore, levels of NLR, IL-1β, IL-17, and TGF-β1 in the COPD+LC group were higher than those in the other groups ($p < 0.001$). The *SERPINA1* methylation levels in the COPD+LC group were higher than that in the other groups ($p < 0.001$)(Figure 1). The *SERPINA1* methylation levels were negatively correlated with the RV/TLC levels in the COPD group ($r = -0.998$, $p = 0.041$), but there was no correlation between *SERPINA1* methylation levels and FEV_1% in the COPD group and in COPD+LC group, respectively ($p > 0.05$). There was no significant difference in CA125, CEA, NSE, SCC, and CY211 among the four groups (Table 3).

Table 2 Clinical Characteristics of Patients in Different Groups (n=147)

Group	COPD+LC n=23	LC n=40	COPD n=56	CON n=28	F/x^2	P
Age, years	66±2	62±1	63±4	57±2	4.556	0.005
Smoking, ex-/smoker/non-smoker	12/5/6	19/10/11	53/2/1	5/18/5	19.095#	<0.001
Smoking Index	35.4±6.1	28.3±3.7	50.6±12.0	4.8±2.3	11.543	<0.001
Pathological type, LUSC/LUAD/SCLC	8/10/5	12/23/5	/	/	1.439#	0.487
TNM stage, I–II/III–IV	2/21	4/36	/	/	0.029#	0.865
FEV_1/FVC,%	60.6±12.7	76.5±4.9	47.3±12.8	82.2±4.9	37.177	<0.001
FEV_1% pred	54.4±17.9	78.8±17.5	54.5±20.6	73.8±18.0	6.671	0.001
RV/TLC%	52.5±10.0	44.9±11.6	47.6±4.6	44.7±14.6	1.512	0.227
DLCO%	57.2±24.4	79.7±15.4	56.3±15.8	65.5±12.9	4.497	0.009

Note: #x^2 value.

Abbreviations: COPD, chronic obstructive pulmonary disease; LC, lung cancer; CON, control; smoking index, average pack number per day multiplied by years of smoking; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; SCLC, small cell lung cancer; TNM, tumor size/lymph nodes/distant metastasis; FEV_1/FVC, the ratio of expiratory volume in the first second to forced vital capacity; FEV_1% pred, percent predicted of forced expiratory volume in the first second%; RV/TLC, residual volume to total lung capacity; DLCO, diffusion capacity (transfer factor) for carbon monoxide.

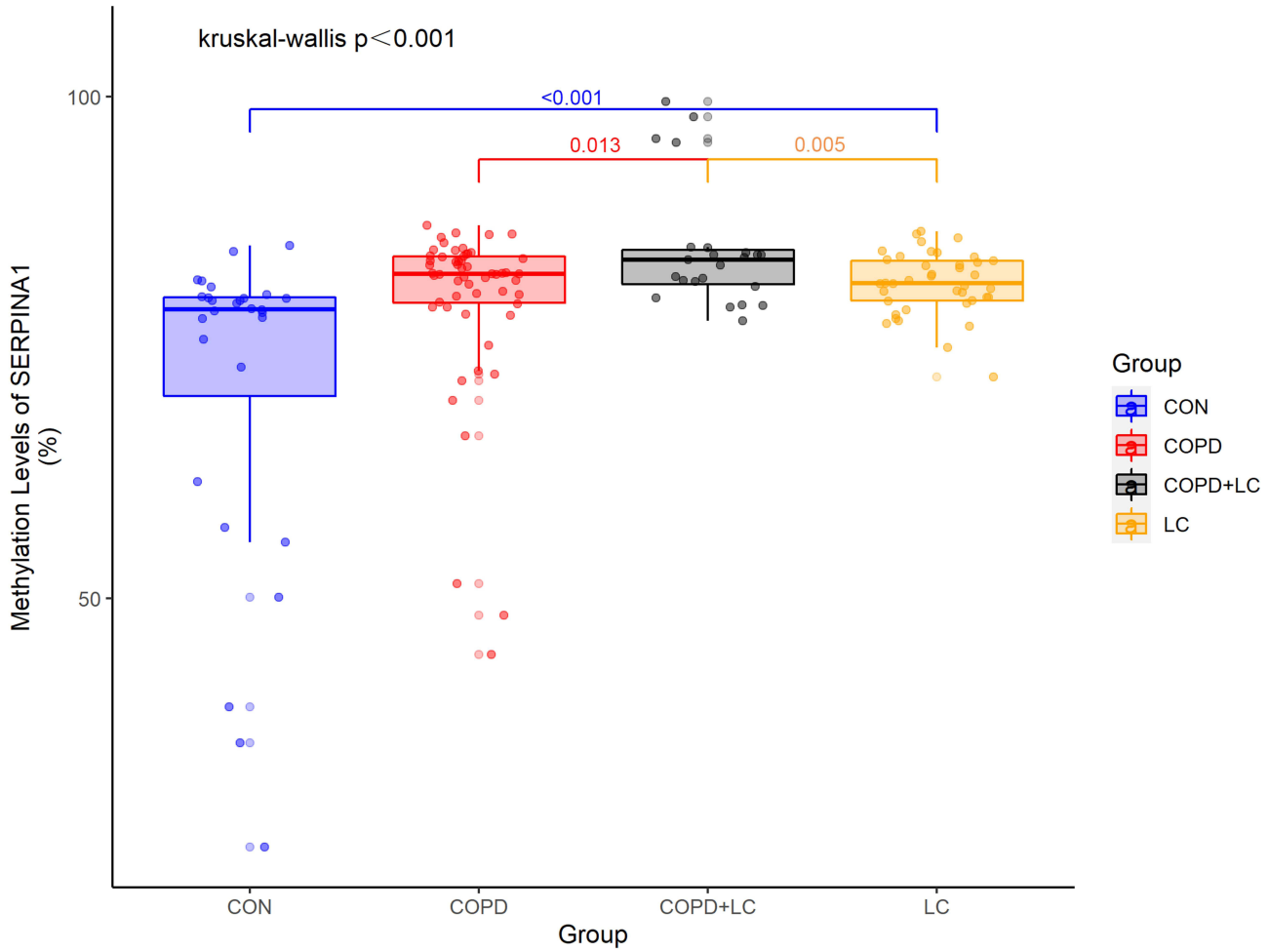

Figure 1 *SERPINA1* methylation levels in four groups. *SERPINA1* methylation levels were higher in the COPD+LC group than those in the COPD group and LC group, respectively. *SERPINA1* methylation levels were higher in the LC group than those in the CON group.
Abbreviations: CON, control; COPD, chronic obstructive pulmonary disease; LC, lung cancer; *SERPINA1*, serine protease inhibitor A1.

The Comparison of Tumor Biomarkers and *SERPINA1* Methylation Levels Between COPD+LC and COPD Groups

To observe the differences between COPD alone and COPD complicated tumor, we compared the differences of tumor biomarkers between the two groups. The Mann–Whitney *U*-test showed that the serum levels of CY211 in COPD+LC group were significantly higher than those in COPD group ($Z = -2.760$, $p = 0.006$). There were no differences in the other tumor biomarkers between the two groups (CA125: $Z = -1.590$, $p = 0.112$; CEA: $Z = -1.650$, $p = 0.099$; SCC: $Z = -1.471$, $p = 0.141$; NSE: $Z = -1.935$, $p = 0.053$) (Figure 2).

SERPINA1 methylation levels were higher in peripheral blood in the COPD+LC group than those in the COPD group ($t = 2.555$, $p = 0.013$). Moreover, the *SERPINA1* methylation levels in the COPD group were higher than those in the CON group ($t = -3.094$, $p = 0.003$) (Figure 1).

The Comparison of Inflammatory Biomarkers and *SERPINA1* Methylation Levels Between COPD+LC and LC Groups

To observe the differences between LC alone and COPD complicated tumors, we compared the differences in inflammatory biomarkers between the two groups. All of the inflammatory levels in the COPD+LC group was higher

Table 3 Biomarkers of Patients in Different Groups (n=147)

Group	COPD+LC n=23	LC n=40	COPD n=56	CON n=28	*F*	P
CA125*(μg/L)	28.3(72.0)	19.6(45.3)	17.0(14.8)	36.8(70.9)	0.611	0.610
CEA*(μg/L)	4.4(42.3)	5.3(30.6)	1.9(3.7)	2.5(2.4)	0.539	0.657
NSE*(ng/mL)	14.6(5.5)	12.6(10.3)	10.2(4.3)	13.3(3.7)	0.673	0.572
SCC*(μg/L)	1.4(1.2)	1.2(1.4)	1.0(0.5)	1.2(1.4)	0.811	0.493
CY211*(μg/L)	2.9(3.1)	2.8(5.6)	1.2(0.4)	2.5(2.1)	0.632	0.597
NLR (%)	7.4±5.4	7.3±4.7	3.6±1.9	3.4±2.3	6.831	<0.001
IL-1β*(pg/mL)	54.8(31.4)	37.7(10.9)	32.2(14.1)	15.3(9.3)	11.848	<0.001
IL-17*(ng/L)	53.2(30.0)	20.9(22.3)	28.6(16.9)	14.3(12.2)	10.253	<0.001
TGF-β1*(ng/L)	603.4(175.7)	377.1(180.2)	458.7(235.0)	160.9(57.6)	10.333	<0.001
SERPINA1(%)	84.8±6.2	81.4±3.1	80.0±8.7	71.3±16.1	10.207	<0.001

Note: *The median (interquartile interval).

Abbreviations: COPD, chronic obstructive pulmonary disease; LC, lung cancer; CON, control; CA125, cancer antigen 125; CEA, carcinoembryonic antigen; NSE, neuron-specific enolase; SCC, squamous cell carcinoma antigen; CY211, cytokeratin 19 fragment antigen 21-1; NLR, neutrophil-to-lymphocyte ratio; IL-1β, interleukin-1β; IL-17, interleukin-17; TGF-β1, transforming growth factor-β1; *SERPINA1*, serine protease inhibitor A1.

than those in the LC group (IL-1β: Z = −3.473, p = 0.001; IL-17: Z = −3.039, p = 0.002; TGF-β1: Z = −2.729, p = 0.006)(Figure 3A–C). Although there was no significant difference in levels of NLR between the COPD+LC group and LC group (p = 0.729), the NLR in the LC group was higher than that in the CON group (t = 3.429, p = 0.001).

SERPINA1 methylation levels were higher in peripheral blood in the COPD+LC group than those in the LC group (t = 2.901, p = 0.005). Moreover, the *SERPINA1* methylation levels in the LC group were higher than those in the CON group (t = −3.858, p < 0.001) (Figure 1).

Multivariate Linear Regression Analysis for LC Development in COPD

The diagnosis of COPD with LC was set as the dependent variable. The age, tumor biomarkers, inflammatory biomarkers, and methylation levels of *SERPINA1* were set as independent variables. The positive results were found in NLR, IL-1β levels and *SERPINA1* methylation levels, which were COPD-related factors associated with LC development (adjusted R^2 = 0.541, DW = 2.073; p < 0.05) (Table 4). Furthermore, the *SERPINA1* methylation levels were positively correlated with the IL-1β levels (r= 0.5188, p = 0.0012) (Figure 3D).

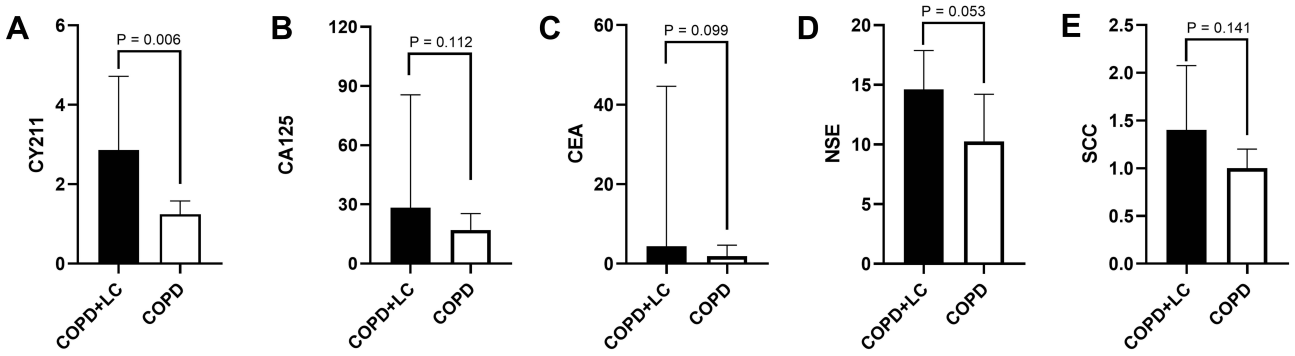

Figure 2 The tumor biomarkers in the COPD+LC group and the COPD group. (**A**) The levels of CY211 in the COPD+LC group were higher than those in the COPD group. (**B**) The levels of CA125 were compared between the COPD+LC group and COPD group. (**C**) The levels of CEA between the COPD+LC group and COPD group. (**D**) The levels of NSE between the COPD+LC group and COPD group. (**E**) The levels of SCC between COPD+LC group and COPD group.

Abbreviations: COPD, chronic obstructive pulmonary disease; LC, lung cancer; CY211, cytokeratin 19 fragment antigen 21-1; CA125, cancer antigen 125; CEA, carcinoembryonic antigen; NSE, neuron-specific enolase; SCC, squamous cell carcinoma antigen.

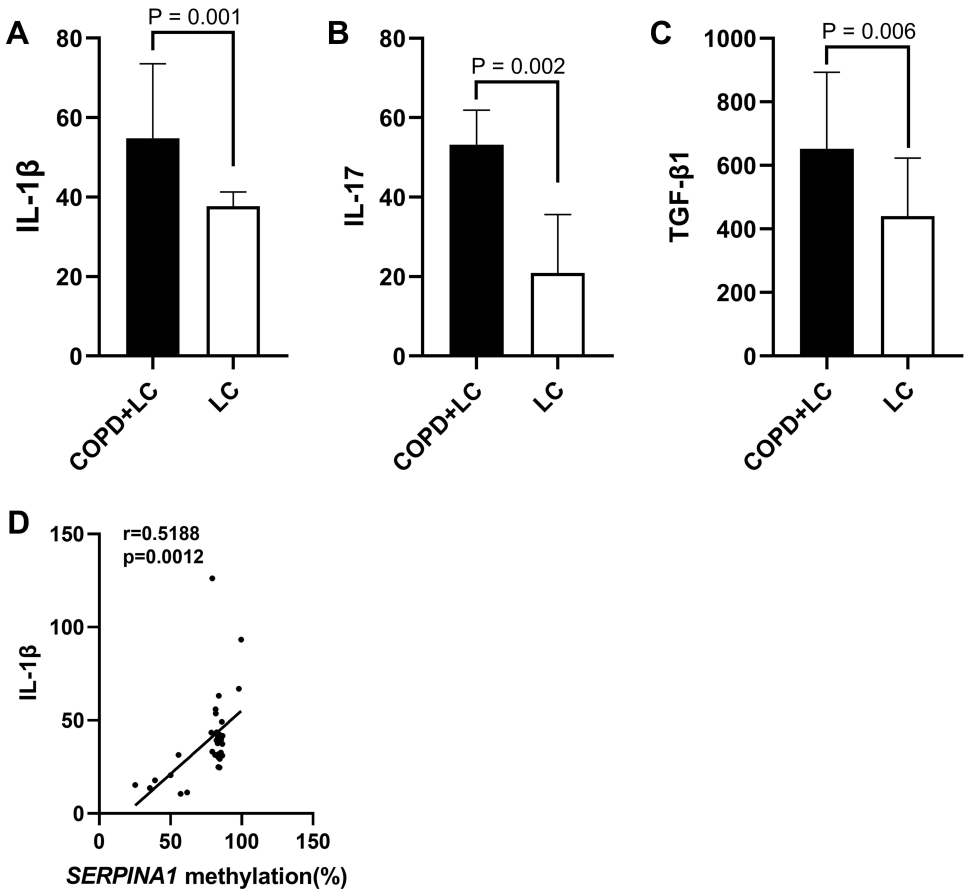

Figure 3 The inflammatory biomarkers in the COPD+LC group and the COPD group. (**A**) The levels of IL-1β in the COPD+LC group were higher than those in the LC group. (**B**) The levels of IL-17 in the COPD+LC group were higher than those in the LC group. (**C**) The levels of TGF-β1 in the COPD+LC group were higher than those in the LC group. (**D**) The association with the *SERPINA1* methylation levels and IL-1β.
Abbreviations: COPD, chronic obstructive pulmonary disease; LC, lung cancer; IL, interleukin; TGF-β1, transforming growth factor-β1; *SERPINA1*, serine protease inhibitor A1.

ROC curves were drawn with *SERPINA1* methylation levels as test variables and the occurrence of LC in COPD as the classification variable. The AUC (area under curve) of *SERPINA1* methylation for the diagnosis of LC in COPD was 0.677 (best critical value = 83.7%, sensitivity = 52.2%, and specificity = 78.2%) (Figure 4).

Discussion

Epigenetic modifications, including DNA methylation, gene silencing, and RNA editing, can increase the risk of lung cancer in COPD.[18] Recently, Various studies have proven that methylation levels could be used as markers for the

Table 4 Multiple Linear Regression Analysis of Biomarkers in COPD with LC Development

Biomarkers	*b*	*Sb*	*P*	*VIF*
NLR	0.111	0.040	0.013	1.473
IL-1β	−0.034	0.015	0.038	3.682
SERPINA1	0.083	0.020	0.001	4.138

Abbreviations: COPD, chronic obstructive pulmonary disease; LC, lung cancer; IL, interleukin; NLR, neutrophil-to-lymphocyte ratio; *SERPINA1*, serine protease inhibitor A1.

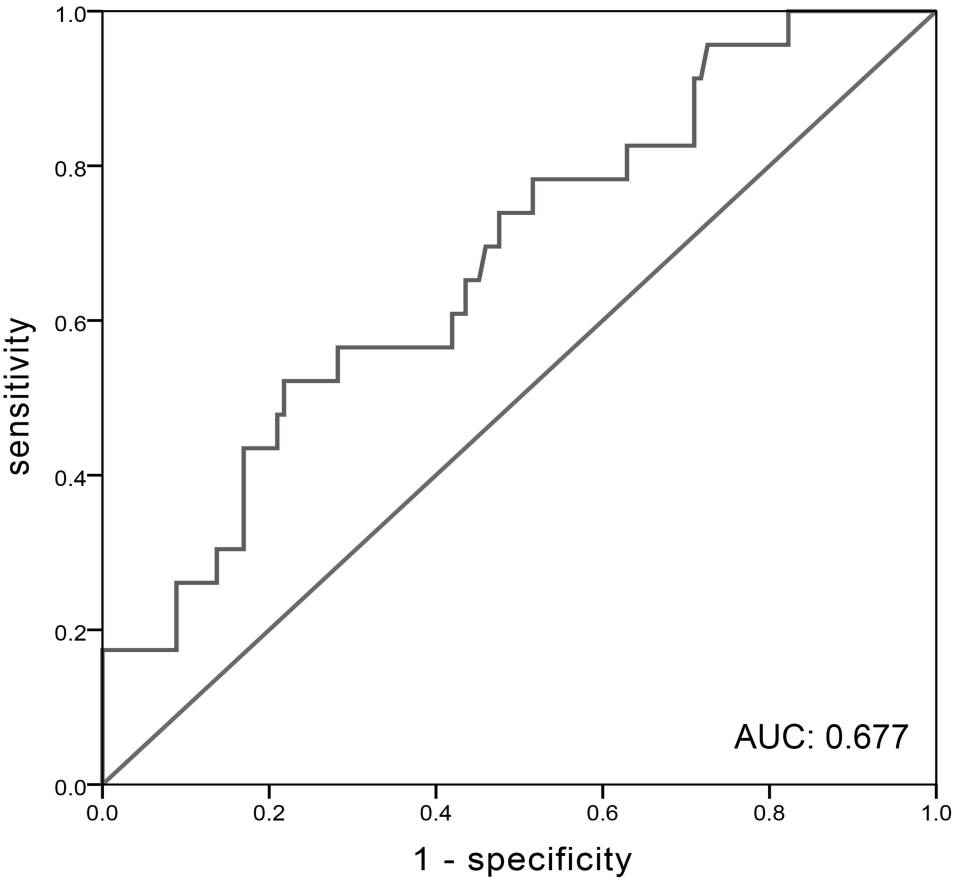

Figure 4 ROC curve for *SERPINA1* methylation levels for diagnosis for lung cancer in patients with chronic obstructive pulmonary disease. The AUC of *SERPINA1* methylation for the diagnosis of LC in COPD was 0.677 (best critical value = 83.7%, sensitivity = 52.2%, and specificity = 78.2%).
Abbreviations: ROC, receiver operating characteristic; AUC, area under curve; *SERPINA1*, serine protease inhibitor A1.

diagnosis of cancer in the early and advanced stages. DNA methylation is a stable alteration that can be easily and rapidly quantified by methylation-specific PCR methods.[19] The present study revealed that male patients in the COPD+LC group have higher levels of serum *SERPINA1* methylation than healthy controls and patients with COPD or LC alone. These results were consistent with those of previous studies showing that *SERPINA1* methylation is positively correlated with lung function and disease severity of COPD patients.[7]

In clinical practice, COPD complicated with LC is remarkably underdiagnosed or diagnosed at an advanced stage, or ineligible for surgery due to poor physical condition. Tumor markers have been mostly used in tumor screening and monitoring, but have not demonstrated accuracy and efficacy in diagnosing lung cancer, especially in COPD patients. The negative comparison results of most tumor biomarkers but CY211 between the COPD+LC and COPD groups might be attributed to the nonspecificity and high sensitivity to various microenvironments in the development of tumor disease.[20] But DNA methylation is a relatively stable modification state as early as embryogenesis until old.[21] The methylation levels of *SERPINA1* could be considered as a good biomarker for the complications via reflecting the change of tumor microenvironment based on COPD.

Smoking exposure results in the infiltration of the mucosa, submucosa, and glandular tissue by inflammatory cells.[22] The resulting inflammation is believed to contribute to LC development in patients with COPD.[23] Chronic inflammation alters the cellular levels of inflammatory mediators and activates proto-oncogenes.[24] IL-1β as an important biological marker of oxidative stress in COPD has been found to be associated with LC.[25,26] Similarly, IL-17 can increase neutrophil capture, and high IL-17 expression is a negative prognostic marker in many cancers, such as gastric, breast, and pancreatic cancer.[27] TGF-β1 is also a useful biomarker of the tumor microenvironment and plays an important role

in epithelial-mesenchymal stability.[28] Thus, we also examined the relationship between inflammatory biomarkers and LC comorbid with COPD in this study. The results showed that IL-1β, IL-17, and TGF-β1 could be considered biomarkers for auxiliary lung cancer diagnosis in COPD, and IL-1β positively correlated with the *SERPINA1* methylation levels. However, the mechanism underlying the interaction between methylation and inflammation needs to be explored further.

This study has a few limitations, such as the small sample size and cross-sectional design. A majority of the study population included smokers and III/IV stage patients. All patients enrolled in this study were male patients to avoid the effect of gender on DNA methylation. Although the results showed that there were no significant differences in the tumor location and pathological types between COPD+LC and LC groups, further detailed investigations should be carried out to explore the mechanisms underlying the regulation of *SERPINA1* methylation in COPD and LC.

Conclusion

The methylation of *SERPINA1* is linked to LC in patients with COPD. The *SERPINA1* methylation levels were positively correlated with the IL-1β levels. These findings may be of diagnostic value.

Data Sharing Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics Statement and Consent to Participate

The studies involving human participants were reviewed and approved by the Ruijin Hospital Ethics Committee (2019-72). The patients/participants provided their written informed consent to participate in this study. Our study was carried out in accordance with the Declaration of Helsinki.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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