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COPD

Comparative Study of Cytokine Levels in Different Respiratory Samples in Mild-to-Moderate AECOPD Patients

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Abstract

Background Matrix metalloproteinase-12 (MMP-12) and Tissue inhibitor of metalloproteinase-4 (TIMP-4) play important roles in the pathophysiology of chronic obstructive pulmonary disease (COPD). Subjects of many previous studies were patients with severe and very severe COPD. However, there are comparatively few studies on patients with mild-to-moderate COPD. Our aim was to measure MMP-12 and TIMP-4 levels and to compare its levels in various materials in patients with mild-to-moderate acute exacerbation of chronic obstructive pulmonary disease (AECOPD). We also compared which of the two materials of these biomarkers was better correlated with disease severity and DODE index.

Methods A total of 39 patients with AECOPD and 25 control subjects were enrolled in our study. MMP-12 and TIMP-4 in different respiratory samples were detected by ELISA.

Results Expression levels of MMP-12 in bronchoalveolar lavage fluid (BALF) and exhaled breath condensate (EBC) and TIMP-4 in BALF were significantly higher in AECOPD patients than that in healthy subjects ($P<0.001$). However, there was no significant difference in TIMP-4 level in EBC of AECOPD patients compared to healthy subjects ($P=0.0527$). The levels of MMP-12 in BALF and EBC and TIMP-4 in BAFL of AECOPD patients were significantly correlated with FEV_1% predicted ($P<0.001$). However, in AECOPD patients, there was no significant correlation between TIMP-4 levels in EBC and BODE index ($r=0.4175$, $P=0.0559$).

Conclusion During mild-to-moderate AECOPD, the levels of MMP-12 and TIMP-4 in BALF were better correlated with FEV_1% predicted and BODE index than that in EBC, indicating that they may be new target interventions for pharmacology to prevent and/or treat AECOPD.

Keywords Matrix metalloproteinase-12 · Bronchoalveolar lavage fluid (BALF) · Exhaled breath condensate · Chronic obstructive pulmonary disease · Tissue inhibitor of metalloproteinase-4

Introduction

Chronic obstructive pulmonary disease (COPD) is a common disease characterized by incomplete reversibility of airflow [1], a pathological feature of chronic inflammation of the airways and lung parenchyma, which is predicted to be the third most frequent cause of death in the world by 2020 [2]. Chronic inflammation of the airways, lung parenchyma, and pulmonary vessels is considered to be an important pathogenesis of COPD [3]. A variety of immune cells and inflammatory mediators are involved in this process.

Inflammatory biomarkers in various body fluid samples are associated with the pathogenesis and clinical outcome of COPD. Matrix metalloproteinases (MMPs) comprise a family of 25 related, yet distinct, zinc-containing enzymes [4]. The role of MMP-12 in lung diseases such as COPD and emphysema has been reported in many pieces of literature [5, 6]. Its production comes from the activation of macrophages, and also from the resident cell synthesis of the alveolar walls of epithelial cells, smooth muscle cells, and endothelial cells [7]. Elastin accounts for about 2.5% (w/w) of lung dry weight and is widely disseminated in the lungs [8], which is essential for the elasticity of alveolar

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and bronchial tubes. Therefore, an increase in the activity of MMP-12 can lead to important elastin degradation and lung parenchymal destruction.

MMP-12 activity can be specifically inhibited by TIMP-4, and the balance of MMP-12/TIMP-4 plays an important role in maintaining normal tissue structure and physiological functions. Serum is the most readily available sample of body fluids in clinical studies and basic research. There are many previous reports on the expression level of MMP-12 in serum [9, 10]. Several studies of MMP-12 in BAL have been reported. One study showed increased expression of heparan sulfate and chondroitin sulfate in BAL during AECOPD. These molecules are significantly associated with MMP-2, MMP-9, and MMP-12 in BALF [11]. Another study suggested that inhibition of MMP-12 with AS111793 reduces airway inflammation in mice exposed to cigarette smoke, strongly suggesting a specific involvement of MMP-12 in lung inflammation following cigarette smoke exposure [12]. Our previous study explored the relationship between the expression levels of MMP-12 and TIMP-4 in peripheral blood and EBC and the disease severity [13]. However, we reviewed literature and found no reports of TIMP-4 expression levels in BALF. Simultaneously, there are not any studies on the difference in expression levels of MMP-12 and TIMP-4 in BALF and EBC.

Thence, our aim was tantamount to detect MMP-12 and TIMP-4 in BALF and EBC, and to explore the differences between COPD patients and healthy controls. We also compared which of the two samples of these cytokines was better correlated with disease severity and DODE index.

Materials and Methods

Study Design and Participants

This was a cross-sectional study conducted in the Affiliated Hospital of Yan'an University, Shaanxi, China. A total of 39 patients with AECOPD were enrolled consecutively in the study between May 2017 and June 2019. Similarly, 25 controlled subjects were from the Health Checkup Center of the Yan'an University Affiliated Hospital. Our study was approved by the Ethics Committee of the Affiliated Hospital of Yan'an University.

The diagnosis of COPD is based on Global Initiative for Chronic Obstructive Lung Disease (GOLD) [14]. After inhaling the bronchodilator, the patient's FEV_1/FVC ratio is less than 0.70, indicating that the airflow is not completely reversible. All included subjects were willing to comply with the regulations to complete the entire program and signed an informed consent form. Exclusion criteria were as follows: (1) COPD patients with interstitial pulmonary fibrosis, asthma, bronchiectasis, tuberculosis, and lung cancer; (2) combined with heart failure, acute myocardial infarction, cerebral infarction, cerebral hemorrhage, or liver and kidney failure; (3) patients with COPD who have mental illness, cognitive dysfunction, or existing exchange disorders.

Expression of MMP-12 and TIMP-4 in BALF and EBC

Bronchoscopy is performed in the morning. All subjects were not allowed to eat before BALF examination, and 5 mL of 2% lidocaine (Grindex, Latvia) was used for topical upper respiratory tract anesthesia. The bronchoscope (Olympus, USA) was wedged into the mid-lobular bronchus and 25 mL × 6 for a total of 150 mL, infused with sterile saline solution (0.9% NaCl). The fluid is gently inhaled immediately after the infusion is completed and collected in a sterile container. The fluid was immediately centrifuged with a 40-μm cell stain (Becton Dickinson, USA) and centrifuged for 15 min below 4 °C. The supernatant was used for ELISA. EBC was gathered and processed according to the American Thoracic Society/ERS recommendations [15], using the TURBO-DECCS 09 system (Medivac, Parma, Italy).

MMP-12 and TIMP-4 in BALF and EBC were analyzed by ELISA using commercial kits (Human MMP-12 and TIMP-4 kits, R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions. The minimum detectable concentrations for MMP-12 and TIMP-4 were 2.5 pg/mL and 1.4 pg/mL, respectively.

BODE Index

The BODE index assigns scores to four variables in order to obtain a score ranging from 0 to 10. It is composed of body mass (B), degree of airflow obstruction (O), level of functional dyspnea (D), and exercise capacity (E) [16]. Body mass is calculated as body mass index (BMI); airflow obstruction is measured as forced expiratory volume in one second (FEV_1%) predicted; dyspnoea is measured using the modified Medical Research Council (mMRC) dyspnoea scale; and exercise capacity is measured as 6-min walk test (6MWT) (Table 1).

Table 1 Multidimensional scoring system for calculation of the BODE index

Variable	Points on BODE index			
	0	1	2	3
Body mass index	> 21	< 21		
FEV_1% predicted	≥ 65	50–64	36–49	≤ 35
MMRC dyspnea scale	0–1	2	3	4
Distance walked in 6 min (m)	≥ 350	250–349	150–249	≤ 149

BMI body mass index, *BMI* Weight (kg)/Height^2 (m^2), *mMRC* modified Medical Research Council, *FEV_1* forced expiratory volume in 1 s

Statistical Analysis

Data are presented as mean ± SD or *n* (%). All data analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL, USA). Pearson's correlation coefficient was used for correlation studies. Baseline differences between different studied groups were determined with unpaired student's t-test and one-way ANOVA. The Spearman correlation coefficient was utilized to analyze the relationship between the studied biomarker variables and the BODE index and FEV_1% predicted. Statistical significance was assumed at a *P* value of < 0.05.

Results

Characteristics and Differences of Study Subjects

Characteristics of the AECOPD patients and control subjects are presented in Table 2. A total of 39 AECOPD patients and 25 control subjects who met the study criteria were eventually enrolled. There were no significant differences in age, sex, body mass index, and smoking status between the two groups. Pulmonary function FEV_1% predicted ($P < 0.001$), FVC% predicted ($P < 0.001$), and FEV_1/FVC ($P < 0.001$) were significantly decreased in the AECOPD group compared with the control subjects.

MMP-12 and TIMP-4 in BALF and EBC

The expression level of MMP-12 in BALF (74.1 ± 43.6) (pg/mL) and EBC (42.7 ± 38.2) (pg/mL) of AECOPD patients was significantly higher than that in control subjects BALF (37.2 ± 38.4) (pg/mL) and EBC (24.5 ± 19.8) (pg/mL) ($P < 0.001$; Fig. 1a, b). Similarly, TIMP-4 levels in BALF (21.5 ± 19.3) (pg/mL) of AECOPD patients were also significantly elevated compared to control subjects (12.1 ± 9.6) (pg/mL) ($P < 0.001$; Fig. 1c). However, there was no significant difference in TIMP-4 level in EBC (6.4 ± 4.7) (pg/mL) of AECOPD patients compared to healthy subjects (5.5 ± 3.8) (pg/mL) ($P = 0.0527$; Fig. 1d).

Relationship Between Inflammatory Biomarkers and FEV_1% Predicted

The concentration levels of MMP-12 in BALF and EBC of AECOPD patients were significantly negatively correlated with FEV_1% predicted ($P < 0.001$, Fig. 2a, b). The expression level of TIMP-4 in BALF of AECOPD patients was also significantly negatively correlated with pulmonary function FEV_1% predicted ($P < 0.001$, Fig. 2c). However, there was no significant correlation between the expression level of TIMP-4 in EBC of AECOPD patients and lung function FEV_1% predicted ($P < 0.001$, Fig. 2d).

Table 2 Baseline characteristics of the AECOPD patients and control subjects

Variable	AECOPD patients (*n* = 39)	Control subjects (*n* = 25)	*P* value
Age (years)	59.3 ± 14.3	57.9 ± 11.8	0.504
Men (%)	33 (85)	21 (84)	0.711
BMI (kg/m^2)	22.6 ± 6.1	23.5 ± 5.7	0.427
Smoking status			
Never-smokers	17 (43.6)	11 (48.0)	0.471
Current smokers	22 (56.4)	14 (52.0)	0.534
Smoking history (pack-years)	18.1 ± 9.4	17.7 ± 12.5	0.457
COPD stages (%)			
Stage 1	15 (38.5)	–	NA
Stage 2	24 (61.5)	–	NA
Spirometry			
FEV_1 (% predicted)	78.5 ± 19.1	102.4 ± 16.8	$P < 0.001$
FVC (% predicted)	86.3 ± 17.4	109.5 ± 12.7	$P < 0.001$
FEV_1/FVC ratio (%)	59.6 ± 9.2	78.3 ± 6.4	$P < 0.001$
mMRC	2.2 ± 0.6	0.8 ± 0.3	$P < 0.001$
6 MWD (m)	339 ± 125	511 ± 128	$P < 0.001$
BODE index scores	2.3 ± 0.7	–	NA

Data are presented as mean ± SD or n (%)

COPD chronic obstructive lung disease, *BMI* body mass index, *mMRC* modified Medical Research Council, *FEV_1* forced expiratory volume in 1 s, *FVC* forced vital capacity, *6 MWD* 6-min walk distance, *BODE* body mass index (B), airflow obstruction (O), dyspnoea (D), and exercise capacity(E)

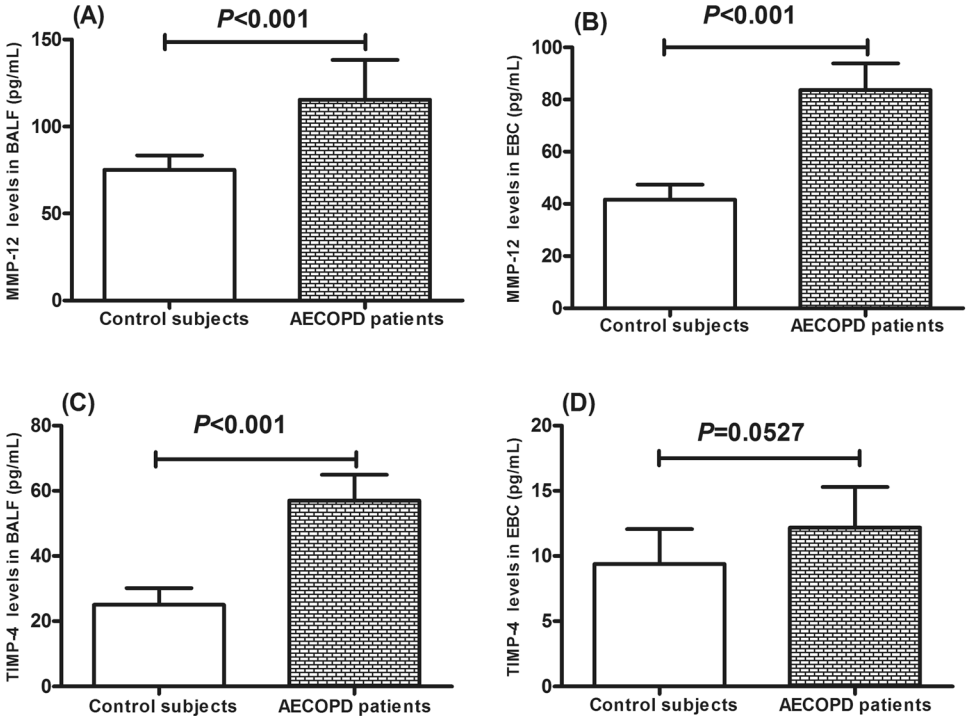

Fig. 1 MMP-12 and TIMP-4 levels in BALF and EBC from AECOPD patients and control subjects. All date presented as mean ± SD

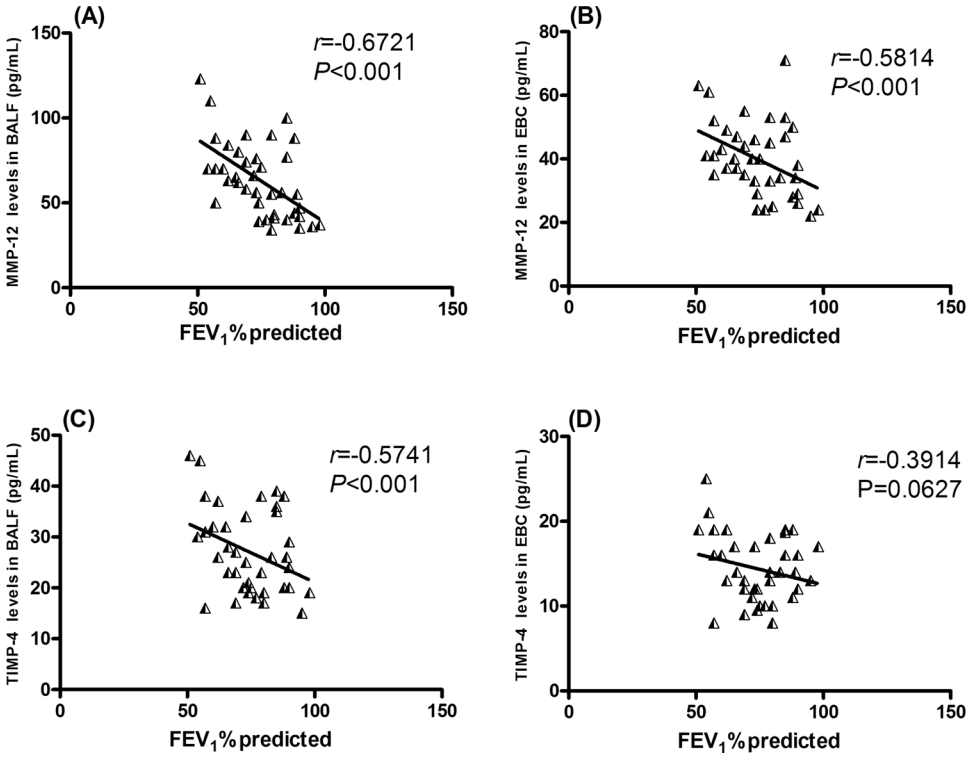

Fig. 2 Relationship between MMP-12 and TIMP-4 levels in different respiratory samples and pulmonary function FEV_1% predicted. **a** MMP-12 in BALF versus FEV_1% predicted; **b** MMP-12 in EBC versus FEV_1% predicted; **c** TIMP-4 in BALF versus FEV_1% predicted; **d** TIMP-4 in EBC versus FEV_1% predicted

Comparison of Cytokine Levels in Different Respiratory Samples

In mild-to-moderate AECOPD patients, the levels of MMP-12 and TIMP-4 in BALF were significantly correlated with the expression levels of MMP-12 and TIMP in EBC ($r=0.5267$, $P<0.001$, Fig. 3a; $r=0.4319$, $P=0.0451$, Fig. 3b), respectively.

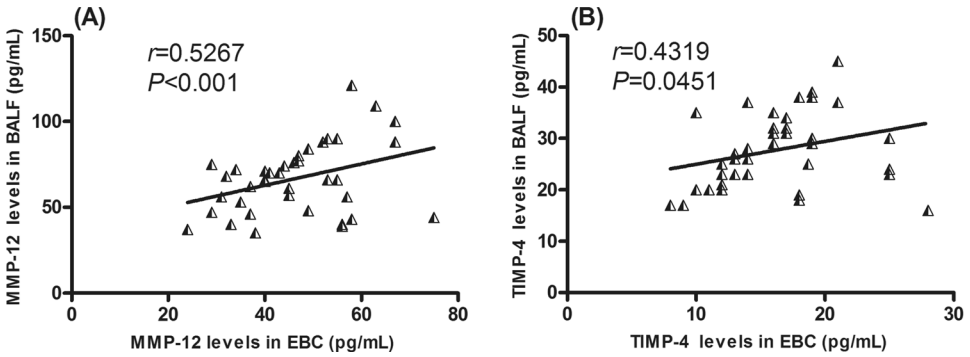

Fig. 3 Correlation analysis of the same biomarker in different respiratory samples. **a** MMP-12 in BALF versus MMP-12 in EBC; **b** TIMP-4 in BALF versus TIMP-4 in EBC

Correlation Between Inflammatory Biomarkers and BODE Index

The expression level of MMP-12 in BALF and EBC was significantly positively correlated with the BODE index ($r = 0.5349$, $P < 0.001$, Fig. 4a; $r = 0.5027$, $P < 0.001$, Fig. 4b). Similarly, the concentration level of TIMP-4 in BALF was also significantly positively correlated with the BODE index ($r = 0.4719$, $P < 0.001$, Fig. 4c). However, the expression level of TIMP-4 in EBC was not significantly correlated with the BODE index ($r = 0.4175$, $P = 0.0559$, Fig. 4d).

Discussion

The aim of present study was to evaluate MMP-12 and TIMP-4 levels and to compare its levels in various materials in patients with mild-to-moderate AECOPD. Then, we utilized the Spearman correlation coefficient to analyze the relationship between inflammatory biomarker parameters and BODE index and predicted FEV_1% predicted. Moreover, we also compared which of the two materials of these biomarkers was better related to disease severity and DODE index.

Our research provides some novel findings compared to previous studies on MMP-12 and TIMP-4. First, this is a study that directly compares MMP-12 and TIMP-4 concentrations in different respiratory samples. Second, we have not found any previous studies to measure TIMP-4 levels in

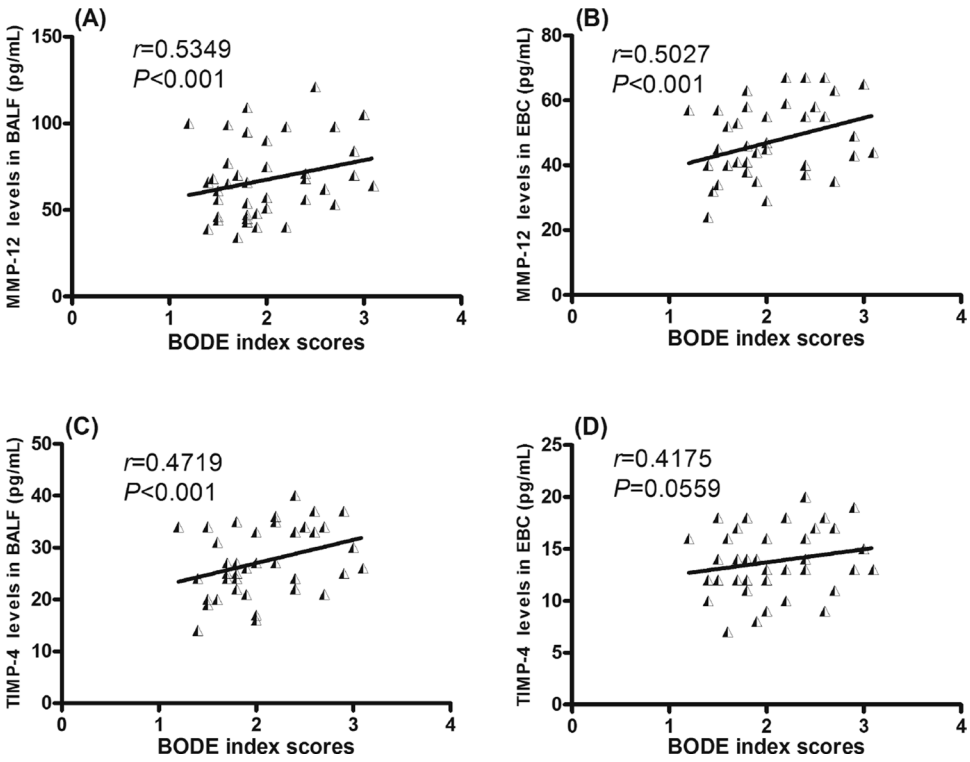

Fig. 4 Scatter plot between inflammatory biomarker levels and BODE index. **a** MMP-12 in BALF versus BODE index; **b** MMP-12 in EBC versus BODE index; **c** TIMP-4 in BALF versus BODE index; **d** TIMP-4 in EBC versus BODE index

BALF. Third, our study concentrated on patients with mild-to-moderate COPD, whereas most of the previous studies used severe and very severe patients. Finally, our COPD subjects did not use glucocorticoids for at least 4 weeks, whereas most previous studies were performed in patients with COPD treated with glucocorticoids.

Our report revealed that expression levels of MMP-12 were significantly higher in BALF of COPD patients compared with control subjects. This is in agreement with the findings of Molet et al. [17]. It analyzes the expression of MMP-12 in BALF cells and bronchial biopsies by immunocytochemistry, immunochemistry, and Western blotting, and found that the number and staining intensity of MMP-12 expressing macrophages in BAL samples of patients with COPD were higher than those of the control group. Another study [11] has suggested a significant increase in the levels of heparan sulfate and chondroitin sulfate in BALF in COPD patients with acute exacerbations, and these molecules are significantly associated with MMP-9, MMP-2, and MMP-12 in BALF. Furthermore, this study showed that MMP-12 level in EBC samples from patients with COPD was negatively correlated with pulmonary function tests. This result is consistent with our previous findings [18]. Moreover, the present report has, for the first time, shown that MMP-12 levels in BALF are inversely correlated to the severity of AECOPD disease.

However, a number of published literature reports are inconsistent with our findings. In a clinical study, the analysis of differential expression of 10 genes in lung tissue revealed that MMP-12 gene expression was not associated with the severity of airflow limitation in COPD patients [19]. In another study [20], macrophages in bronchoalveolar lavage fluid from emphysema patients were maintained in vitro for 24 h, and then mRNA transcription levels of matrix metalloproteinase (MMP) gelatinase A and B were assessed semi-quantitatively. Finally, no difference in the transcriptional level of MMP-12 was observed between the patient and the control sample, and no MMP-12 protein was detected in the macrophage culture medium from either group. There are many literature reports on MMP-12, but the results are not completely consistent. The reason may be that not only MMP-12 is inhibited by tissue inhibitors of metalloproteinases (TIMPs), but also the activity of MMP-12 is tightly regulated at the level of gene transcription and protein translation. Furthermore, COPD is a highly heterogeneous disease, and the biological activities of MMPs in the pathogenesis of pulmonary emphysema and airway remodeling of COPD are extremely complex, requiring researchers to comprehensively evaluate various factors.

It is widely accepted that the biological activities of MMPs are regulated by TIMPs, and the imbalance of TIMPs/MMPs in the lung plays an important part in the pathophysiology of COPD. TIMP-4 expression is restricted to heart, kidney, and adipose tissue. This restricted expression suggests specific and different physiological functions. Therefore, some scholars have pointed out that TIMP-4 is more specific to particular MMPs than TIMP-1, -2, and -3 [21]. Firstly, we tested the concentration level of TIMP-4 in the subject's EBC sample. However, we found that TIMP-4 expression levels in EBC were elevated in mild-to-moderate AECOPD patients compared with healthy subjects, but were not statistically significant. In addition, there was not any significant correlation between the concentration level of TIMP-4 in EBC and lung function FEV_1% predicted. This is not consistent with our previous study [13], possibly because previous reports included patients with severe-very severe COPD. However, our current study only recruited mild-to-moderate COPD patients. COPD is a highly heterogeneous disease with different clinical phenotypes in terms of symptoms, drug response, inflammatory biomarkers, and prognosis [22]. Secondly, our data have suggested that TIMP-4 levels in BALF are significantly elevated in mild-to-moderate AECOPD patients compared with healthy subjects. Furthermore, the concentration level of TIMP-4 in BALF was significantly correlated with lung function FEV_1% predicted. One animal experiment demonstrated that neutrophils and macrophages in BALF of rat COPD model with cigarette smoke exposure (CSE) were markedly elevated, and expression levels of MMP-8, MMP-9, MMP-12, TIMP-1, and TIMP-4 in lung tissue were also significantly increased [23]. Another report indicated that TIMP-4 in patients with COPD not only inhibits the protein activity of MMP-9, but is also considered to be a potent inhibitor of MMP-12 [24]. Additionally, by reviewing literature, we found that there are numerous reports on TIMP-4 in malignant tumors [25], pulmonary fibrosis [26], and pulmonary hypertension [27]. These reports above implied that TIMP-4 plays a critical role in the pathophysiology of COPD or emphysema.

To our knowledge, the loss of functional capacity in COPD patients is associated with frequent acute exacerbations and mortality, so there are increasing reports on the assessment of functional activity in patients with COPD [28]. Because COPD is a systemic disease characterized by airway and pulmonary parenchymal lesions, its diagnosis should be based on several evaluation parameters associated with multiple factors. The BODE index is a multidimensional scoring system that is significantly more accurate in predicting mortality in COPD patients than in pulmonary function FEV_1% predicted alone [29]. In our present study, our findings showed that MMP-12 expression levels in BALF and EBC samples in mild-to-moderate AECOPD patients are significantly positively correlated with the BODE index. Furthermore, the level of TIMP-4 in BALF was significantly associated with the BODE in patients with mild-to-moderate AECOPD. However, there was not any significant correlation between TIMP-4 levels in EBC

sample and the BODE index. The differential expression of TIMP-4 in different respiratory samples of patients with mild-to-moderate AECOPD may be related to high heterogeneity of COPD, disease severity, and inconsistency of international operating regulations for EBC testing.

In conclusion, during mild-to-moderate AECOPD, there is increased expression of MMP-12 and TIMP-4 in BALF. These molecules are significantly correlated with severity of airflow limitation and BODE index, indicating that these molecules may be new target interventions for pharmacology to prevent and/or treat AECOPD.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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