Elevated plasma cytochrome *c* levels in patients with chronic obstructive pulmonary disease

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Apoptosis and inflammation are involved in the pathogenesis of chronic obstructive pulmonary disease (COPD). Mitochondrial damage-associated molecular patterns (DAMPs) are released during cell apoptosis and cause inflammation. Cytochrome *c* (CYC) is an important mitochondrial DAMP and participates in the process of apoptosis, but the relationship between circulating CYC levels and COPD remains unclear. 54 COPD patients and 36 healthy controls were included in the present study. Basic characteristics along with plasma CYC and systemic inflammatory factors were collected and analysed in all subjects. In addition, the COPD patients were required to complete the COPD assessment test (CAT). CYC was detected in 40.74% of COPD patients but was undetectable in 30 (83.3%) of 36 healthy individuals. The levels of CYC were higher in COPD compared with controls, and associated inversely with FEV_1% predicted ($r = –0.321$, $P = 0.018$) and FEV_1/FVC ($r = –0.353$, $P = 0.009$). A positive correlation was found between levels of CYC and CAT score ($r = 0.463$, $P = 0.000$). However, no correlations were found between CYC levels and inflammatory biomarkers, pack-years or body mass index (BMI). Our study showed that plasma levels of CYC were elevated in COPD patients and correlated with lung function and CAT score, suggesting that CYC may play a role in COPD.

Keywords: Apoptosis, chronic obstructive pulmonary disease, cytochrome *c*, inflammation, lung function.

CHRONIC obstructive pulmonary disease (COPD) is a worldwide health problem affecting 5–10% of the population and estimated to be the fifth burden of disease and the third leading cause of mortality globally in 2020 (refs 1, 2). COPD was recently regarded as a disease affecting not only the lungs, but is also related to the comorbidities associated with the disease, such as cardiovascular disorders, skeletal muscle dysfunction, and diabetes since many inflammatory components were considered to be released into the circulation of COPD patients[3,4].

Apoptosis is a strictly regulated mechanism of cellular suicide which has been evolved to eliminate unnecessary or unhealthy cells from the body. It plays an important role in the maintenance of homeostasis and keeping the balance of proliferation and differentiation[5,6]. The role of apoptosis in the pathogenesis of lung diseases, such as COPD and emphysema, has been widely studied. It has been reported that apoptosis increased in endothelial, alveolar epithelial and inflammatory cells in COPD[7] patients.

Cell death can lead to the release of mitochondrial damage-associated molecular patterns (DAMPs) which contribute to inflammation[8]. Cytochrome *c* (CYC), an important mitochondrial damage-associated molecular patterns (DAMPs), is a hemoprotein present in the intermembrane space of mitochondria with concentrations as high as 0.5–1.0 mM (ref. 9). It participates in the energy-linked processes and is also considered as a marker of apoptosis. CYC is a key factor in the mitochondrial intrinsic pathway which is an important pathway of apoptosis. Under certain conditions of stimulation, CYC released from damaged mitochondria promotes the formation of apoptosome which participates in the activation of apoptosis[10–12]. In addition, recent studies showed that during cell injury, CYC – a mitochondrial molecule, becomes DAMPs and can be recognized by immune cells[8]. It has also been shown that CYC levels were increased in the plasma of patients with chronic hepatitis C[13] and systemic inflammatory response syndrome[14], indicating the capacity of CYC releasing into extracellular spaces.

Based on the important role of CYC in apoptosis and inflammation, we suppose that CYC could be a potential biomarker of COPD. Therefore, this study was performed to detect the plasma levels of CYC in COPD patients and healthy controls, and to analyse the correlations of CYC with lung functions, inflammatory biomarkers and COPD assessment test (CAT) score.

The study design followed the principles of the Declaration of Helsinki and the approval was obtained from the Institutional Review Board for Human Studies of West China Hospital of Sichuan University. During March 2013 and February 2014, patients with COPD were enrolled from the outpatient department of West China Hospital and the control subjects were enrolled from the hospital's physical examination center. Consents were obtained from all subjects.

Diagnoses of COPD were determined by the standard lung function test using a Spirotel® spirometer (MIR Medical International Research Srl, Rome, Italy), following the guidelines of the Global Initiative for Obstructive Lung Disease (GOLD)[2]. Patients were included if the ratio of forced expiratory volume in 1 second/forced vital capacity (FEV_1/FVC) was less than 70% and less than 12% increase of FEV_1 after the inhalation of salbutamol (200 mg) and had not received any standard treatment for COPD before. Based on clinical history, patients with acute exacerbation of COPD (AECOPD) during the three

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Table 1. Characteristics of subjects

	COPD	Control	*P*-value*
n	54	36	
Age (year)	63.70 ± 10.53	61.11 ± 9.71	0.241
Sex (M/F)	33/21	21/15	0.948
BMI (kg/m^2)	22.45 ± 2.59	23.07 ± 2.44	0.262
Smoker/nonsmoker	29/26	13/23	0.120
Smoking (packing years)	15.25 ± 20.15	9.16 ± 14.95	0.111
FEV_1 (L)	1.66 ± 0.59	2.86 ± 0.73	0.000
FVC (L)	3.16 ± 0.84	3.57 ± 0.97	0.037
FEV_1/FVC%	53.23 ± 12.31	81.00 ± 5.38	0.000
FEV_1% predicted	69.83 ± 21.17	112.33 ± 17.98	0.000

COPD, Chronic obstructive pulmonary disease; M, Male; F, Female; BMI, Body mass index; FEV_1, Forced expiratory volume in one second; FVC, Forced vital capacity. *Unpaired Student's *t*-test, Chi-square test; Mann–Whitney *U*-test.

months prior to the study, autoimmune diseases, infectious, cancers and metabolic diseases were excluded.

Subjects were asked to fast overnight from 21 : 00 h. Venous blood taken from all subjects was centrifuged at 400 g, 4°C for 10 min. The plasma was collected and stored at –80°C until measurement. CYC was measured by ELISA (Diaclone, France). Inflammatory biomarkers, including C-reactive protein (CRP), tumour necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1), interleukin-8 (IL-8), interleukin-1β (IL-1β) and interleukin-6 (IL-6), were determined by a Luminex Bio-Plex 200 system (Bio-rad, California, USA) according to the manufacturer's instructions. The detection sensitivities were 0.05 ng/ml for CYC, 116 pg/ml for CRP, 1.2 pg/ml for TNF-α, 9.9 pg/ml for MCP-1, 1.8 pg/ml for IL-8, 0.8 pg/ml for IL-1β and 1.7 pg/ml for IL-6. All patients were required to complete the CAT. Clinical features and other parameters, such as body mass index (BMI), were also collected. Investigators performing the analysis were not made aware of the details of the subjects.

SPSS (version 17.0, SPSS Inc., USA) was used to perform data analysis. The results were shown as mean ± standard deviation (SD). Comparisons of characteristics between groups were performed by unpaired Student's *t*-test, Mann-Whitney rank sign or Chi-square tests as appropriate. Spearman rank test was used to perform the correlation analysis. $P < 0.05$ was set to be statistically significant.

Fifty four patients with COPD and thirty six healthy individuals were included in the study. The basic characteristics of subjects, including demographic data, smoking statuses and lung functions were collected (Table 1). The two groups were of the same age and gender and BMI was similar between the groups. Although the percentage of smokers and smoking pack years was higher in the COPD group, these differences did not show any statistical significance.

CYC could be detected in 22 (40.74%) of 54 COPD patients with the median concentration of 0.18 ± 0.35 ng/ml. In contrast, CYC was within the detection limit in 30 (83.3%) of 36 healthy individuals. The levels of CYC in COPD were significantly higher than those in the control group ($P = 0.017$; Figure 1). However, there were wide variations in levels of CYC. Significant differences were also found between the groups of COPD and control in CRP, IL-8, IL-1β and IL-6, whereas the levels of TNF-α and MCP-1 were similar between the two groups.

We studied the correlations between plasma CYC levels and characteristics related to other diseases in the COPD group. There was an inverse correlation between plasma CYC and lung function in COPD patients, based on the FEV_1% predicted ($r = –0.321$, $P = 0.018$; Figure 2 *a*) and FEV_1/FVC ($r = –0.353$, $P = 0.009$). Moreover, plasma levels of CYC were positively correlated with CAT score ($r = 0.463$, $P = 0.000$; Figure 2 *b*). We also compared CYC levels with inflammatory biomarkers, pack-years and BMI; however, no relationship was found. The correlations of CYC levels with parameters mentioned above were also analysed in a multivariate linear regression model (Table 2), which showed a positive association of circulating CYC levels with CAT score.

The present study examines the plasma levels of CYC in COPD patients and the correlations with lung function, inflammatory biomarkers and symptoms.

The results indicate that COPD patients had higher levels of CYC in the plasma compared with healthy controls. In the COPD group, the CYC levels correlated with lung function and CAT score. Numerous studies from both animal models of COPD and human patients indicate that apoptosis participated in the pathogenesis of COPD. An increase of apoptosis was found in endothelial and alveolar epithelial cells, associated with higher activated subunits of caspase-3 and less anti-apoptotic protein Bcl-2 (refs 5, 7). Apoptosis, as a pattern of cell death, induces the release of CYC from cells into the circulation. Elevated levels of serum CYC were observed in diseases in which apoptosis was regarded as one mechanism[13–15]. Studies *in vivo* also suggested that serum

levels of CYC might reflect the extent of apoptotic cell death[15–17]. In our study, plasma levels of CYC were higher in COPD patients than healthy controls. CYC levels were also inversely correlated with lung function in COPD, indicating that CYC may be associated with disease severity, which might be in parallel with the extent of apoptotic death of endothelial and alveolar epithelial cells. A previous study[18] has suggested that mitochondria, isolated from skeletal and respiratory muscles of patients with COPD, released higher levels of CYC. However, their study only examined the CYC levels in mitochondria and not in circulation. Our study explored the levels of CYC in the plasma and patients did not take any standard treatment for COPD which can reduce interference of drugs.

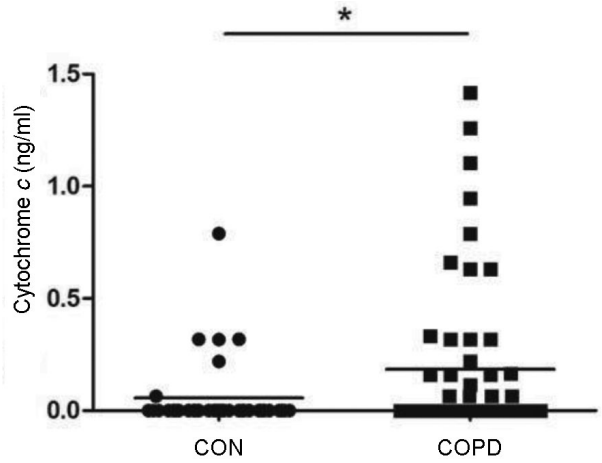

Figure 1. Plasma levels of cytochrome *c* in COPD patients and healthy controls.

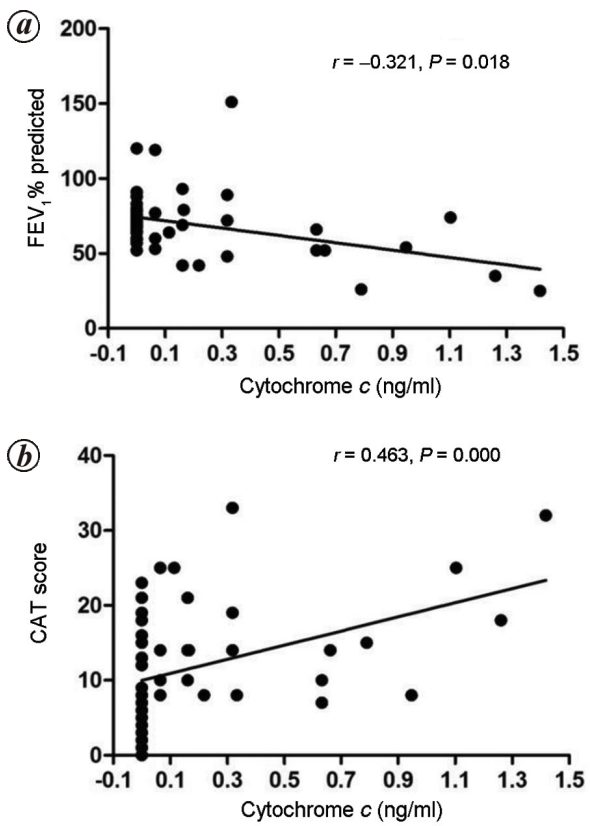

Figure 2. Correlation of plasma levels of cytochrome *c* and (***a***) FEV_1% predicted and (***b***) COPD assessment test (CAT) score.

CYC is a member of DAMPs released from mitochondria[8]. Mitochondrial DAMPs are released during apoptosis and considered to be involved in inflammatory reactions and the pathogenesis of human diseases. Extracellularly added CYC in the culture medium increased the percentage of apoptotic neurons[19], and activated nuclear factor-κB to increase the release of proinflammatory cytokines and chemokines in mouse splenocytes *in vitro*, while injection of recombinant CYC into arthrosis could cause arthritis and accumulation of $Mac1^+$ cells in mice[20]. In hemodialysis patients, CYC levels were found to be positively associated with IL-6 levels[21]. These studies suggest that CYC may participate not only in apoptosis but also in inflammatory reactions. In our study, higher plasma levels of CYC were found in COPD patients. We also analysed the levels of systemic inflammation biomarkers, including CRP, TNF-α, MCP-1, IL-8, IL-1β and IL-6, in all subjects. The concentrations of CRP, IL-8, IL-1β and IL-6 were significantly elevated in patients with COPD, whereas TNF-α and MCP-1 levels did not show statistical significance, which is consistent with a previous study[22]. This might be explained by the sample size and the enrolled patients were all in stabilized phase without exacerbations for three months before the study. The associations between CYC and those biomarkers were not significant. Whether extracellular CYC is involved in the pathogenesis of COPD and induces inflammatory reactions in the disease can be known by further studies.

Table 2. Associations of parameters with plasma cytochrome *c* levels in a multivariate linear regression model

Parameter	Standardized β coefficient	*P*-value
Age	–0.067	0.621
Gender	–0.055	0.742
BMI	–0.016	0.897
Smoking (pack years)	0.042	0.782
CRP	–0.141	0.324
TNF-α	–0.072	0.764
MCP-1	0.074	0.580
IL-8	0.200	0.789
IL-1β	–1.677	0.163
IL-6	1.543	0.356
FEV_1/FVC%	–0.299	0.134
FEV_1% predicted	–0.234	0.274
CAT score	0.279	0.042

BMI, Body mass index; CRP, C-reactive protein; FEV_1, Forced expiratory volume in one second; FVC, Forced vital capacity; CAT, Chronic obstructive pulmonary disease assessment test.

CAT is a comprehensive questionnaire consisting of 8 items for assessment of symptoms and health status of COPD patients[2]. The present study showed that levels of CYC were positively associated with CAT scores, indicating that patients with more symptoms had higher levels of CYC. Thus, we speculate that higher circulating CYC may be linked to worse lung functions and emphysema. Many studies indicated that apoptosis contributed to alveolar destruction and development of lung emphysema[5,23–27] and blockade of apoptosis by a broad caspase inhibitor reduces emphysema[28]. More studies on functions of CYC in COPD are warranted to unravel the underlying mechanisms.

Some limitations should be considered when analysing the data. A limited number of subjects were included in the study and most patients were with moderate COPD. Future studies should include more subjects and focus on patients with more severe COPD. Additionally, no long-term follow-up has been performed. So we do not know whether levels of CYC correlate with AECOPD and the prognosis of patients, which still needs further studies. Finally, it would be worth evaluating the relationship between CYC and emphysema which could be assessed by high-resolution computed tomography (HRCT).

In conclusion, the present study demonstrated that the levels of plasma CYC were higher in the COPD patients than those in the healthy controls and correlated inversely with lung function and positively with CAT score. Due to the limitations mentioned above, further studies are needed to confirm the role of CYC in patients of COPD.

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ACKNOWLEDGEMENTS. This study was supported by grants (31171103, 81230001 and 81470236) from the National Natural Science Foundation of China and grants (2014BAI08B04 and 2012BAI05B02) from National Key Technology Research and Development Program of the Ministry of Science and Technology of China.

Received 17 August 2015; revised accepted 29 November 2015

doi: 10.18520/cs/v110/i8/1532-1535