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TGF- β 1, IL-6, and TNF- α in Bronchoalveolar Lavage Fluid: Useful Markers for Lung Cancer?

SUBJECT AREAS:
LUNG CANCER
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Changes of cytokines in bronchoalveolar lavage fluid (BALF) reflect immunologic reactions of the lung in pulmonary malignancies. Detection of biomarkers in BALF might serve as an important method for differential diagnosis of lung cancer. A total of 78 patients admitted into hospital with suspected lung cancer were included in our study. BALF samples were obtained from all patients, and were analyzed for TGF- β 1, IL-6, and TNF- α using commercially available sandwich ELISA kits. The levels of TGF- β 1 in BALF were significantly higher in patients with lung cancer compared with patients with benign diseases ($P = 0.003$). However, no significant difference of IL-6 ($P = 0.61$) or TNF- α ($P = 0.72$) in BALF was observed between malignant and nonmalignant groups. With a cut-off value of 10.85 pg/ml, TGF- β 1 showed a sensitivity of 62.2%, and a specificity of 60.6%, in predicting the malignant nature of pulmonary disease. Our data suggest that TGF- β 1 in BALF might be a valuable biomarker for lung cancer. However, measurement of IL-6 or TNF- α in BALF has poor diagnostic value in lung cancer.

A major problem in lung cancer is the lack of clinically useful tests for early diagnosis and screening patients with lung lump by noninvasive diagnostic procedures¹. It is reported that approximately two-thirds of lung cancer patients is the presence of metastatic tumors at the time of diagnosis². Lung cancer screening by chest X-ray and sputum cytology have proven ineffective in improving patient survival rate^{3,4}, leading to the search for more sensitive and specific tests. One promising approach is the identification of lung cancer-specific biomarkers and detection of them at an early stage.

Considering that tumor biomarkers are produced directly by the tumor or by non-tumor cells as a response to the presence of tumor cells, the elevation of tumor biomarkers can be detected earlier than radiographic abnormalities⁵. Investigating specific molecular markers in airways might serve as an important adjunct to routine examination for lung cancer diagnosis. The utility of cytokines in bronchoalveolar lavage fluid (BALF) for differential diagnosis of lung cancer has been described in several studies^{6–12}. In our previous reports, we also observed that the levels of vascular endothelial growth factor (VEGF) and neuron-specific enolase (NSE) were significantly higher in BALF of lung cancer patients than in that of patients with benign diseases^{13,14}. Recently, transforming growth factor (TGF)- β 1, interleukin (IL)-6, and tumor necrosis factor (TNF)- α were suggested in published studies as possible diagnostic biomarkers of lung cancer due to their higher concentrations in serum of lung cancer patients^{15–17}. However, up to data, little study has performed to study these cytokines in BALF of lung cancer. In this study, we performed a prospective study to investigate TGF- β 1, IL-6, and TNF- α expression in airways by comparing levels of them in benign diseases and lung cancer.

Results

The BAL fluid of TGF- β 1 concentration was significantly higher in patients with lung cancer compared with patients with benign diseases (18.2 [8.4–46.4] pg/ml *versus* 8.4 [3.5–17.0] pg/ml, $P = 0.003$; Figure 1A). However, there was no significant difference in BAL fluid IL-6 (4.3 ± 0.4 pg/ml *versus* 4.1 ± 0.4 pg/ml, respectively, $P = 0.61$; Figure 1B) or TNF- α (1.4 ± 0.2 pg/ml *versus* 1.2 ± 0.3 pg/ml, respectively, $P = 0.72$; Figure 1C) between lung cancer patients and non-cancer controls. However, no significant difference in BALF TGF- β 1, IL-6, and TNF- α levels were observed between smokers and nonsmokers in any group (data not shown).

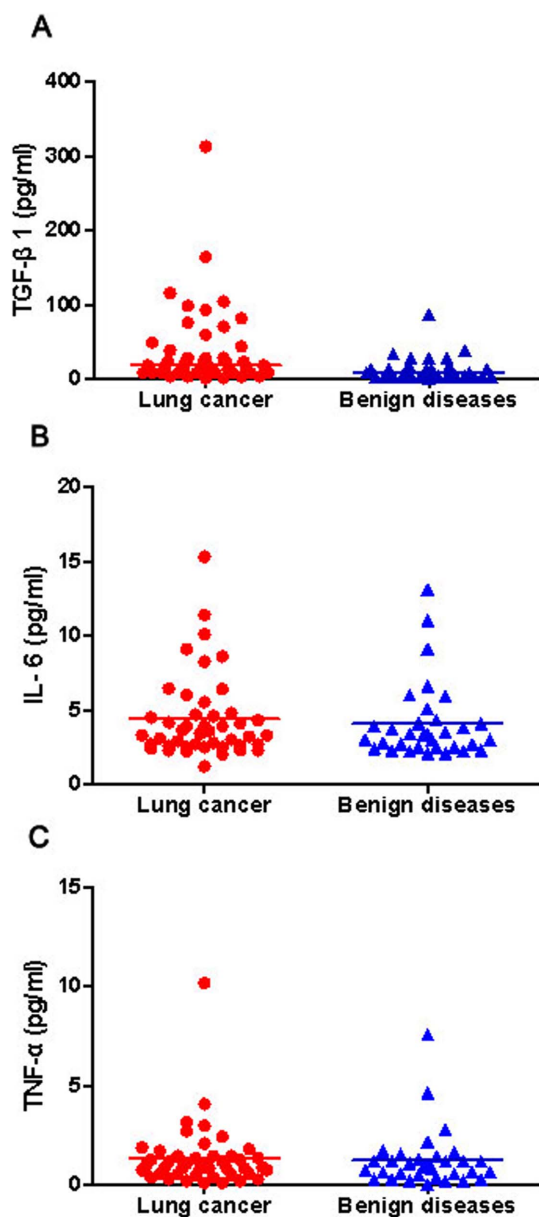


Figure 1 | Comparison of TGF- β 1, IL-6, and TNF- α levels in bronchoalveolar lavage fluid between benign and malignant groups. The levels of TGF- β 1 were significantly higher in lung cancer patients than those in benign diseases ($P = 0.003$, Mann-Whitney U -test) (A); no significant difference of IL-6 ($P = 0.61$, Student's t -test) (B) or TNF- α ($P = 0.72$, Student's t -test) (C) levels was found between malignant and nonmalignant groups. Horizontal lines represent the median values for TGF- β 1; Horizontal lines represent the mean values for IL-6 and TNF- α .

We evaluated the correlation of TGF- β 1, IL-6, and TNF- α in BALF by the Pearson correlation analysis. A significant correlation was found between TGF- β 1 and IL-6 in BALF ($r = 0.337$, $P = 0.003$; Figure 2A). Nevertheless, the levels of TGF- β 1 in BALF were not relevant to that of TNF- α ($r = 0.121$, $P = 0.290$; Figure 2B). Similarly, there was no significant correlation was observed between IL-6 and TNF- α in BALF ($r = -0.022$, $P = 0.847$; Figure 2C).

Considering TGF- β 1 was statistical differences between lung cancer patients and non-cancer controls, ROC analysis was further conducted to examine the diagnostic ability of the TGF- β 1 for predicting lung cancer. As shown in Figure 3, the area under the receiver operating characteristic curve (AUC) was 0.695 ($P = 0.003$). With a cutoff value of 10.85 pg/ml, TGF- β 1 had a sensitivity of 62.2%, a specificity

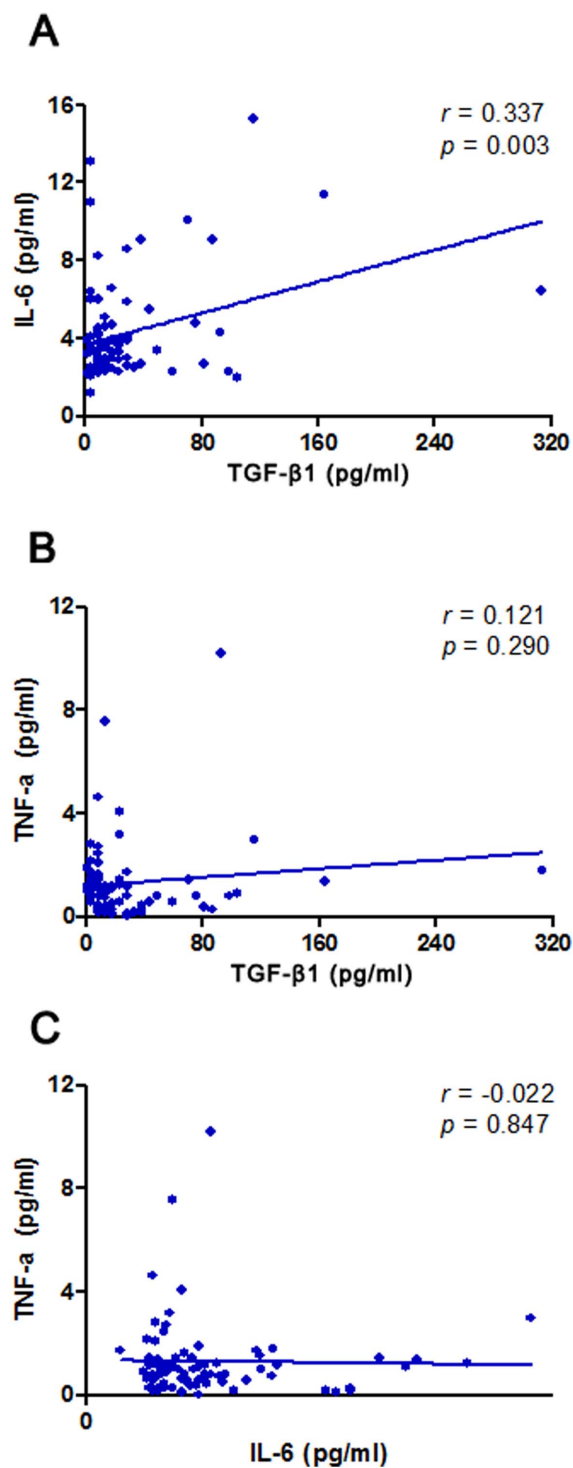


Figure 2 | Correlation of TGF- β 1, IL-6, and TNF- α levels in bronchoalveolar lavage fluid. Statistically correlation was observed between TGF- β 1 and IL-6 ($r = 0.337$, $P = 0.003$) (A); no significant correlation was found between TGF- β 1 and TNF- α ($r = 0.121$, $P = 0.290$) (B) or between IL-6 and TNF- α ($r = -0.022$, $P = 0.847$) (C).

of 60.6%, a positive predictive value of 67.5%, and a negative predictive value of 52.6%, in predicting the malignant nature of pulmonary disease.

Discussion

Distinguishing benign lung diseases from lung cancer through non-invasive approaches such as useful tumor markers is very important

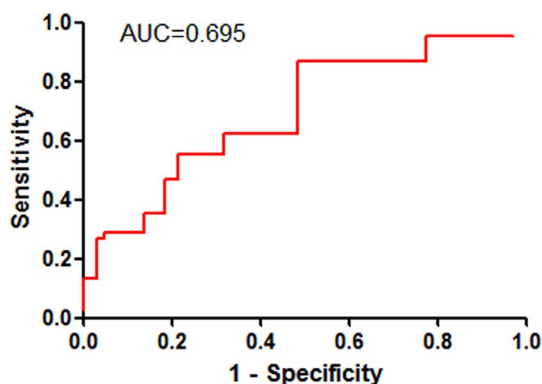


Figure 3 | Receiver operating characteristic (ROC) curve was performed to evaluate the threshold value of TGF- β 1 in differentiating malignant from benign pulmonary diseases. TGF- β 1 reached a sensitivity of 62.2%, a specificity of 60.6%, a positive predictive value of 67.5%, and a negative predictive value of 52.6% (cut-off value: 10.85 pg/ml; area under the curve: 0.695).

as to avoid patients undergoing surgery for a benign condition¹⁸. In our previous reports, we found levels of cancer-specific cytokines in BALF elevated much earlier and present at a higher concentration than those in peripheral blood¹³. Thus, detection of biomarkers in BALF can serve as an important method for lung cancer diagnosis^{13,14}. In the present study, we conducted a prospective study to investigate whether levels of BAL fluid TGF- β 1, IL-6, and TNF- α could be useful in distinguishing malignant from benign pulmonary diseases.

A total of 78 patients with suspected lung cancer were enrolled in our study. TGF- β 1, IL-6, and TNF- α were determined by commercially available sandwich ELISA kits. The results showed that the levels of TGF- β 1 in BALF were higher among patients with lung cancer than in that of patients with benign diseases. In addition, a significant correlation was found between TGF- β 1 and IL-6 in BALF. However, no significant difference in BAL IL-6 or TNF- α was observed between lung cancer patients and non-cancer controls.

TGF- β 1 expression was significantly higher in lung cancer patients compared with patients with benign diseases, indicating TGF- β 1 in BALF might be a useful biomarker of lung cancer. ROC analysis was further conducted to examine the diagnostic ability of the TGF- β 1 for predicting lung cancer. The result showed that the diagnostic threshold afforded by the ROC analysis was 10.85 pg/ml. The area under the ROC was 0.695. With a threshold value of 10.85 pg/ml, TGF- β 1 had a sensitivity of 62.2%, a specificity of 60.6%, a positive predictive value of 67.5%, and a negative predictive value of 52.6%, in predicting the malignant nature of pulmonary disease. Therefore, the presence of low levels of TGF- β 1 in BALF indicated a low probability of malignancy.

Numerous studies provided indirect evidence for a functional link between TGF- β 1 and IL-6 in many human diseases. Chen and colleague demonstrated that activated TGF- β 1/IL-6 pathways could be responsible for more aggressive tumor growth and resistance to treatment in oral cancer¹⁹. In another report, Yamada *et al.* showed that crosstalk between IL-6 and TGF- β 1 was associated with key features of malignancy²⁰. None the less, there was evidence that the levels of IL-6 in serum was good marker of lung cancer and might serve as a predictive biomarker for the efficacy of therapy^{21,22}. However, in our study, no elevated concentrations of IL-6 in BALF were observed in lung cancer patients, although a significant correlation was found between TGF- β 1 and IL-6 in BALF.

TNF- α is a multifunctional cytokine playing a key role in apoptosis and cell survival as well as in inflammation and immunity²³. Dalaveris *et al.* have analyzed the levels of TNF- α in exhaled breath condensate (EBC) and serum of patients with primary lung cancer²⁴.

They observed that the levels of TNF- α was higher in both serum and EBC of lung cancer patients than age and gender-matched healthy controls²⁴. Nevertheless, we failed to find a significant difference in the levels of TNF- α in BALF between benign and malignant groups. In addition, no significant correlation between TNF- α and TGF- β 1 in BALF was found. Our results demonstrated that determination of the BAL fluid TNF- α level in flexible bronchoscopy may be useless in the diagnosis of lung cancer.

Some limitations of our study are worth discussing. First, there was significant difference on smoking status between lung cancer patients and non-cancer controls. Tobacco smoking is an established risk factor for lung cancers and may have an effect on human immune response. Although no differences in cytokine levels between smokers and non-smokers were detected in our study, environmental factors should also be considered in the development of lung cancer. Second, the prognostic value of TGF- β 1, IL-6, and TNF- α in BALF were not described in our study. Some cytokines may provide information about the clinical outcome of lung cancer patients²⁵. On the other hand, biomarkers can also help clinicians in selecting the most effective anticancer treatments for each patient²⁶. Future studies with well-matched controls are needed to detect these cytokines and their prognostic values, which might lead to better understanding biological characteristics of them in lung cancer.

In summary, our study showed that the levels of TGF- β 1 were significantly higher in BALF in lung cancer patients than in that of patients with benign diseases. This work may only suggest that TGF- β 1 in BALF is a valuable biomarker for lung cancer and further investigation is needed. However, measurement of TNF- α or IL-6 in BALF has poor diagnostic value in lung cancer.

Methods

Study subjects. A prospective study was conducted in Affiliated Hospital of Ningbo University in China from February 2011 to July 2013. The study protocol was approved by the Institutional Review Board for Human Studies of Affiliated Hospital, School of Medicine, Ningbo University (Ningbo, China). Written informed consent was obtained from all participants. The experiments were performed in accordance with American College of Chest Physicians Evidence-Based Clinical Practice Guidelines²⁷. 78 patients admitted into this hospital with suspected lung cancer were included. Clinical information regarding patient characteristics was based on patient records and registries. All patients had histological confirmed and were excluded if they had received preoperative chemotherapy or radiotherapy. Basic characteristics for patients are summarized in Table 1. There were 45 lung cancer patients (60.8 ± 1.2 years) and 33 patients with noncancerous diseases (58.2 ± 1.7 years). The pathologic types included 18 squamous cell carcinomas, 11 adenocarcinomas, 10 small cell carcinomas, and 6 of other cell types. There were 31 patients with pneumonia, 2 with tuberculosis, and 1 with pulmonary sarcoidosis in the control group. Smoking habits were defined at 1 year prior to diagnosis for cases or 1 year prior to interview for controls.

Bronchoalveolar lavage. BALF samples were collected and the methods were referred to previous studies^{9,12-14}. The lavage was done prior to brushing or biopsies to avoid contamination with blood. The bronchus on the disease side was washed with two 50-ml aliquots sterile physiological saline. The fluid was gently withdrawn into a

Table 1 | The characteristics of the patients

	Lung cancer	Benign group	P value
Age, years	60.8 ± 1.2	58.2 ± 1.7	0.20
Gender			0.68
Male	28	19	
Female	17	14	
Smoking status			0.55
Smokers	20	12	
Non-smokers	25	21	
Pack/years	66.1 ± 9.3	29.6 ± 5.3	0.001
Histological type			
Squamous cell carcinoma	18		
Adenocarcinoma	11		
Small-cell lung cancer	10		
Others	6		



siliconized container placed in iced water. The recovered volume of BALF > 60-ml was considered to be an acceptable level of quality. The chilled lavage fluid was filtered through a nylon filter to remove mucus and centrifuged at 3,000 rpm for 10-min. The cell pellets were separated from the supernatants and stored at -80°C .

Measurements of TGF- β 1, IL-6, and TNF- α . The levels of TGF- β 1 (pg/ml), IL-6 (pg/ml), and TNF- α (pg/ml) were measured using sandwich enzyme linked immunosorbent assays (ELISA). All reagents used for the experiments were standard high-quality chemicals from international companies (TGF- β 1: R&D systems, Minneapolis, MN, USA; IL-6 and TNF- α : eBioscience, San Diego, CA, USA). The assays were conducted according to the manufacturer's guidelines. The samples were analyzed in batches to minimize interassay variability.

Statistical analysis. Data was presented as means \pm standard error of the mean (SEM). TGF- β 1 levels were not normally distributed and were expressed as medians and interquartile ranges (IQR). If the data distribution was normal, comparison between different groups was done using the Student's *t*-test; otherwise, the nonparametric Mann-Whitney *U*-test was applied. The relationships between different markers were determined using Pearson correlation. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated to evaluate diagnostic performance for TGF- β 1. Receiver operating characteristic (ROC) analysis was conducted to examine the diagnostic ability of the TGF- β 1 for predicting lung cancer. All hypothesis tests were 2-sided, with statistical significance defined as having a *P* value of less than 0.05. Statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) or SPSS for windows (version 13; SPSS, Chicago, IL).

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Author contributions

C.C., Z.D., Z.C. and Z.X. designed the experiments. C.C., Z.D., Z.C., Z.X., S.S., Y.Y. and D.L. carried out the experiments and calculations. C.C., Z.D., Z.C. and Z.X. wrote and edited the paper.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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