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*Original Article*

# $CD4^+CD25^+Foxp3^+$ regulated T cells and expression of IL-37 and chemokine ligand 2 in lung cancer and their clinical significance

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**Abstract:** Objective: To explore the expression of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs), IL-37 and chemokine (CC motif) ligand 2 (CCL2) in the peripheral blood mononuclear cells (PBMC), and its relationship with the clinical and pathological features of lung cancer. Methods: Flow cytometry (FCM) was utilized to detect the expression of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) in $CD4^+$ Treg cells of the PBMC from 128 cases of lung cancer patients and 119 cases of normal healthy individuals. ELISA was adopted to detect IL-37 and CCL-22 expression. Results: The expressions of $CD4^+CD25^+FOXP3^+$ Tregs and CCL-22 in patients PBMC with lung cancers were greater than that of in healthy controls, while the expression of IL-37 was lower. There were great diagnostic value of sensitivity and specificity of the expression level of $CD4^+CD25^+FOXP3^+$ Treg, IL-37 and CCL-22 in PBMC of lung cancer, which were associated with the tumor size, histological types, differentiation degree, TNM grading, lymphatic metastasis and distance metastasis. The results of follow-ups showed the 5-year survival rate of patients with high expression of $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs and CCL-22, as well as low expression of IL-37 was significant lower. The results of Kaplan-Meier and Cox multivariate analysis model indicated that distance metastasis, expressions of $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs, IL-37 and CCL-22 in PBMCs were the independent risk factors of prognosis of lung cancer. Conclusion: The expression of $CD4^+CD25^+FOXP3^+$ Tregs, IL-37 and CCL-22 was closely related to lung cancer, which could contribute to the diagnosis in early stage, prognosis evaluation and immunization therapy of lung cancer.

**Keywords:** Lung cancer, $CD4^+CD25^+Foxp3^+$ Treg, IL-37, CCL-22, pathological features, prognosis

## Introduction

Lung cancer is one of the most common fatal malignant tumors and this combined with its very grave prognosis makes it a very deadly disease worldwide [1]. The incidence of lung cancer in Europe in 2008 was estimated to be 390,900 cases, and 342,100 patients died of the disease [2]. The vast majority of patients with lung cancer are resulted from long-term exposure to tobacco smoke, and other factors such as genetic factors or exposure to asbestos, radon gas, or other forms of air pollution would also lead to lung cancer [3]. The symptoms of fever, fatigue, poor appetite and weight loss are not specific for lung cancer; the patients may also suffer from bone pain and other neurological symptoms, such as fainting, convulsions, headaches, or limb weakness [4]. It is reported that although the introduction of new chemotherapeutic agents and several molecularly targeted drugs in the past decade, outcomes remain poor, with overall cure rates less than 20% [5, 6]. The high mortality and poor prognosis of lung cancer are mainly due to the difficulty of early diagnosis [7]. Several onco-

genes, anti-oncogenes and certain lung cancer-specific proteins have been found to be candidate biomarkers for lung cancer, while they are not suitable for its early detection [8]. Thus, it is important to find the biomarkers which have prognosis and therapeutic effects on lung cancer.

It has been clarified that both tumor characteristics and patient's immune responses affect tumor development or metastasis [9]. Tregs are usually considered as a major cell population involved in immune tolerance that protects cancer cells from antitumor immunity, and are immune-suppressive subsets of T cells [10]. $CD4^+CD25^+$ Treg cells are the most studied cells that have regulatory functions in tumor immunology [11]. The forkhead family protein Foxp3 is a transcription factor highly expressed in $CD4^+$ Tregs and it is a regulator of T-cell tolerance and is necessary for the development and function of Tregs [12, 13]. FOXP3 expression in humans is not confined to functionally regulatory T cells, and it can also be momently expressed in conventionally activated $CD4^+$ $CD25^+$ effector T cells [10]. CCL22 chemokines derived from a tumor induce the migration of Tregs through CCR4, which is a chemokine receptor for CCL22, and impair anti-tumor immunity in cancers [14]. Furthermore, IL-37 is the newest anti-inflammatory cytokine in regulating inflammatory responses [15]. It is reported that IL-37 thus acts as a natural suppressor of both innate inflammatory along with immune responses [5]. Zhu et al. suggested a close relationship between changes in $CD4^+CD25^+$ $Foxp3^+$ Treg cells and aging and lung tumor genesis and development [16]. However, the associations of IL-37 and CCL-22 with lung cancer have not been discovered. Hence, the aim of this study is to discuss the expression of $CD4^+CD25^+FOXP3^+$ regulatory Tregs, IL-37 and CCL-22 in the peripheral blood of lung cancer patients, and its relationship with the clinical and pathological features.

## Material and methods

### *Ethnic statements*

The study was approved by the Institutional Review Boards of Affiliated Hospital of Guangdong Medical College. Written informed consent was obtained from each eligible participant and the study was performed in accordance with the Declaration of Helsinki [17]. All the participants or their agents were signed the informed consent with self-willingness and the study was approved by the Ethics Committee of our hospital.

### *Research subjects*

A total of 128 patients with lung cancer confirmed pathologically after surgery in Affiliated Hospital of Guangdong Medical College were collected in this study form December 2009 to December 2010. There were 102 males and 26 females aged 38~75 years with median age of 59 years and average age of 59.13 ± 11.97 years. Among them, 88 patients were smoking patients while 40 patients were not. According to the pathological types, 49 patients were diagnosed with adenocarcinoma, 59 patients with squamous cell carcinoma, 22 patients with small cell carcinoma, 73 patients with peripheral lung cancer, 55 patients with central lung cancer, 52 patients with left lung cancer and 76 patients with right lung cancer. There are 79 patients with tumor diameter equal or greater than 3 cm and 49 patients with tumor diameter less than 3 cm. Based on different differentiation grades, 57 patients were poor differentiation, while 71 patients were middle to well differentiation. Referring to tumor metastasis, 62 patients were lymph node metastasis and 47 patients were distant metastasis. In the light of TNM stage drafted by American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC) in 2009 [18], 38 patients were diagnosed with I stage, 12 patients with II stage, 56 patients with III stage and 55 patients with IV stage. The inclusion criteria were as follows: 1. Undergo lobectomy or lung resection and store biopsy in our hospital; 2. All the patients were diagnosed with lung cancer confirmed pathologically; 3. Patients have detailed and completed clinical follow-up survey and pathological diagnosis data; 4. Before the resection operation, the patients were not taken any treatments, including radiotherapy, chemotherapy and Chinese traditional treatment; 5. The death cause of patients were lung cancer; 6. The survival information of patients were gathered by follow-ups. The 119 cases of peripheral blood from health checkup people were selected in the study as control group, which were not showed diseases of heart, liver, lung and stomach or other organs with normal liver and renal

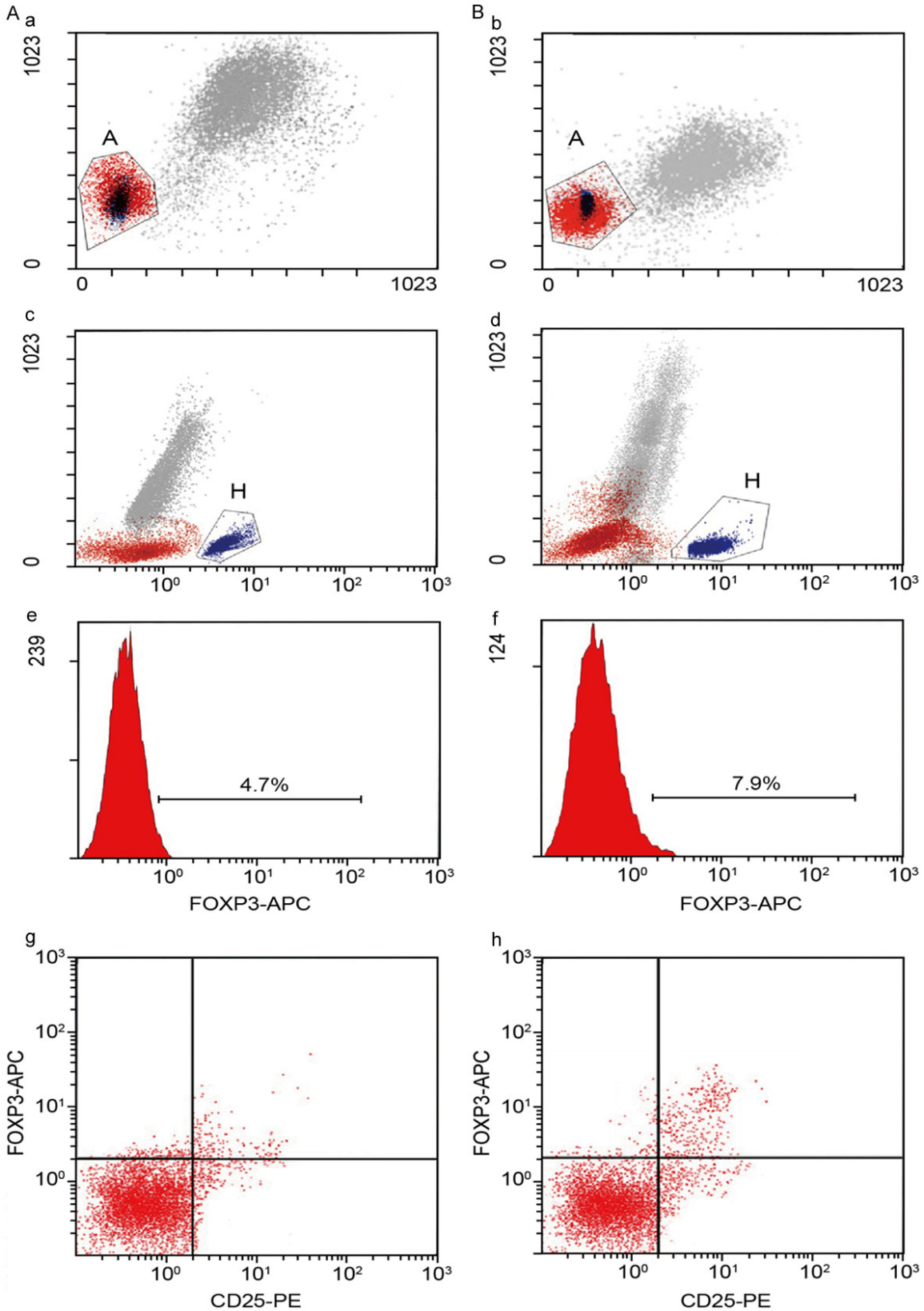


**Figure 1.** The presentation of $CD4^+CD25^+Foxp3^+$ Tregs in PBMCs. Note: A in (a/b), lymphocytes; H in (c/d), $CD4^+$ lymphocytes; (e/f) Showed the expression levels of Foxp3; (g/h) Showed the $CD4^+CD25^+Foxp3^+$ Tregs; Group (A) was healthy control and (B) was patients with lung cancer.

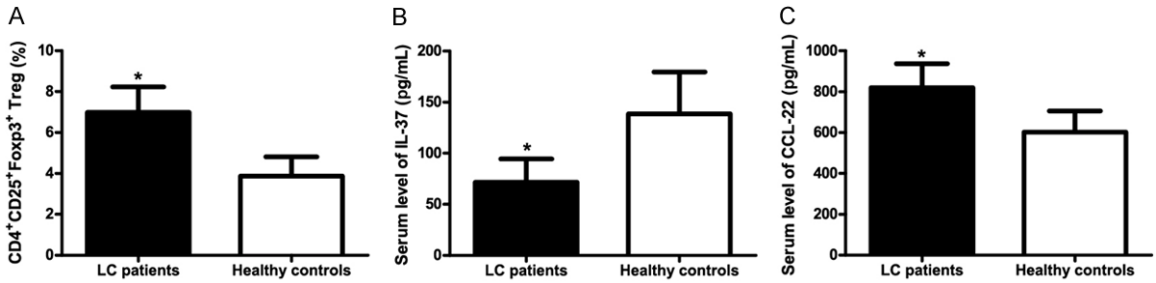


**Figure 2.** The expression levels of $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22 in PBMC. Note: A. $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs; B. IL-37; C. CCL-22.

functions. Among them, there are 97 males and 21 females aged 35~68 years with median age of 50 years and average age of 50.93 ± 10.07 years. The smoking patients were 70 cases while the other 49 patients without smoking habits. The ages, gender and smoking status between the two groups were showed no statistical differences (all $P > 0.05$).

*Disposal of specimens*

The peripheral bloods (2 tubes, 2 ml per tube) of participants with empty stomach were drawn before the operation The whole blood from 1 tube with EDTA anticoagulant were kept at the room temperature and the FCM examination was conducted within 4 h; the other tube were used for serum collection by centrifuged centrifuge (3000 rpm, 5 min). The samples from the two tubes were marked and kept in the ice box (-80°C) waiting for enzyme linked immunosorbent assay (ELISA) detection.

*Detection on expression of $CD4^+CD25^+Foxp3^+$ regulatory T cell (Tregs) in the peripheral blood of lung cancer patients by flow cytometry technique (FCM)*

The ratios of $CD4^+CD25^+Foxp3^+$ Tregs in $CD4^+$ T cells in peripheral blood of participants were detected using coulterEPicsXL FCM (BECKMANCOULTER Company, American). Manipulation of FCM was strictly complied with the instruction of agents of regulatory T cells from whole blood (eBioscience Company, American). PBMC were separated by human lymphocytes. The 20 μL mixture of anti-human CD4 antibody marked with FITC and anti-human CD25 antibody marked with APC were added in the 100 μL PBMC. The CD4 and CD25 molecules were stamped in the cell membrane, and the Foxp3 molecules were tabbed with Foxp3 antibody marked with PE.

*Detection of the IL-37 and CCL-22 expression by ELISA method*

The serum level of IL-37 and CCL-22 were detected by ELISA method in both case group and control group. All the detection agents were purchased from Haiyuan leaf Biotechnology Co. Ltd and the manipulations were strictly complied with the instruction.

*Follow-up status*

The survival status was followed up by telephone-visit or household registration investigation in public security bureau. The duration of follow-up was 5 years and these patients who were lost were regarded as censored data. A total of 114 patients were followed up while 14 patients were lost, and the follow-up rate 89.06%.

*Statistical analysis*

Software SPSS 19.0 was used for data arrangement and statistical analysis. Measurement data were expressed by average value and standard deviation (SD). The t text was adopted for comparison among groups and the pairwise comparison was carried out with least significant difference (LSD) method; comparison among groups was conducted with enumeration data by $\chi^2$ text. Pearson correlation were applied for the analysis of relationships between two variables; ROC curves were used for evaluating diagnostic effects and area under curve (AUC) was calculated to assess the diagnostic effects of $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs, IL-37 and CCL-22 in peripheral blood on patients with lung cancer. Survival analysis was performed with Kaplan-Meier method and multivariate analysis were conducted with Cox proportional hazards model. All of the statistical data were received two-

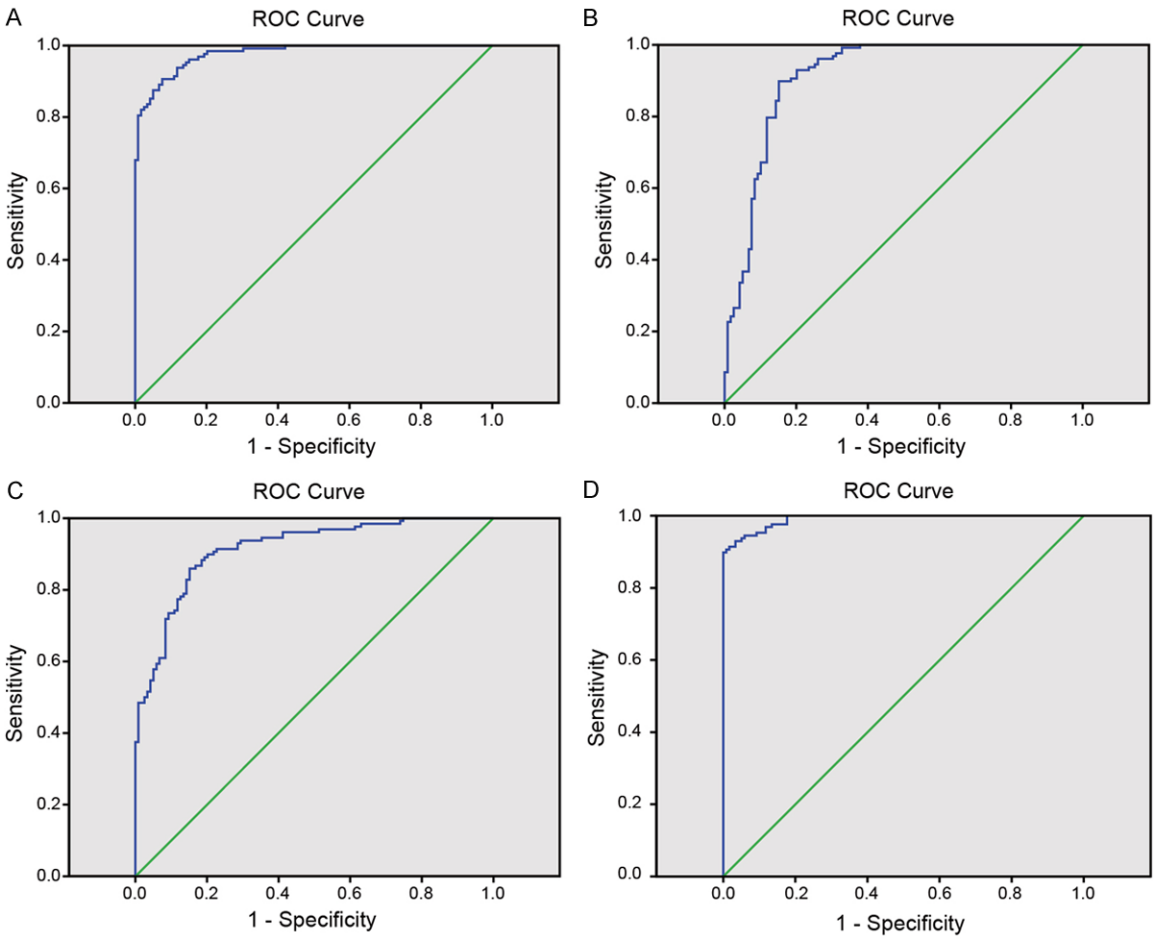


**Figure 3.** The diagnostic value of $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22 in lung cancer. Note: A. $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs; B. IL-37; C. CCL-22; D. $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22.

sided test, which showed statistical significance when $P < 0.05$.

## Results

*Comparison of expression levels of $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22 between case and control group*

The expression of $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs in peripheral blood mononuclear cells (PBMCs) of patients with lung cancer was (6.99 ± 1.24)%, while the expression in control group was (3.87 ± 0.94)% (t = 9.866, $P < 0.001$), showed in **Figures 1** and **2A**. The expression level of IL-37 in patients with lung cancer was (71.71 ± 22.68) pg/mL, while the expression in control group was (138.53 ± 41.02) pg/mL in control group (t = 5.90, $P < 0.001$), presented in **Figure 2B**. The expression level of CCL-22 in patients with lung cancer was (820.62 ± 117.65) pg/mL, while the expression in control group was (602.17 ± 103.95) pg/mL in control group (t = 5.90, $P < 0.001$), showed in **Figure 2C**.

*The diagnostic value of the expression level of $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22 in lung cancer*

The AUC of the diagnostic value of the expression level of $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs in PBMC of lung cancer was 0.977 (95% confidence interval (CI) = 0.963-0.991, $P < 0.001$). When the cut-off value was 5.29, the sensitivity (90.60%) and specificity (92.44%) presented the maximum value (**Figure 3A**). The AUC of the diagnostic value of the expression level of IL-37 in PBMC of lung cancer was 0.913 (95% CI = 0.875-0.951, $P < 0.001$). When the cut-off value was 97.95, the sensitivity (89.80%) and specificity (84.87%) presented the maxi-

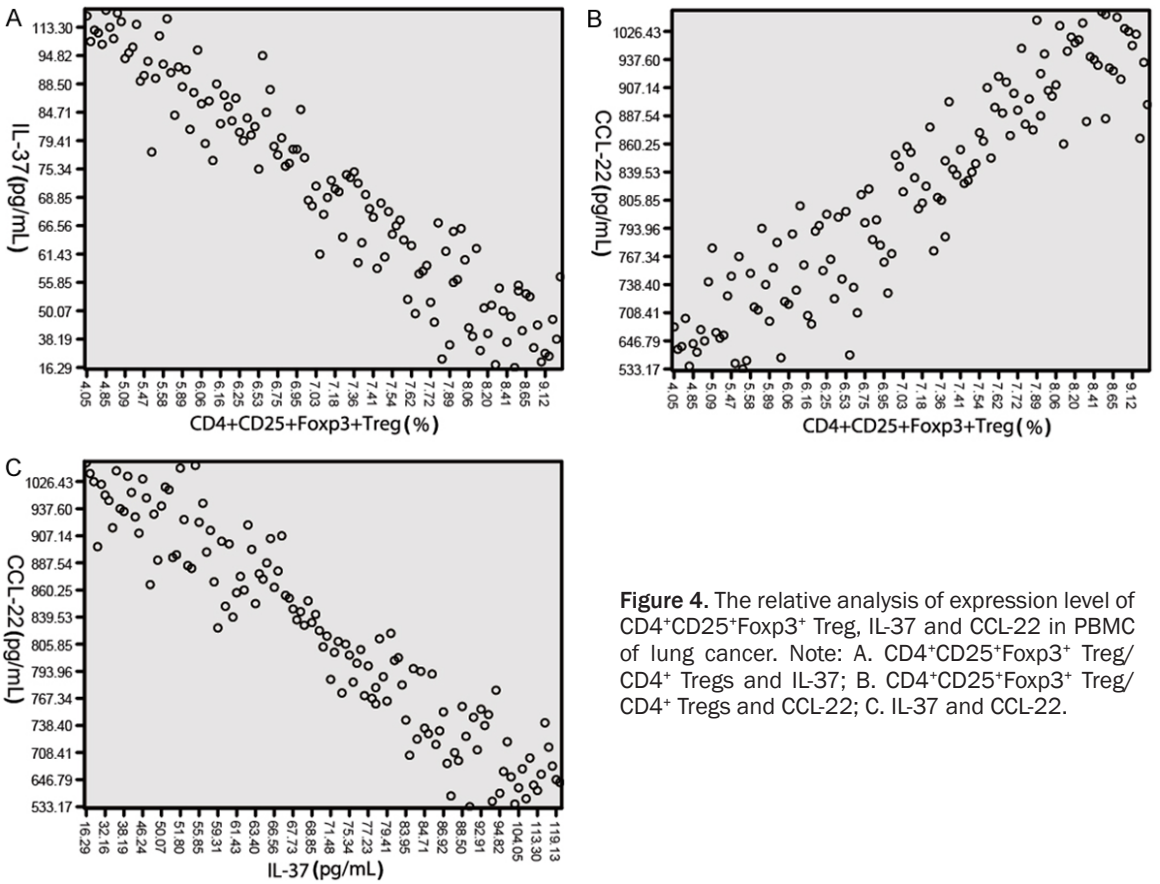


**Figure 4.** The relative analysis of expression level of $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22 in PBMC of lung cancer. Note: A. $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs and IL-37; B. $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs and CCL-22; C. IL-37 and CCL-22.

mum value (**Figure 3B**). The AUC of the diagnostic value of the expression level of CCL-22 in PBMC of lung cancer was 0.914 (95% CI = 0.879-0.948, $P < 0.001$). When the cut-off value was 97.95, the sensitivity (85.90%) and specificity (84.87%) presented the maximum value (**Figure 3C**). The AUC of the co-diagnostic value of the expression levels of $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs, IL-37 and CCL-22 was 0.991 (95% CI = 0.983~0.998, $P < 0.001$), and the sensitivity and specificity was 90.6% and 99.16%, respectively (**Figure 3D**).

*The correlation analysis of expression level of $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22 in PBMC of lung cancer*

There was a significant negative correlation between the expression levels of $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs and IL-37 in PBMC of lung cancer (r = -0.911, $P < 0.001$, **Figure 4A**), while the correlation between the expression levels of $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs and CCL-22 was positive (r = 0.860, $P < 0.001$, **Figure 4B**), as well as the negative correlation between the expression levels of IL-37 and CCL-22 (r = -0.891, $P < 0.001$, **Figure 4C**).

*The clinical features of lung cancer with the expression of $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22*

**Table 1** showed the clinical features of lung cancer with the expression of $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22. The expression levels of $CD4^+CD25^+Foxp3^+$ Tregs, IL-37 and CCL-22 in patients with lung cancers were associated with the tumor size, histological types, differentiation degree, TNM grading, lymphatic metastasis and distance metastasis (all $P < 0.001$). However, the gender, age, smoking history, tumor location and diseased region were without significant relations with these expressions (all $P > 0.05$). Furthermore, the expression levels of $CD4^+CD25^+Foxp3^+$ Tregs, IL-37 and CCL-22 in patients with squamous cell carcinoma were significant higher than that of in adenocarcinoma of lung as well as small cell carcinoma (all $P < 0.05$).

**Table 1.** The relationship of $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22 expression with clinical features of lung cancer

| Parameters | N | $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs (%) | | | IL-37 (pg/mL) | | | CCL-22 (pg/mL) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Expression level | *t/F* | *P* | Expression level | *t/F* | *P* | Expression level | *t/F* | *P* |
| Gender | | | | | | | | | | |
| Male | 102 | 6.93 ± 1.26 | | | 72.51 ± 23.48 | | | 817.00 ± 114.37 | | |
| Female | 26 | 7.21 ± 1.14 | 1.004 | 0.317 | 68.56 ± 19.31 | 0.790 | 0.431 | 835.00 ± 131.1 | 0.695 | 0.488 |
| Age (years) | | | | | | | | | | |
| ≥ 55 | 85 | 7.04 ± 1.19 | | | 71.34 ± 21.46 | | | 821.33 ± 114.35 | | |
| < 55 | 43 | 6.90 ± 1.34 | 0.583 | 0.561 | 72.43 ± 25.17 | 0.256 | 0.798 | 819.32 ± 125.30 | 0.091 | 0.927 |
| Smoking history | | | | | | | | | | |
| Smoking | 88 | 6.95 ± 1.21 | | | 71.45 ± 23.25 | | | 818.55 ± 119.72 | | |
| Without smoking | 40 | 7.09 ± 1.31 | 0.602 | 0.548 | 72.27 ± 21.65 | 0.188 | 0.851 | 825.29 ± 114.32 | 0.299 | 0.765 |
| Diseased region | | | | | | | | | | |
| Central type | 55 | 7.12 ± 1.2 | | | 70.91 ± 22.81 | | | 822.46 ± 126.57 | | |
| Peripheral type | 73 | 6.89 ± 1.27 | 1.013 | 0.313 | 72.3 ± 22.72 | 0.343 | 0.732 | 819.29 ± 111.34 | 0.150 | 0.881 |
| Tumor location | | | | | | | | | | |
| Left side | 52 | 7.04 ± 1.23 | | | 70.49 ± 22.22 | | | 815.12 ± 119.34 | | |
| Right side | 76 | 6.96 ± 1.25 | 0.373 | 0.710 | 72.53 ± 23.1 | 0.498 | 0.619 | 824.44 ± 117.12 | 0.439 | 0.662 |
| Tumor size | | | | | | | | | | |
| ≥ 3 cm | 79 | 7.76 ± 0.8 | | | 57.8 ± 15.1 | | | 890.55 ± 80.77 | | |
| < 3 cm | 49 | 5.75 ± 0.7 | 14.525 | < 0.001 | 94.12 ± 12.61 | 14.064 | < 0.001 | 707.96 ± 70.82 | 13.019 | < 0.001 |
| Histological types | | | | | | | | | | |
| SCC | 59 | 8.05 ± 0.65 | | | 52.33 ± 13.49 | | | 921.31 ± 69.17 | | |
| Adenocarcinoma | 47 | 6.1 ± 0.91* | | | 88.26 ± 13.14* | | | 728.89 ± 74.86* | | |
| SCLC | 22 | 6.06 ± 0.93* | 105.691 | < 0.001 | 88.29 ± 16.53* | 105.928 | < 0.001 | 746.77 ± 72.05* | 108.025 | < 0.001 |
| Differentiation degree | | | | | | | | | | |
| Low-differentiated | 57 | 8.08 ± 0.64 | | | 51.78 ± 13.4 | | | 924.18 ± 68.58 | | |
| Middle and high differentiated | 71 | 6.12 ± 0.85 | 14.373 | < 0.001 | 87.7 ± 14.38 | 14.472 | < 0.001 | 737.55 ± 74.95 | 14.537 | < 0.001 |
| TNM grading | | | | | | | | | | |
| I+II | 50 | 7.78 ± 0.78 | | | 57.55 ± 15.04 | | | 891.68 ± 80.67 | | |
| III+IV | 78 | 5.76 ± 0.7 | 14.944 | < 0.001 | 93.78 ± 12.71 | 14.107 | < 0.001 | 709.67 ± 71.37 | 13.002 | < 0.001 |
| lymphatic metastasis | | | | | | | | | | |
| N0 | 66 | 8.01 ± 0.66 | | | 53.11 ± 13.61 | | | 917.5 ± 69.57 | | |
| N1-3 | 62 | 6.03 ± 0.81 | 15.136 | < 0.001 | 89.17 ± 13.84 | 14.852 | < 0.001 | 729.68 ± 71.79 | 15.015 | < 0.001 |
| Distance metastasis | | | | | | | | | | |
| M0 | 81 | 8.24 ± 0.59 | | | 49.1 ± 13.18 | | | 939.76 ± 64.99 | | |
| M1 | 47 | 6.27 ± 0.89 | 14.973 | < 0.001 | 84.82 ± 15.56 | 13.233 | < 0.001 | 751.54 ± 79.84 | 13.729 | < 0.001 |

Note: *, compared with SCC, $P < 0.05$; SCC, squamous cell carcinoma; SCLC: Small Cell Lung Cancer.

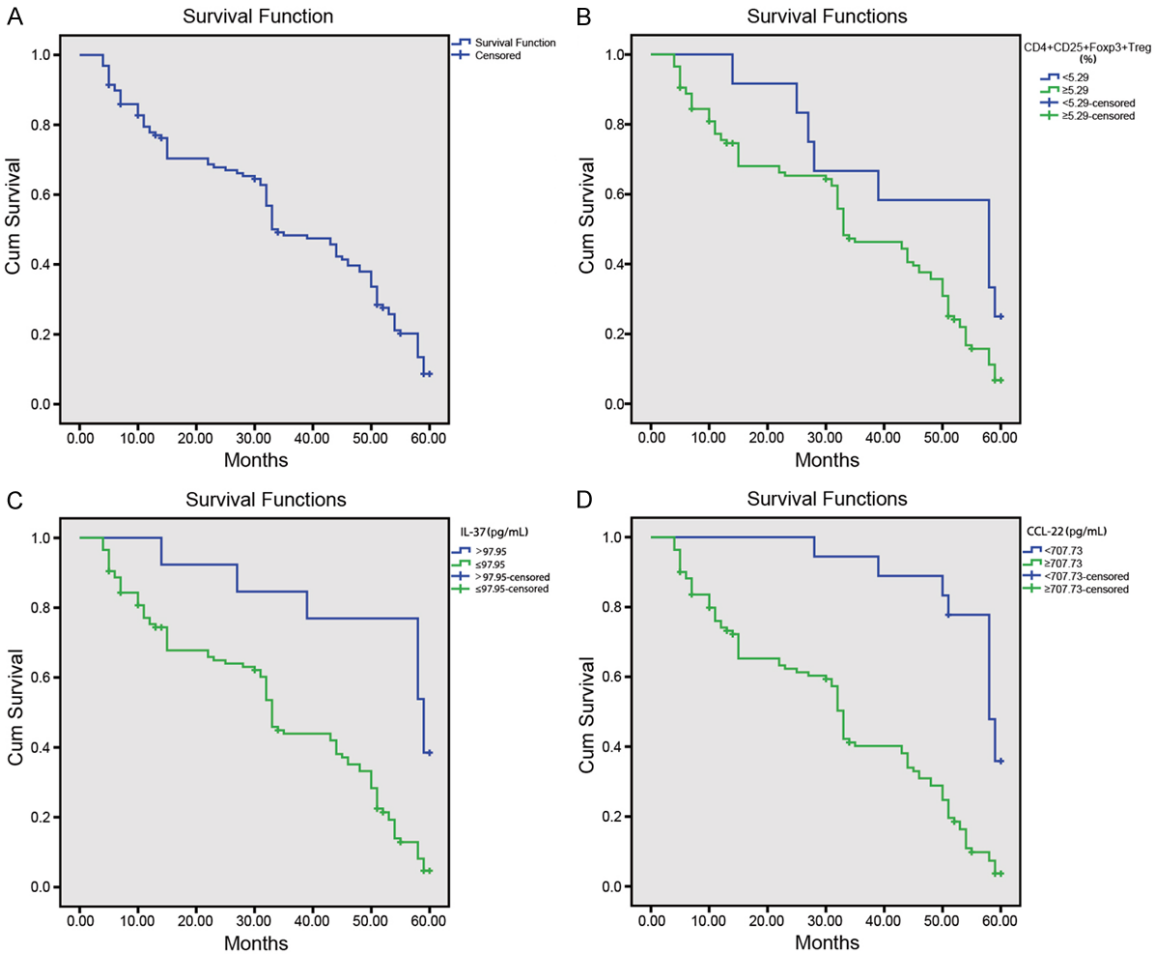


**Figure 5.** Associations of expression level of $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22 with the prognosis of patients with lung cancer. Note: A. General survival curve; B. Associations of expression level of $CD4^+CD25^+Foxp3^+$ Treg with the prognosis of patients with lung cancer; C. Associations of expression level of IL-37 with the prognosis of patients with lung cancer; D. Associations of expression level of CCL-22 with the prognosis of patients with lung cancer.

*Associations of expression level of $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22 with the prognosis of patients with lung cancer*

Showed in **Figure 5A**, 114 patients with lung cancer were followed up with the time of 4~60 months (median time: 34.00 months), and the results showed the 5-year survival rate was 6.14% (7/114). The 5-year survival rate of patients with high expression of $CD4^+$ $CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs (≥ 5.29%) was 3.57% (4/112), which was significant lower than the patients with low expression (< 5.29%; 5-year survival rate: 25.00%, 3/12) ($\chi^2$ = 4.843, $P$ = 0.028, **Figure 5B**). The 5-year survival rate of patients with low expression of IL-37 (> 97.95 pg/mL) was 1.98% (2/101), which was significant lower than the patients with high expression (> 97.95 pg/mL; 5-year survival rate: 1.03%, 1/97) ($\chi^2$ = 14.486, $P$ < 0.001, **Figure 5C**). The 5-year survival rate of patients with high expression of CCL-22 (≥ 704.73 pg/mL) was 1.03% (1/97), which was significant lower than the patients with low expression (< 704.73 pg/mL; 5-year survival rate: 35.29%, 6/17) ($\chi^2$ = 23.438, $P$ < 0.001, **Figure 5D**). The results of Kaplan-Meier method indicated that the expression of $CD4^+CD25^+Foxp3^+$ Treg, IL-37 and CCL-22 were related to the prognosis of patients with lung cancer.

*Analysis of influences parameters in survival time of patients with lung cancer*

Showed in **Table 2**, the results of Cox multivariate analysis model indicated that distance metastasis, expressions of $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs, IL-37 and CCL-22 in PBMCs

**Table 2.** Detection of survival time in patients with lung cancer by Cox multivariate analysis model

| | B | SE | Wald | df | Sig. | ExP (B) | 95.0% CI for ExP (B) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lower | Upper |
| Gender (female vs. male) | 0.354 | 0.246 | 2.057 | 1 | 0.151 | 0.702 | 0.433 | 1.138 |
| Age (≥ 55 vs. < 55) | 0.139 | 0.227 | 0.373 | 1 | 0.541 | 1.149 | 0.736 | 1.792 |
| Smoking history (smoking vs. without smoking) | 0.448 | 0.237 | 3.591 | 1 | 0.058 | 0.639 | 0.402 | 1.015 |
| diseased region (central type vs. Peripheral type) | 0.108 | 0.214 | 0.253 | 1 | 0.615 | 1.114 | 0.732 | 1.696 |
| Tumor location (Left side vs. Right side) | 0.352 | 0.220 | 2.560 | 1 | 0.110 | 0.703 | 0.457 | 1.082 |
| Tumor size (≥ 3 cm vs. < 3 cm) | 2.074 | 1.164 | 3.173 | 1 | 0.075 | 7.954 | 0.812 | 77.898 |
| Histological types (SCC vs. Adenocarcinoma/SCLC) | 0.482 | 1.167 | 0.171 | 1 | 0.679 | 0.617 | 0.063 | 6.079 |
| differentiation degree (low vs. middle-high) | 0.338 | 1.055 | 0.103 | 1 | 0.749 | 1.402 | 0.177 | 11.080 |
| TNM grading (III+IV vs. I+II) | 1.138 | 1.113 | 1.045 | 1 | 0.307 | 3.120 | 0.352 | 27.637 |
| lymphatic metastasis (metastasis vs. no metastasis) | 1.078 | 0.693 | 2.417 | 1 | 0.120 | 2.938 | 0.755 | 11.428 |
| Distance metastasis (metastasis vs. no metastasis) | 2.134 | 0.456 | 21.935 | 1 | 0.000 | 8.449 | 3.459 | 20.637 |
| CD4 (≥ 5.29% vs. < 5.29%) | 1.748 | 0.463 | 14.226 | 1 | 0.000 | 5.743 | 2.316 | 14.244 |
| IL37 (≤ 97.95 pg/mL vs. > 97.95 pg/mL) | 4.211 | 1.260 | 11.170 | 1 | 0.001 | 67.399 | 5.705 | 796.228 |
| CCL22 (≥ 704.73 pg/mL vs. < 704.73 pg/mL) | 1.261 | 0.613 | 4.234 | 1 | 0.040 | 3.527 | 1.062 | 11.718 |

Note: CI, confidence interval; SCC, squamous cell carcinoma; SCLC: Small Cell Lung Cancer.

were the independent risk factors of prognosis of patients with lung cancer. The protective factors of prognosis in patients with lung cancer were low expression of $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ ($P < 0.001$) and CCL-22 ($P < 0.000$), high expression of IL-37 ($P = 0.001$) and no metastasis ($P = 0.004$).

## Discussion

Our findings suggested that the expressions of $CD4^+CD25^+Foxp3^+$ Tregs and CCL-22 in patients PBMC with lung cancers were greater than that of in healthy controls, while the expression of IL-37 in lung cancer was lower. Additionally, our study demonstrated that the expression level of $CD4^+CD25^+Foxp3^+$ Tregs, IL-37 and CCL-22 in patients with lung cancers were associated with the tumor size, histological types, differentiation degree, TNM grading, lymphatic metastasis and distance metastasis, indicating that the expressions of $CD4^+CD25^+FOXP3^+$ Tregs, IL-37 and CCL-22 were closely related to lung cancer. Tregs were initially characterized as having a $CD4^+CD25^+$ phenotype, and these cells are considered to adjust the antitumor immune response, and Tregs can also suppress the activity of cytotoxic T cells via the direct cell-to-cell contact or through the release of cytokines [19]. Treg cells with increased activation can mediate immune suppression by direct contact, and affect the activation and proliferation of $CD8^+$ T and NK cells; it can also inhibit the expression of APC in combination with stimulatory molecules, thus inhibiting the proliferation of effector T cells [20]. Presented in Nasrollah's study, the cells with a phenotype of $CD4^+CD25^+$ as well as intracellular FoxP3 serve as the main inhibitory phenotype in the immune system, which reported the expression of $CD4^+CD25^+Foxp3^+$ Tregs are higher in the patients with lung cancer [21]. Chen C et al. indicated that the percentage of $CD4^+CD25^+FOXP3^+$ Tregs correlated with the pathological stage in NSCLC and tumor burden [22]. Pan XD et al. suggested that the up-regulation of Tregs may play an essential role in oncogenesis and development of lung cancer in an elderly population [23]. CCR4, a chemokine receptor, is highly expressed on Tregs, and it mediates the recruitment of Tregs in vivo through its ligand, called CCL22 [24]. As macrophage-derived chemokine, CCL22 plays an important role in growth, progression and metastasis of tumors [25]. Previously reported associations of CCL22 with lung cancer, and studies of high expression of CCL22 and their respective receptors in lung tumors all lend biologic support for our observed associations [26, 27]. IL-37 is an anti-inflammatory cytokine with several biological functions and acts through an intracellular mechanism translocating to the nucleus [28]. The expression of IL-37 in epithelial cells or macrophages practically suppressed the production of pro-inflammatory cytokines, while in human blood cells, the abundance of these cytokines enhanced with the silencing of endogenous IL-37 [5].

Our study also indicated that there are great diagnostic value of sensitivity and specificity of the expression level of $CD4^+CD25^+FOXP3^+$ Treg, IL-37 and CCL-22 in PBMC of lung cancer, distance metastasis, and the expressions of $CD4^+CD25^+Foxp3^+$ Treg/$CD4^+$ Tregs, IL-37 and CCL-22 in PBMCs were the independent risk factors of prognosis of lung cancer, implying that $CD4^+CD25^+Foxp3^+$ Tregs, IL-37 and CCL-22 selectively inhibit the host immune response and therefore could contribute to the diagnosis in early stage, prognosis evaluation and immunization therapy of lung cancer. Andreas et al. showed that Tregs are significantly affected by therapy and Tregs changes in PB are positively correlated with clinical response to therapy, and thus Tregs measurements in PB early during therapy as immunological biomarker predicting clinical response to therapy [29]. Tumor-infiltrating $Foxp3^+$ Tregs could be related to the favorable prognosis in some types of human carcinomas in an unexpected observation [10]. It has been reported that regulatory T cells migrate toward the malignant tumor microenvironment in a process mediated by chemokines CCL22 [14]. CCL22 can promote the accumulation of EOS and TH2 cells by the receptor ligand signaling pathway, which can lead to the clinical symptoms of lung cancer patients and it can lay a theoretical foundation for exploring the pathogenesis and treatment of lung cancer [30]. IL-37 is a strong anti-inflammatory cytokine that plays an important role in limiting tissue injury during infections by limiting the duration and intensity of immune and inflammatory reactions and is protective in animal models [15]. One study suggested that the level of IL-37 was positively correlated with the pulmonary function, and the conclusion was that IL-37 was correlated with the severity of bronchial asthma, indicating that IL-37 can be used as an index to evaluate the severity of lung disease [5].

In conclusion, expressions of $CD4^+CD25^+$ $Foxp3^+$ Tregs, IL-37 and CCL-22 in patients with lung cancers is up-regulated in PB, which demonstrated that $CD4^+CD25^+Foxp3^+$ Tregs, IL-37 and CCL-22 selectively restrain the host immune response and thereof might contribute to the diagnosis in early stage, prognosis evaluation and immunization therapy of lung cancer.

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## Disclosure of conflict of interest

None.

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## References

[1] Grunnet M and Sorensen JB. Carcinoembryonic antigen (CEA) as tumor marker in lung cancer. Lung Cancer 2012; 76: 138-143.

[2] Ferlay J, Parkin DM and Steliarova-Foucher E. Estimates of cancer incidence and mortality in Europe in 2008. Eur J Cancer 2010; 46: 765-781.

[3] Thun MJ, Hannan LM, Adams-Campbell LL, Boffetta P, Buring JE, Feskanich D, Flanders WD, Jee SH, Katanoda K, Kolonel LN, Lee IM, Marugame T, Palmer JR, Riboli E, Sobue T, Avila-Tang E, Wilkens LR and Samet JM. Lung cancer occurrence in never-smokers: an analysis of 13 cohorts and 22 cancer registry studies. PLoS Med 2008; 5: e185.

[4] Collins LG, Haines C, Perkel R and Enck RE. Lung cancer: diagnosis and management. Am Fam Physician 2007; 75: 56-63.

[5] Nold MF, Nold-Petry CA, Zepp JA, Palmer BE, Bufler P and Dinarello CA. IL-37 is a fundamental inhibitor of innate immunity. Nat Immunol 2010; 11: 1014-1022.

[6] Sandler A, Yi J, Dahlberg S, Kolb MM, Wang L, Hambleton J, Schiller J and Johnson DH. Treatment outcomes by tumor histology in Eastern Cooperative Group Study E4599 of bevacizumab with paclitaxel/carboplatin for advanced non-small cell lung cancer. J Thorac Oncol 2010; 5: 1416-1423.

[7] Hori S, Nishiumi S, Kobayashi K, Shinohara M, Hatakeyama Y, Kotani Y, Hatano N, Maniwa Y, Nishio W, Bamba T, Fukusaki E, Azuma T, Takenawa T, Nishimura Y and Yoshida M. A metabolomic approach to lung cancer. Lung Cancer 2011; 74: 284-292.

[8] Han MK, Oh YH, Kang J, Kim YP, Seo S, Kim J, Park K and Kim HS. Protein profiling in human sera for identification of potential lung cancer biomarkers using antibody microarray. Proteomics 2009; 9: 5544-5552.

[9] Grivennikov SI, Greten FR and Karin M. Immunity, inflammation, and cancer. Cell 2010; 140: 883-899.
[10] Ladoire S, Martin F and Ghiringhelli F. Prognostic role of FOXP3+ regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer. Cancer Immunol Immunother 2011; 60: 909-918.
[11] Karagoz B, Bilgi O, Gumus M, Erikci AA, Sayan O, Turken O, Kandemir EG, Ozturk A and Yaylaci M. CD8+CD28- cells and CD4+CD25+ regulatory T cells in the peripheral blood of advanced stage lung cancer patients. Med Oncol 2010; 27: 29-33.
[12] Kwon HK, Lee CG, So JS, Chae CS, Hwang JS, Sahoo A, Nam JH, Rhee JH, Hwang KC and Im SH. Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. Proc Natl Acad Sci U S A 2010; 107: 2159-2164.
[13] Zhang L and Zhao Y. The regulation of Foxp3 expression in regulatory CD4(+)CD25(+)T cells: multiple pathways on the road. J Cell Physiol 2007; 211: 590-597.
[14] Mizukami Y, Kono K, Kawaguchi Y, Akaike H, Kamimura K, Sugai H and Fujii H. CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3+ regulatory T cells in gastric cancer. Int J Cancer 2008; 122: 2286-2293.
[15] Tete S, Tripodi D, Rosati M, Conti F, Maccauro G, Saggini A, Cianchetti E, Caraffa A, Antinolfi P, Toniato E, Castellani ML, Conti P and Theoharides TC. IL-37 (IL-1F7) the newest anti-inflammatory cytokine which suppresses immune responses and inflammation. Int J Immunopathol Pharmacol 2012; 25: 31-38.
[16] Zhu LJ, Hou PF, Wang L, Zhang GB, Xie Y, Pan XD and Chang TT. Changes in CD4+ CD25+ Foxp3+ Regulatory T Cells in Relation to Aging and Lung Tumor Incidence. Int J Gerontol 2012; 6: 187-191.
[17] World Medical Association Inc. Declaration of Helsinki. Ethical principles for medical research involving human subjects. J Indian Med Assoc 2009; 107: 403-405.
[18] Edge SB and Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol 2010; 17: 1471-1474.
[19] Shimizu K, Nakata M, Hirami Y, Yukawa T, Maeda A and Tanemoto K. Tumor-infiltrating Foxp3+ regulatory T cells are correlated with cyclooxygenase-2 expression and are associated with recurrence in resected non-small cell lung cancer. J Thorac Oncol 2010; 5: 585-590.
[20] Banerjee DK, Dhodapkar MV, Matayeva E, Steinman RM and Dhodapkar KM. Expansion of FOXP3high regulatory T cells by human dendritic cells (DCs) in vitro and after injection of cytokine-matured DCs in myeloma patients. Blood 2006; 108: 2655-2661.
[21] Erfani N, Mehrabadi SM, Ghayumi MA, Haghshenas MR, Mojtahedi Z, Ghaderi A and Amani D. Increase of regulatory T cells in metastatic stage and CTLA-4 over expression in lymphocytes of patients with non-small cell lung cancer (NSCLC). Lung Cancer 2012; 77: 306-311.
[22] Chen C, Chen D, Zhang Y, Chen Z, Zhu W, Zhang B, Wang Z and Le H. Changes of CD4+CD25+FOXP3+ and CD8+CD28- regulatory T cells in non-small cell lung cancer patients undergoing surgery. Int Immunopharmacol 2014; 18: 255-261.
[23] Pan XD, Mao YQ, Zhu LJ, Li J, Xie Y, Wang L and Zhang GB. Changes of regulatory T cells and FoxP3 gene expression in the aging process and its relationship with lung tumors in humans and mice. Chin Med J (Engl) 2012; 125: 2004-2011.
[24] Montane J, Bischoff L, Soukhatcheva G, Dai DL, Hardenberg G, Levings MK, Orban PC, Kieffer TJ, Tan R and Verchere CB. Prevention of murine autoimmune diabetes by CCL22-mediated Treg recruitment to the pancreatic islets. J Clin Invest 2011; 121: 3024-3028.
[25] Ma J, Liu L, Che G, Yu N, Dai F and You Z. The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. BMC Cancer 2010; 10: 112.
[26] de Chaisemartin L, Goc J, Damotte D, Validire P, Magdeleinat P, Alifano M, Cremer I, Fridman WH, Sautes-Fridman C and Dieu-Nosjean MC. Characterization of chemokines and adhesion molecules associated with T cell presence in tertiary lymphoid structures in human lung cancer. Cancer Res 2011; 71: 6391-6399.
[27] Shiels MS, Pfeiffer RM, Hildesheim A, Engels EA, Kemp TJ, Park JH, Katki HA, Koshiol J, Shelton G, Caporaso NE, Pinto LA and Chaturvedi AK. Circulating inflammation markers and prospective risk for lung cancer. J Natl Cancer Inst 2013; 105: 1871-1880.
[28] McNamee EN, Masterson JC, Jedlicka P, McManus M, Grenz A, Collins CB, Nold MF, Nold-Petry C, Bufler P, Dinarello CA and Rivera-Nieves J. Interleukin 37 expression protects mice from colitis. Proc Natl Acad Sci U S A 2011; 108: 16711-16716.
[29] Pircher A, Gamerith G, Amann A, Reinold S, Popper H, Gachter A, Pall G, Woll E, Jamnig H, Gastl G, Wolf AM, Hilbe W and Wolf D. Neoadjuvant chemo-immunotherapy modifies CD4(+)CD25(+) regulatory T cells (Treg) in non-small cell lung cancer (NSCLC) patients. Lung Cancer 2014; 85: 81-87.
[30] Nakanishi T, Imaizumi K, Hasegawa Y, Kawabe T, Hashimoto N, Okamoto M and Shimokata K. Expression of macrophage-derived chemokine (MDC)/CCL22 in human lung cancer. Cancer Immunol Immunother 2006; 55: 1320-1329.