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Th17 cell frequency and IL-17A production in peripheral blood of patients with non-small-cell lung cancer

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Abstract

Objective: This study investigated the frequency of T-helper (Th)17 lymphocytes and production of cytokine interleukin (IL)-17 in peripheral blood of patients with non-small-cell lung cancer (NSCLC) and their use as a marker of clinical value.

Methods: Sixty patients with NSCLC and 60 healthy volunteers were enrolled in the study. Flow cytometry was used to detect the frequency of Th17 lymphocytes in peripheral blood, and enzyme-linked immunosorbent assay (ELISA) was used to detect serum levels of IL-17. We analyzed the association of Th17 lymphocytes and IL-17 levels in the peripheral blood of patients with their clinicopathological features.

Results: Frequency of Th17 lymphocytes and production of IL-17 were significantly higher in the NSCLC group than in the control group and were higher in patients with a smoking history compared with non-smokers. Moreover, Th17 lymphocyte and IL-17 expression levels were higher in patients with squamous cell carcinoma than in patients with adenocarcinoma, and significantly higher in patients with stage III and IV cancers than in patients at stage I or II.

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Conclusion: Th17 lymphocytes and IL-17 play an important role in the development of NSCLC in patients and may have clinical value as markers for treatment of NSCLC.

Keywords
Non-small-cell lung cancer, T-helper17 lymphocyte, interleukin-17 cytokine, flow cytometry, enzyme linked immunosorbent assay, clinical marker

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Introduction

The incidence of lung cancer is presently the highest of all cancers worldwide, and non-small-cell lung cancer (NSCLC) accounts for 80% to 85% of lung cancers. Globally, approximately 80% of newly diagnosed NSCLCs are accompanied by metastatic disease and thus have a poor prognosis; the average survival time is approximately 12 months. A previous study confirmed that immune dysfunction is an important cause of tumor development.[1] Furthermore, a number of studies have identified that T-helper (Th)17 lymphocytes and the cytokine interleukin (IL)-17 are highly expressed in patients with NSCLC.[1–3] These promote microvasculogenesis in the tumor microenvironment and induce tumor cell growth and metastasis.[4,5]

The present study aimed to investigate the expression of Th17 lymphocytes and cytokine IL-17 in peripheral blood of patients with NSCLC and determine whether these have clinical value. We hypothesized that the elevated expression of Th17 lymphocytes and cytokine IL-17 in the peripheral blood of patients with NSCLC plays an important role in the development of NSCLC.

Materials and methods

Patients

Sixty patients with NSCLC (experimental group) in whom the primary tumor was untreated and 60 healthy volunteers (control group) were enrolled in the present study. The clinical assessment of the collected clinical samples was approved by the Institutional Review Committee of Shanxi Provincial Cancer Hospital, and all participants provided signed informed consent before participation.

Inclusion criteria were (1) patients diagnosed with primary lung cancer by cytology, pathology, or radiographic examination (without radiotherapy, chemotherapy, or immunotherapy); (2) patients >18 years old; (3) patients who provided signed informed consent.

Exclusion criteria were (1) patients with a long history of cigarette smoking (more than 10 years); (2) patients with chronic obstructive pulmonary disease; (3) patients with bronchial asthma or other pulmonary diseases; (4) patients with acute and chronic infections; (5) patients with multiple sclerosis, rheumatoid arthritis, or other autoimmune diseases.

Reagents

Stimulant phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma (St. Louis, MO, USA). Protein transport inhibitor Brefeldin A (BFA), monoclonal antibodies CD3-PerCP, CD4-PE-Cy7, and IL-17-APC, and the fixation/permeabilization rupture agent were purchased from BD Biosciences (San Jose, CA, USA). The 1640 medium containing 10% fetal bovine serum and human

peripheral blood lymphocyte separation solution were purchased from Hao Yang Biological Products (Tianjin, China).

Flow cytometry

Samples were collected by venipuncture into heparin sodium tubes. For flow cytometry analysis, 250 μL of whole blood was mixed with 750 μL of 1640 medium containing 10% fetal bovine serum; then, 100 ng/mL PMA, 1 μg/mL ionomycin, and 10 μg/mL BFA were added, mixed, and incubated at 37°C with 5% CO_2 for 5 hours. Then, the cells were centrifuged at $250 \times g$ for 5 minutes, the supernatant was discarded, CD3 (10 μL) and CD4 (10 μL) were added, mixed, and placed in the dark for 15 minutes. Two milliliters of BD hemolysis reagent was added, incubated for 10 minutes at room temperature, and centrifuged at $250 \times g$ for 5 minutes. Then, 1 mL of rupture agent was added, and the cells were incubated at room temperature for 20 minutes. Next, 2 mL of phosphate-buffered saline (PBS) was added and the mixture was centrifuged at $250 \times g$ for 5 minutes. Finally, to stain the cells, IL-17 (10 μL) was added and mixed for 30 minutes. After incubation, 2 to 3 mL of washing solution was added to the cells, the mixture was centrifuged at $250 \times g$ for 5 minutes, and 300 μL of PBS was added for the flow cytometry test.

ELISA

Serum was separated from 5 mL of whole blood collected from all participants and stored at −80°C. We used ELISA kits to quantify serum concentrations of IL-4, interferon (IFN)-γ, and IL-17 (Human IL-4 Quantikine ELISA Kit D4050, Human IFN-γ Quantikine ELISA Kit DIFNB0, Human IL-17 Quantikine ELISA Kit D1700, R&D Systems, Minneapolis, MN, USA) and followed the manufacturer's instructions for the kits. Briefly, a 96-well plate was coated with antibody in advance and blocked to reduce non-specific binding. Then, standards and plasma samples were added to the plate. Following a 1-hour incubation and five washes of the plate, we added streptavidin–horseradish peroxidase (HRP)-labeled antibody to the plate. After an additional incubation, we added tetramethylbenzidine (TMB) substrate and stopped the reaction by adding sulfuric acid. Concentrations of IL-4, IFN-γ, and IL-17 were measured using a spectrophotometer Epoch (BioTek Instruments Inc., Winooski, VT, USA) at 450 nm within 15 minutes. All ELISA results are expressed as cytokine concentrations (ng/L). A standard curve was generated using known amounts of the respective purified recombinant cytokines.

Statistical analysis

All data were recorded and analyzed using Excel 2016 (Microsoft Corp., Redmond, WA, USA) and SPSS version 21.0 (IBM Corp., Armonk, NY, USA). Quantitative data were expressed as mean ± standard deviation. A P-value of <0.05 was considered significant.

Results

Patient characteristics

In the experimental group, 39 patients were men and 21 patients were women, and their average age was 60.63 ± 9.45 years old. In the control group, 35 patients were men and 25 were women, and their mean age was 58.72 ± 9.23 years old (Table 1). According to the TNM staging criteria, 28 cases were rated as stage I–II, 14 cases were rated as stage III, and 18 cases were rated as stage IV. According to the World Health Organization (WHO) classification criteria, 30 patients had squamous cell carcinoma

Table 1. The levels of Th17 lymphocytes and IL-17 in peripheral blood of two groups (mean ± standard deviation).

Index	Experimental group	Control group	*P*-value
N	60	60	
Age (years)	60.63 ± 9.45	58.72 ± 9.23	>0.05
Sex (male/female)	39/21	35/25	>0.05
Th17 lymphocytes (%)	1.74 ± 1.18*	0.89 ± 0.56	<0.05
IL-17 (pg/mL)	8.32 ± 2.52*	5.36 ± 1.18	<0.05

Compared with the control group, *$P < 0.05$.

Table 2. Relationship between clinical parameters and Th17 lymphocyte and IL-17 levels in patients with lung cancer (mean ± standard deviation).

Variable	Group	Number	Th17 lymphocytes (%)	IL-17 (pg/mL)
Sex	Male	39	1.77 ± 1.19	9.23 ± 4.54
	Female	21	1.68 ± 1.18	7.18 ± 3.43
Smoking history	No	23	1.61 ± 1.16	7.95 ± 2.19
	Yes	37	1.82 ± 1.20*	8.76 ± 3.71*
Pathological type	Squamous cell carcinoma	30	1.84 ± 1.23*	9.41 ± 4.72*
	Adenocarcinoma	30	1.65 ± 1.14	7.32 ± 3.92
TNM staging	I–II	28	1.10 ± 0.73	6.54 ± 3.49
	III	14	1.95 ± 1.30*	9.64 ± 3.76*
	IV	18	2.57 ± 1.12*	14.49 ± 6.84*

*Two groups of patients with different clinical indicators were compared, $P < 0.05$.

and 30 had adenocarcinoma (Table 2). There were no significant differences in clinical profiles between the experimental group and control group (Table 1).

Levels of Th17 lymphocytes and IL-17 in peripheral blood

The frequency of Th17 lymphocytes was 1.74 ± 1.18% in the experimental group and 0.89 ± 0.56% in the control group. The expression level of IL-17 was 8.32 ± 2.52 pg/mL in the experimental group and 5.36 ± 1.18 pg/mL in the control group. The frequency and expression of Th17 lymphocytes and IL-17 were higher in peripheral blood of patients in the experimental group than in the control group ($P < 0.05$, Table 1).

Association of clinical parameters with Th17 lymphocyte and IL-17 levels

The frequency of Th17 lymphocytes and expression of IL-17 were higher in patients with a history of smoking than in non-smoking patients. In addition, levels were higher in patients with squamous cell carcinoma than in those with adenocarcinoma and higher in patients at stages III and IV than in those at stage I–II ($P < 0.05$, Table 2).

Discussion

The major cell types with an anti-tumor effect are $CD4^+$ T cells, $CD8^+$ T cells, and natural killer (NK) cells. Th17 lymphocytes are a newly identified subgroup of $CD4^+$ T cells that secrete IL-17, recruit granulocytes, and are involved in a variety of

tumors.[6] At present, there is no clear consensus on the anti-tumor effect of Th17 lymphocytes or IL-17 in the tumor microenvironment. However, a number of studies have confirmed that Th17 lymphocytes and IL-17 exist in a variety of tumors and participate in tumor angiogenesis or tumor immunity.[7–9] A study conducted by Du et al.[10] reported that the number of IL-17-secreting $CD4^+$ T lymphocytes was positively correlated with tumor microvessel density. Chen et al. and Zhang et al.[11,12] reported that expression of IL-17 was correlated with tumor progression, clinicopathological features, smoking status, and TNM staging of NSCLC patients but not with age, sex, differentiation, or pathological type.

The levels of Th17 lymphocytes and IL-17 in the peripheral blood were significantly higher in the experimental group than in the control group, which is consistent with previous studies. Our results indicated that Th17 lymphocytes and IL-17 play an important role in the development of NSCLC and may have clinical value as markers of disease progression.

Previous studies have reported that Th17 lymphocytes and IL-17 are highly expressed in patients with NSCLC.[13] However, unlike these previous studies, we showed that frequency of Th17 lymphocytes and expression of IL-17 were higher in patients with a smoking history than in non-smokers, higher in patients with squamous cell carcinoma than in patients with adenocarcinoma, and higher in patients with stage III and IV cancers than in patients at stage I–II. Therefore, our results extend the findings of these previous studies. However, it is necessary to further explore the synergistic effect of IL-17 and other cytokines and chemokines in the tumor microenvironment in tumorigenesis, and to perform an in-depth study to determine whether different IL-17 sources in the body have different effects on tumors. In addition, because of the limited sample size, our data were insufficient to determine the difference in Th17 and IL-17 production by cancer stage; this warrants further research.

The present study has several limitations. First, this was not a randomized controlled trial. Second, the study was conducted at a single center and the sample size was limited. Third, we reported the frequency of Th17 cells in the present study, but the number of Th17 cells was not measure and should be determined in a future study. Fourth, patients who had undergone radio-, chemo-, or immunotherapy were excluded from this study. Thus, the effect of chemotherapy on expression of Th17 and IL-17 should be researched in the future.

In summary, exploring the immune mechanism of Th17 lymphocytes and related cytokines in patients with NSCLC is valuable for the early diagnosis and assessment of prognosis of NSCLC. Suppressing the high expression of Th17 lymphocytes may become a target for lung cancer immunotherapy.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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