Relationship between CD4+CD25+ Treg and expression of HIF-1α and Ki-67 in NSCLC patients

L.-Y. HE, L. LI, M.-L. GUO, Y. ZHANG, H.-Z. ZHANG

People's Hospital of Hebei Province, Shijiazhuang, Hebei Province, China

Hong-Zhen Zhang is engaged in clinical treatments of and basic research on malignant tumor

Abstract. – **OBJECTIVE:** To investigate the relationship between the CD4+CD25+ Treg cell proportion in the peripheral blood and the clinicopathologic features of non-small cell lung cancer (NSCLC) patients and hypoxia-inducible factor-1α (HIF-1α), Ki-67 expression.

PATIENTS AND METHODS: Flow cytometry was used to measure the CD4+CD25+ Treg cell level in peripheral blood and immunohistochemical staining used to detect the expression of HIF-1α and Ki-67 protein in cancer tissue of each of 50 NSCLC patients.

RESULTS: The level of CD4+CD25+ Treg cell in peripheral blood was related to pathologic grades (t = 3.265, p = 0.006) and clinical stage (t = 4.417, p = 0. 001) of NSCLC instead of to patient's gender and pathologic type of tumor (p > 0.05). The level of CD4+CD25+ Treg cell was positively correlated with the expression of HIF-1α (r = 0.711, p = 0.003) and Ki-67 (r = 0.517, p = 0.04), respectively.

CONCLUSIONS: CD4+CD25+ Treg cell can be used as a predictor of immune status and prognosis of NSCLC patients and the levels of HIF-1α and Ki-67 protein expression may relate to inhibition of immune cells.

Key Words:
CD4+CD25+ regulatory T lymphocyte, Non-small-cell lung carcinoma/biology, Hypoxia-inducing Factor-1α, Ki-67.

Introduction

Tumor microenvironment is a niche where tumor tissue develops, and acidosis, tissue hypoxia and malnutrition exist at different levels. Hypoxia-inducible factor-1α (HIF-1α) is a key regulatory factor for the adaptation of tumor to tissue hypoxia in the microenvironment and involves in tumor growth, angiogenesis, body's metabolism, and other biological processes. Ki-67 represents the proliferative activity of tumor cells. The CD4+CD25+ regulatory T lymphocytes (Treg cells) in the body behave for low immune response and immune suppression and at the same time play a regulatory role for immunological function[1]. This study is designed to investigate the significance of the Treg cells, or the relationship between the Treg cell proportion in peripheral blood and the expression of HIF-1α and Ki-67 in tissues of patients of non-small-cell lung carcinoma (NSCLC), in order to provide reference for clinical treatments.

Patients and Methods

Background of NSCLC Patients

The samples of peripheral blood and paraffin-embedded cancer tissue are collected from each of 50 cases of non-small-cell lung carcinoma pathologically diagnosed in the People's Hospital of Hebei Province before treatment from July 20, 2011 to Dec. 31, 2012. The patients, 33 male and 17 females and aged from 26 to 77 with a median of 64 years, included 25 cases of squamous cell carcinoma, 21 cases of adenocarcinoma, and four cases of adenosquamous carcinoma, which all together showed 27 cases of highly differentiated cells and 23 cases of poorly differentiated cells. The cases can be classified into stages of I-II in 12 cases and III-IV in 38 cases according to the latest TNM staging criteria of the International Association for the Study of Lung Cancer (IASLC) in 2009.

Major Reagents

CD4-FITC monoclonal antibodies and CD25-PE monoclonal antibodies (Beckman Coulter, Brea, CA, USA), were produced by Beckman Coulter Company. Rabbit anti-human HIF-1α and Ki-67 monoclonal antibodies were purchased from Epitomics Company in the USA. Human plasma anticoagulant protein S (SP) kit and diaminobenzidine stain (DAB) were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China.

Corresponding Author: Zhang Hong-Zhen, MD; e-mail: hongzhenzhang456@sohu.com

Methods

Flow Cytometry

The peripheral blood sample of 5 ml, with heparin for anticoagulation, was taken from each patient of NSCLC a day before anti-tumor therapy, treated with whole blood hemolysis and labeled by direct immunofluorescence, and tested for CD4+CD25+ Treg cells by flow cytometry. In details, a quantity of 20 []l of monoclonal antibody against CD4+CD25+ Treg cells was added into the test tubes for flow cytometry, and 100 []l of blood sample treated with heparin added into the same test tube, followed by mixing. The tube stood for 30 min at dark at room temperature, and then the hemolysin was added twice each at 0.5 ml. The suspension was mixed and stood at dark at room temperature for 5 min before detection. The intensity of each kind of fluoresce on cell surface was detected via flow cytometry and saved as two-dimensional and scattering dot-plot in a computer. After automatic analysis of the number and percentage of positive cells of each monoclonal antibody, the percentages of CD4+CD25+ Treg cells were calculated from the FITC-CD4/PE-CD25 two-parameter diagrams.

Immunohistochemistry

According to S-P method, phosphate buffered saline (PBS) instead of primary antibody was used as negative control, and the known positive sections stained with the same procedure as positive control.

The results of HIF-1α and Ki-67 expression were analyzed by semi-quantitative scoring method. The criteria for expression intensity of HIF-1α: Score of "0" for no positive cells or positive cell percentage ≤1%, "1" for positive cell percentage of 2-10%, "2" for positive cell percentage of 11-50%, "3" for positive cell percentage of 51-75%, "4" for positive cell percentage of ≥76%. The criteria for expression intensity of Ki-67: "0" for positive cell percentage of ≤5%, "1" for positive cell percentage of 6-25%, "2" for positive cell percentage of 26-50%, "3" for positive cell percentage of 51-75%, "4" for positive cell percentage of ≥76%. The score for color rendering of stained cells: "0" for cells without coloration, "1" for light yellow staining, "2" for brown-yellow staining, "3" for brown staining. The multiplication of the score for expression intensity and that for color rendering is used as the results of immunohistochemistry, and 0-4 scores are defined as negative result, 5-12 scores as positive results.

Statistical Analysis

The experimental data of this research were expressed as mean ± standard deviation and subjected to t-test and ANOVA by using statistical package SPSS13.0 (SPSS Inc., Chicago, IL, USA), and the correlation is analyzed using linear correlation analysis. The statistical difference is designed at $p < 0.05$.

Results

Expression of HIF-1α and Ki-67 in NSCLC Tissues

Immunohistochemical staining showed that the proteins of HIF-1α and Ki-67 were expressed in NSCLC tissues, respectively, at 70% and 61.1% (Table I), and both detected by staining of brown granules mainly in the nucleus (Figure 1A-D).

Clinical Meaning of CD4+CD25+ Treg Cell Proportion in NSCLC Patients

Before treatment, the content of CD4+CD25+ Treg lymphocytes in NSCLC patients was 12.1 ± 3.1%. As shown in Table II, the proportion of the CD4+CD25+ Treg lymphocytes related to the results of pathological differentiation ($t = 3.265$, $p = 0.006$) and the results of TNM staging ($t = 4.417$, $p = 0.001$). The more advanced the cells differentiated and the stages were, the higher the proportion of the Treg cells was. However, this proportion did not relate to the age, gender, and histological types of cancer of patients (all at $p > 0.05$).

The Relationship between CD4+CD25+ Treg Cells in Peripheral Blood and HIF-1α Expression in NSCLC Tissue

The proportion of CD4+CD25+ Treg cell in peripheral blood of NSCLC patients with HIF-1α positive cancer tissue was 12.38 ± 1.93% while that proportion of CD4+CD25+ Treg cell in blood

Table I. Cases of protein expression of HIF-1α and Ki-67 in NSCLC tissues (n = 50).

	Negative expression	Positive expression	Positive rates
HIF-1α	15	35	70
Ki-67	12	38	76

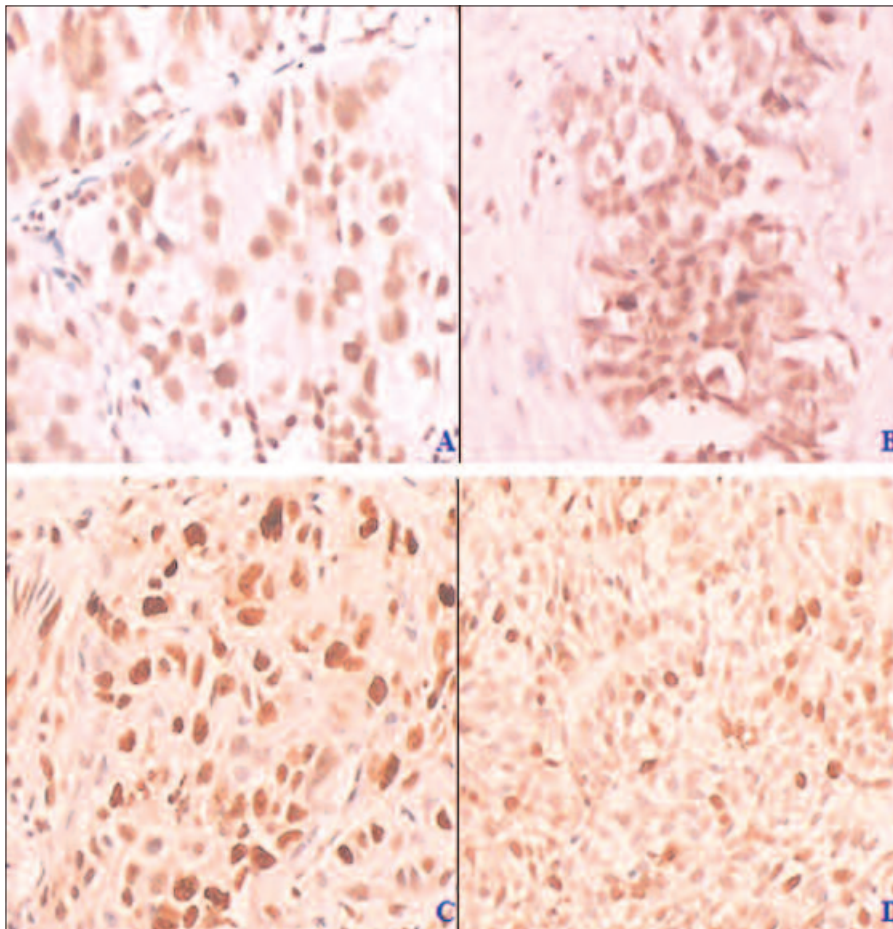**Figure 1. A,** HIF-1α positive in squamous cell carcinoma (SP × 200). **B,** HIF-1α expression in adenocarcinoma (SP × 200). **C,** Ki-67 expression in squamous cell carcinoma (SP × 200). **D,** Ki-67 expression in adenocarcinoma (SP × 200).

sample of NSCLC patients with HIF-1α negative cancer tissue was 6.58 ± 1.51% (Table III). And there was a positive correlation between the HIF-1α protein expression in cancer tissue and the proportion of CD4⁺CD25⁺ Treg cell in peripheral blood ($r = 0.711$, $p = 0.003$).

The Relationship Between CD4⁺CD25⁺ Treg Cell in Peripheral Blood and Ki-67 Expression in NSCLC Tissue

The proportion of CD4⁺CD25⁺ Treg cell in peripheral blood of NSCLC patients with Ki-67 positive cancer tissue was 11.83 ± 2.65%, while

Table II. Clinical meaning of CD4⁺ CD25⁺ Treg cell level ($\bar{x} \pm s$, %) in NSCLC patients.

Clinical meanings of cancer	n	CD4⁺ CD25⁺ Treg cells (%)	p
Pathological categories			
Squamous cell carcinoma	25	9.53 ± 3.90	0.476
Adenocarcinoma	21	9.09 ± 3.44	
Adenosquamous carcinoma	4	8.10 ± 3.09	
Pathological differentiation			0.006
Highly differentiated	27	7.34 ± 2.66*	
Poor differentiated	23	11.68 ± 2.65*	
TNM stage			
Stage I-II	12	6.95 ± 2.14*	0.001
Stage III-IV	38	12.02 ± 2.44	

Table III. Relationship between CD4+ CD25+ Treg cell in peripheral blood ($\bar{x} \pm s$, %) and HIF-1α in NSCLC tissue.

	HIF-1α (+) (n = 35)	HIF-1α (−) (n = 15)	r	ρ
CD4+ CD25+ Treg cell	12.38 ± 1.93 (%)	6.58 ± 1.51 (%)	0.711	0.003

that proportion of CD4+CD25+ Treg cell in blood sample of NSCLC patients with Ki-67 negative cancer tissue was 7.01 ± 2.53% (Table IV). And there was a positive correlation between Ki-67 protein expression in cancer tissue and the proportion of CD4+CD25+ Treg cell in peripheral blood ($r = 0.517$, $p = 0.04$).

Discussion

The incidence of lung cancer is on the increase in recent years. The surgery, radiotherapy, and chemotherapy cannot solve the problem of recurrence and metastasis[2]. Resistance to anticancer agents is one of the primary impediments to effective cancer therapy. Chemoresistance occurs not only to clinically established therapeutic agents but also to novel targeted therapeutics[3,4]. The recurrence and metastasis may result from the changes of cytogenetic nature of tumor cells and the effects of the immune cell activities and endogenous factors in tumor microenvironment. Studies showed that the immune escape of tumor cells from the immune surveillance and the immune suppression may be the main reason for proliferation, invasion, and metastasis of tumor cells.

It was found in immunological studies of recent years that the regulatory T lymphocytes (Treg cells) are closely related to body's immune functions. One reason for lower cellular immune function in cancer patients may be the increase of the proportion of CD4+CD25+ Treg cells because the changes of the proportion of these lymphocytes have an important role to play in cancer patients. So far, a number of studies have confirmed a significant increase of CD4+CD25+ Treg cells in pathological tissues of gastrointestinal cancer, NSCLC[5,6], breast cancer and nasopharyngeal

cancer[7], and other tumors. The study of Ju et al[8] has shown a positive correlation between the CD4+CD25+ Treg cells and the clinical stage of NSCLC. This study showed that the proportion of CD4+CD25+ Treg cells in peripheral blood was related to pathological grades and clinical stages of NSCLC, regardless of gender of patients and histological type of tumors. The partial reason for the malignant cell differentiation and clinical progress of pathological stage of lung cancer and the increase of the proportion of CD4+CD25+ Treg cells is the induction of chemokines invaded from the tumor microenvironment into the local cancer, and a variety of inhibition factors, such as TGF-β, COX-2, CD70, Galectin-1 and IDO, which can induce CD4+CD25− Treg cells into CD4+CD25+ Treg cells, and that the antigen-presenting tumor-associated antigen induced the proliferation of the CD4+CD25+ Treg cells.

The reason why anti-tumor immune mechanism in the tumor microenvironment cannot effectively kill the solid tumors includes the hypoxic, acidosis, and low nutrition. HIF-1α plays a central role in the adaptation of tumor cells to hypoxic environment because the HIF-1α expression is significantly increased especially in the area of apparent tumor necrosis and the edge of tumor infiltrating area. According to hypoxia experiments of A549 and BE2, the HIF-1α-CCR7-ERK1/2 can regulate the invasion and migration of cancer cells, and increase the metastatic ability of lung cancer cells[9]. Mainly, Ki-67 is widely expressed in proliferating tissues instead of in resting cells[10], and is a symbol of tumor cells with high proliferative activity and so considered to be an ideal target antigen for detection of tumor cell proliferation. Studies showed that the higher expression of Ki-67 indicated higher chance of proliferation, metastasis, and recurrence of cancer cells[11]. This study showed that

Table IV. Relationship between CD4+CD25+ Treg cell in peripheral blood ($\bar{x} \pm s$, %) and Ki-67 in NSCLC tissue.

	in Ki-67(+) tissue (n = 38)	in Ki-67(−) tissue (n = 12)	r	ρ
CD4+ CD25+ Treg cell	11.83 ± 2.65 (%)	7.01 ± 2.53 (%)	0.517	0.04

the protein expression of HIF-1α and Ki-67 in lung cancer was related to the level of CD4⁺CD25⁺ Treg cells in peripheral blood, and the increase of HIF-1α and Ki-67 protein expression was correlated to higher proportion of CD4⁺CD25⁺ Treg cells. These results may indicate that the insufficient oxygen and nutrient in microenvironment resulted in low immune responses to tumor cells, facilitating the proliferation and metastasis of tumor cells. Up to now, there have been few domestic and international researches on the relationship between immune cells and hypoxic microenvironment and the related mechanism remains unclear.

Conclusions

The level of CD4⁺CD25⁺ Treg cells in peripheral blood of NSCLC patients was related to the clinical and pathological features and could be used as a predictor of immune status and prognosis of the lung cancer patients. And the level of CD4⁺CD25⁺ Treg cells was positively correlated with the protein expression of HIF-1α and Ki-67, providing a hopeful reference for clinical application.

―――――――――――
Conflict of Interest
The Authors declare that there are no conflicts of interest.

References

1) Jusufovic E, Iljazovic E, Kosnik M, Keser D, Korosec P, Zukic E, Prnjavorac B, Sejdinovi R, Ajanovi E. Local D4⁺, CD8⁺ and CD56⁺ reactions to lung cancer in regard to pathohistological type and clinical stage. Medicinski Glasnik 2011; 8: 101-108.

2) Ma GF, Zhang RF, Ying KJ, Wang D. Effect evaluation of cisplatin-gemcitabine combination chemotherapy for advanced non-small cell lung cancer patients using microarray data. Eur Rev Med Pharmacol Sci 2015; 19: 578-585.

3) Zhang B, Liu ZM, Hao FG, Wang M. siRNA-directed clusterin silencing promotes cisplatin antitumor activity in human non-small cell lung cancer xenografts in immunodeficient mice. Eur Rev Med Pharmacol Sci 2014; 18: 1595-1601.

4) Li J, Shi R, Yu P. Effects of chemotherapy on global gene expression in non-small cell lung cancer. Eur Rev Med Pharmacol Sci 2014; 18: 126-131.

5) Ruffini E, Asioli S, Filosso PL, Lyberis P, Bruna MC, Macrì L, Daniele L, Oliaro A. Clinical significance of tumor-infiltrating lympocytes in lung neoplasms. Ann Thorac Surg 2009; 87: 365-372.

6) Lv Y, Shen Y, Huang J. Analysis of the peripheral blood lymphocyte subsets and their correlation with the clinical properties of lung cancer patients. J Clin Pulmon Med 2011; 9: 1420-1422.

7) Qin J, Teng J, Feng G. Changes of regulatory T lymphocyte level in peripheral blood of nasopharyngeal patients. Pract Oncol 2010; 4: 416-418.

8) Ju S, Qiu H, Zhou X, Zhu B, Lv X, Huang X, Li J, Zhang Y, Liu L, Ge Y, Johnson DE, Ju S, Shu Y. CD13+CD4+CD25hi regulatory T cells exhibit higher suppressive function and increase with tumor stage in non-small cell lung cancer patients. Cell Cycle 2009; 8: 2578-2585.

9) Zhang W, Zhang H, Xing L. Antisense oligonucleotide of hypoxia-inducible factor-1alpha suppresses growth and tumorigenicity of lung cancer cells A549. J Huazhong Univ Sci Technolog Med Sci 2006; 26: 448-450.

10) Kuratomi K, Yano H, Tsuneoka M, Sakamoto K, Kusukawa J, Kojiro M. Immunoohistochemical expression of mina53 and ki-67 proteins in human primary gingival squamous cell c-arcinoma. Kurume Med J 2006; 53: 71-78.

11) Gentile V, Viclni P, Glacomellii, Cardillo MR, Pierangeli A, Degener AM. Detection of human papillomavirus DNA, p53 and ki-67 expression penile carcinomas. Int J Immunopathol Pharmacol 2006; 19: 209-215.