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Case Report

CD4$^+$CD25$^+$Foxp3$^+$ T regulatory cells in the peripheral blood of non-small cell lung cancer patients

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Abstract: In recent years, much progross have made on the mechanism of tumor tumorigenesis and development. Research has showed the tumor cell expressed the inhibitory molecules and formed a suppression microenvironment to avoid the cytotoxicity from the effctor immune cells. In this circumstance, tumor cell was able propagate limitless. In this study, we investigated the frequency of inhibitory cell CD4$^+$CD25$^+$ Treg cell, the mRNA expression in plasma and the serum concentration of TGF-β in the 40 cases of non-small cell lung cancer patients and 40 health physical examination volunteers. As it expected, in the peripheral blood, the frequence of Treg cell, the FOXP3 mRNA in the cancer tissue cell and the serum TGF-β in the NSCLC patients is significant higher than the health voluteer. In addition, Foxp3 expression was interfered in human lung cancer cell line A549, which were co-cultured with CD4+ T cells, to investigate the effect of Foxp3 on the proliferation of lung cancer specific T cells. Compared with control group, A549 cells with reduced expression of Foxp3 significantly promoted the proliferation of CD4$^+$ T cells and resulted in a significantly low level of TGF-β (P<0.05). In conclusion, the CD4$^+$CD25$^+$Foxp3$^+$ Treg cells and TGF-β were significantly increased in NSCLC patients. Elevated expression of Foxp3 in lung cancer cells was involved in the inhibition of the proliferation of tumor-specific T cells.

Keywords: CD4$^+$CD25$^+$Foxp3$^+$, TGF-β, non-small cell lung cancer, Foxp3

Introduction

Lung cancer is one of the most common malignancies around the global. In recent years, the incidence and mortality of lung cancer have risen rapidly, and the mortality of the lung cancer is the highest one, regardless of the gender [1]. Epidemiology studies have showed that risk factors closely associated with lung cancer development mainly comprises environmental pollution, cigarette smoking and occupation [2-5]. However, the mechanism behind the pathogenesis of lung cancer remain further elucidated.

Regulatory T cells (Treg) play an important role in tumor escaping. The development and the function of immunosuppressive of Treg cells were mainly regulated by the fork-head box P3 (Foxp3) transcription factor [6]. A large body of evidence has shown that Foxp3 was closely associated with tumor formation and development. Currently, it has believed that T cells could mediate anti-tumor immune response and inhibit tumor through recognizing antigens, which presented on the surface of the antigen presenting cells. In that, tumor cell could express the inhibitory molecule and recruit the supressive cells, including the Treg cell, and develop the T cell exclusion microenvironment. In this surrounding, the function of the effector T cells was restrained [7].

In this study, we investigated the frequency of CD4$^+$CD25$^+$Foxp3$^+$ Treg cell and the transforming growth factor beta (TGF-β) in the NSCLC patients' peripheral blood. We also explored the inhibitory function of Foxp3 in the lung cancer cell line A549 on the tumor specific T cells. These results could enlighten on the NSCLC and provide experimental evidence for the treatment and prognosis of NSCLC.

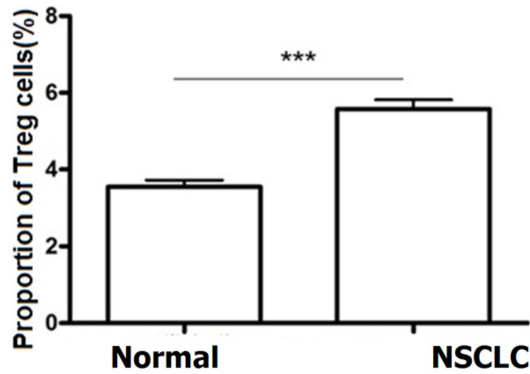

Figure 1. Percentage of Treg cells in the peripheral blood of NSCLC patients and normal subjects. Independent samples t test was used to the statistic. ***t=6.276, P<0.001.

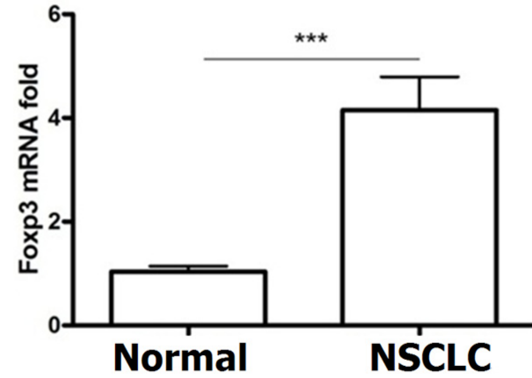

Figure 2. Levels of Foxp3 mRNA in the peripheral blood. Independent samples t test was used to the statistic. ***t=9.125, P<0.001.

Material and methods

Clinical data

From March 2012 to March 2014, lung cancer tissues were collected from 40 NSCLC patients undergoing surgeries at the Fist People's Hospital of Yunnan Province. Of these 40 patients, 23 were males and 17 were females, with a mean age of 62.11±8.41 years old with a range of 54-73. All the patients had not received any radiotherapy or chemotherapy prior to surgery. Forty people (19 males and 21 females, mean age 61.23±7.51 years old, range from 54 to 72 years old) undertaked health check-up during the same time regard as controls.

Reagents

RNA isolation was performed using Trizol RNA purification kit (Invitrogen, Shanghai, China). RNA was reverse transcribed using cDNA reverse transcription kit (Fermantes, USA). PCR kit was purchased from Sunshine Biotechnology (Nanjing Co., Ltd., Nanjing, China). Rat anti-human CD4-FITC, rat anti-human CD25-APC and rat anti-human FoxP3-PE antibodies were purchased from eBioscience, USA. Erythrocyte lysis solution was purchased from Boster Bio-engineering, Wuhan, China. Fix/Perm Cell Permeabilization Kit was purchased from Invitrogen, USA. GolgiStop protein transport inhibitor was purchased from BD-biosciences, USA. Incomplete RPMI 1640 medium used for lymphocyte culture, fetal bovine serum (FBS) and penicillin-streptomycin (P-S antibiotic mixture) were purchased from Boster Bio-engineering, Wuhan, China, and these solutions were mixed at a ratio of 89:10:1 to obtain a complete RPMI 1640 medium.

Methods

Peripheral blood samples for the following experiments were collected in the morning from lung cancer patients and healthy controls respectively.

Flow cytometry

Peripheral red blood cells (RBCs) were lysed and diluted in complete RPMI 1640 medium to a concentration of 2×10^6 cells/mL. After 800 g, 5 min centrifugation, cell pellets were resuspended in 100 μL PBS (PH=7.4), incubated with anti CD25-APC (0.2 mg/mL) and anti CD4-FITC (0.3 mg/ml) antibodies in the dark for 30 min at room temperature. Subsequently, samples were washed once with PBS and incubated with 1 mL Fix/Perm Cell Permeabilization solution at 4°C in the dark for 40 min. After centrifugation, cell pellets were resuspended in 100 μL permeabilization buffer and incubated with 2 μL FC-receptor block at 4°C in the dark for 20 min, followed by incubation with anti Foxp3-PE antibodies (0.15 mg/mL) at 4°C in the dark for 40 min. Finally, cells were washed once with PBS and resuspended in 500 μL PBS for analysis. Cells were acquired using FACS Calibur flow cytometer (BD Bioscience, USA). Data were analyzed by using CELLQUEST program.

Elisa

Serum concentration of TGF-ß was measured using ELISA kits (eBioscience, USA) following the manufacturer's instruction.

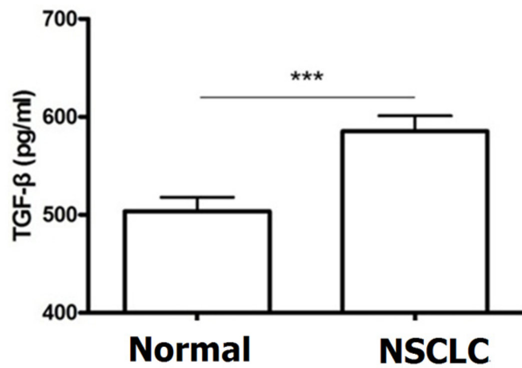

Figure 3. TGF-β levels in the peripheral blood of NSCLC patients and normal subjects. Independent samples t test was used to the statistic. ***t=9.125, P<0.001.

RT-PCR

RT-PCR was performed to determine levels of Foxp3 mRNA in the plasma. 5 mL peripheral venous blood sample was collected from each subject in the morning. The blood sample was treated with EDTA (Sunshine Biotechnology (Nanjing) Co, Ltd., Nanjing, China) and immediately (within two hours) centrifuged at 4,000 g for 5 min at 4°C. Subsequently, the upper plasma layer was collected in an EP tube and stored at -80°C for further analysis.

A total of 200 μL plasma was homogenized in 1 mL Trizol and total RNA was isolated using Trizol RNA purification kit following manufacturer's instructions. The isolated RNA was dissolved in 20 μL DEPC-treated water, and the quantity and the purity of RNA were determined using NanoDrop ND-1000 spectrophotomete (Thermo Scientific, USA). The resulting RNA was reverse transcribed into cDNA by using reverse transcription kit (Fermantes, USA) and stored at -20°C. RT-PCR reaction conditions are as follows: denaturation at 95°C for 20 s, followed by 40 cycles of 60°C for 20 s and 70°C for 1 s. The primers sequence of Foxp3 were as follows: F: 5'-CACGCATGTTTGCCTTCTTCAGA-3', R: 5'-GTAGGGTTGGAACACCTGCTGGG-3'. The primers sequence of GAPDH were listed as follows: F: 5'-ATCTGGCACCACACCTTC-3', R: 5'-AGCCAGGTCCAGACGCA-3'. RT-PCR was performed on ABI 7300 RT-PCR system (Applied Biosystems, USA). Relative quantitative analysis of data was performed using $2^{-\Delta\Delta Ct}$ method [8].

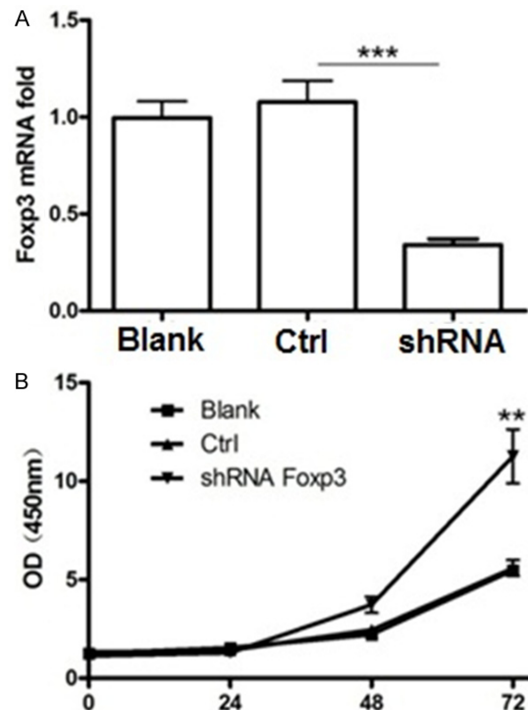

Figure 4. Influence of A549 cells interfered by shRNA-Foxp3 on T cell proliferation. qPCR result for Foxp3 mRNA level in A549 cells aftered transfected with siRNA-Foxp3, scramble RNA and control (A), and CCK8 result for the proliferation of $CD4^+$ T cells (B). One way ANOVA was used for the statistic.

Cell transfection

Foxp3 expression was interfered by introducing siRNA targeted at Foxp3 gene into human lung cancer cell line A549 using Lipofectamine 2000 (Invitrogen, USA). The sequence of Foxp3-specific shRNA was CACTATCACACATAGGTGT, and the sequence of scramble RNA was 5'-UCAUAAGUGAUGCUGGAGCTT-3'. Transfection efficiency was assessed by RT-PCR.

Cell counting kit-8 (CCK-8) assay of specific T cell proliferation

A549 lung cancer cells grown to ~50% confluence were transfected with siRNA-Foxp3 followed by co-culture with sorted $CD4^+$ T cells (95% purity, Miltenyi, catalogue no. 130-094-131). Dulbecco's Modified Eagle's Medium (DMEM) and CCK-8 kit at a ratio of 1:10 were mixed and added to each well of cultures at 0 h, 24 h, 48 h and 72 h after transfection, respectively. Optical density (OD) of samples was

measured at 450 nm using a spectrophotometer and a cell growth curve was generated.

Statistical analysis

All statistical were analyzed by using the SPSS17.0 software. Graphs were generated by Graphpad Prism5 software. Quantitative data were expressed as mean ± SD and differences between groups were analysed using independent samples t test or one way ANOVA. $P<0.05$ was considered statistically significant.

Results

Frequncy of the Treg cells in the peripheral blood of NSCLC

Flow cytometry study showed that frequency of Treg cells in the peripheral blood of control group was significantly lower than that in patients with NSCLC (3.71±0.41)% vs. (5.12±0.62)% ($P<0.001$). This result indicating that the aberrant levels of Treg cells in the peripheral blood of NSCLC patients might be associated with the development and progression of lung cancer (**Figure 1**).

Foxp3 mRNA levels augmented in plasma of NSCLC

RT-PCR analysis demonstrated that the Foxp3 mRNA level in the plasma of NSCLC patients was significantly higher than that of control group ($P<0.01$). This suggesting that the abnormal expression of Foxp3 in the plasma of NSCLC patients might promote the development and progression of NSCLC (**Figure 2**).

Increased TGF-ß level in the serum of NSCLC

Cytokines associated with the secretion function of Treg cells were investigated via ELISA. The results showed that TGF-ß level in the serum of NSCLC patients was significantly higher than that of control group (592.41±41.47) pg/mL vs. (504.47±38.41) pg/ml ($P<0.001$) (**Figure 3**).

Down-regulated Foxp3 in A549 cells promote proliferation of $CD4^+$ T cells

The function of Foxp3 in tumor cells was investigated by *in vitro* down regulated the Foxp3 in A549 cells by transfected the Foxp3 siRNA. And then co-culture of the A549 cells with purified $CD4^+$ T cells. The results revealed that compared with that of control cells, Foxp3 mRNA level was significantly decreased in A549 cells aftered transfected with siRNA-Foxp3 (**Figure 4A**), and the proliferation of $CD4^+$ T cells was significantly increased (**Figure 4B**), thereby indicating that Foxp3 expression in A549 tumor cell line could inhibit the tumor specific T cells.

Discussion

Currently, lung cancer is one of the most life-threatening malignancies disease. With the acceleration of industrialization and increasing smokers, the incidence rates of lung cancer have been rising rapidly in urban areas of China. Lung cancer has become the leading cause of cancer deaths in urban populations of China.

NSCLC is the most common lung cancer, accounting for 80% of all types of lung cancers, and ~75% patients were at mid-or late-stage at diagnosis. The morality of NSCLC is higher and prone to local infiltration and distant metastasis. The development and progression of NSCLC is a complex process involving multiple factors, genes, signal pathways and other steps [9]. Comprehensive therapeutic strategy include surgery, radiotherapy and chemotherapy. All of these are recommended for the treatment of lung cancer to prolong the survival of lung cancer patients. However, resulted in unfavorable outcomes. With the advancing of tumor immunology and molecular biology, increasing number of research have shown that the development, progression, metastasis and recurrence of malignant tumors are associated with immune dysfunction and the suppression of cellular immunity.

$CD4^+CD25^+Foxp3^+$ Treg cell is a class of and an important subtype of T lymphocytes. It's a group of regulatory T cells and is differ from Th1 and Th2 cells. The immunosuppressive function of Treg cells has gained widespread attention. Numerous studies have shown that Treg cells play critical roles in maintaining homeostasis, mediating tolerance to allo-grafts and surveilling of tumors [10-13]. Li Feng [14] had shown that the higher frequency of Treg cell in the peripheral blood of hepatocarcinoma patients with a lower survival rates. This indicated that Treg cells might play a significant role in promoting cancer progression. In addition, Ramos RN had reported that macrophages

in breast cancer patients could induce increase the percentage of Treg cell. However, few studies had reported the Treg cells in NSCLC and its effect on the effector T cells.

In the present study, the levels of Treg cells in the peripheral blood of NSCLC patients were investigated to explore the implication of Treg cells in the treatment of NSCLC patients. The results demonstrated that the frequency of Treg cell in the peripheral blood of NSCLC patients were significantly higher than health voluteers and levels of Foxp3 mRNA were significantly higher in the plasma of NSCLC patients. These results suggested that Treg cells were involved NSCLC supressive microenvironment. And it had a certain impact on the development and progression of NSCLC. As TGF-ß is a specific regulatory cytokine for the Tregs. Next, we analyzed the concentration of TGF-ß in the serum between in the two groups. Results showed that serum TGF-ß in NSCLC patients were significantly higher than the control group. Vazquez PF have reported that serum TGF-ß levels were elevated in both murine NSCLC and human NSCLC cell line, confirming the results of the present study [15].

Studies have suggested that T cells can mediate antitumor immune response and tumor killing function via recognizing the antigens presented on the surface of tumor cells. However, many studies have shown that tumor-specific T cells are often not able to be activated effectively [7]. In further step, Foxp3 expression was knockdown by siRNA in lung cancer cell line A549. Then we investigated the influence of Foxp3 on A549 cells on the proliferation of $CD4^+$ T cells. Results demonstrated that reduced Foxp3 expression in A549 cells could promote the proliferation of $CD4^+$ T cells. Thereby indicating that the proliferation of tumor specific $CD4^+$ T cells may be suppressed in NSCLC through high expression of Foxp3. Studies in recent years have shown that some tumor cells with high expression of Foxp3 exhibit similar immunosuppressive function as Treg cells, for example, human lymphoma cells [16] and pancreatic cancer cells [17] inhibited T cell proliferation, which was consistent with the results of the present study.

In summary, Treg cells play an important role in the suppressive microenvironment of NSCLC, and high expression of Foxp3 in lung cancer cells was involved in the regulate the tumor microenvironment by reducing the T cells. In conclusion, assessment the Treg cells in the peripheral blood is helpful for NSCLC patients in the treatment and prognostic evaluation.

Disclosure of conflict of interest

None.

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