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Original Article

B7-H6 expression in non-small cell lung cancers

Xiuqin Zhang[1,3], Guangbo Zhang[2], Yan Qin[3], Ruizhen Bai[3], Jianan Huang[1,2]

[1]*Department of Respiratory Medicine, The First Affiliated Hospital of Soochow University, 188 Shizi Street, Suzhou 215006, China;* [2]*Clinical Immunology Laboratory of Jiangsu Province, 708 Renmin Road, Suzhou 215006, China;* [3]*The First Affiliated Hospital of Jiangnan University, 200 Huihe street, Binhu District, Wuxi 214000, China*

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Abstract: B7 family has been known to be a negative regulator of immunity response in patients with lung cancer. B7-H6 as a novel identified member of B7 family is found to trigger natural killer (NK) cell cytotoxicity and cytokine secretion by binding natural cytotoxicity receptor NKp30. Up until now, no investigations have been made about B7-H6 expression in lung cancer. We present the result of the B7-H6 prognostic value in 65 non-small cell lung cancer (NSCLC) tissues and 65 matched adjacent non-tumor tissues by Immunohistochemistry (IHC). Meanwhile, fluorescence activated cell sorter (FACS) analysis was used to detect B7-H6 receptor NKp30 expression in 7 non-small cell lung cancer tissues and 7 adjacent non-tumor tissues. Here, the result showed B7-H6 immunoreactivity in 6/65 (9.23%) lung cancer patients and 4/65 (6.15%) in adjacent non-tumor tissues. No relationship was found between B7-H6 expression and clinic pathological features. Similarly, no relevance was found for NKp30 expression in lung cancer tissues and non-tumor tissues. However, B7-H6 positive carcinomas were significantly correlated with degree of differentiation ($P = 0.044$). Three year survival rate after operation did not show the prognostic value for B7-H6 expression. Our study suggests that B7-H6 has a limited value as a prognostic marker in the patients of lung cancer.

Keywords: B7-H6, NKp30, lung cancer, immunohistochemistry

Introduction

Worldwide, lung cancer is a leading cause of cancer death in men, and it is the second cause of cancer death for women. Roughly 85% patients are non-small cell lung cancer, over half of lung cancer is diagnosed at an advanced stage, 16% are diagnosed at the early stage. In spite of many available adjuvant therapies are available, however, the overall five-year survival rate remain low for lung cancer patients, a major reason is diagnosis of lung cancer at advanced stage [1, 2]. Therefore, we would make great efforts to explore novel markers and contribute to early diagnosis of lung cancer.

The B7 family members control T cell mediated immune response by binding their CD28 receptors on activated T cells, the inhibitory members of B7 family can yield regulatory signals to terminate or weaken functions of activated T cells in tumor environment [3-5]. At present, several B7 family numbers have been found in lung cancer. B7-H6 known as NGR3LG1 is a newly identified member in the B7 family. B7-H6 mRNA and protein expression are not detected in normal tissues, and expressed mainly on the cell surface of various tumor cells such as hematological malignancies, it seems that its expression maybe associate with tumor prognosis in a large number of tumor patients [6, 7]. B7-H6 triggers antitumor of natural killer cell cytoxicity and cytokine secretion by binding NKp30 receptor which is a natural cytotoxicity receptor expressed mainly on the surface of natural killer cells [6-8]. However, there are no data about the clinical significance of B7-H6 expression in patients of lung cancer.

In this article, we investigated B7-H6 expression in lung cancer tissues and adjacent non-tumor tissues by immunohistochemistry (IHC), then explored the relationship between B7-H6 expression and clinic pathological features to

Table 1. The percent of B7-H6 expression in lung cancer tissues and non-tumor tissues

B7-H6 expression	Tumor (N ratios [%])	Non-tumor (N ratios [%])
Positive (n = 10)	6/9.23	4/6.15
Negative (n = 120)	59/90.77	61/91.85

Table 2. Relationship between B7-H6 expression and clinicopathological parameters in lung carcinoma patients

Characteristic parameters	cases	B7-H6 positive cases	B7-H6 negative cases	*P* value
Age (years)				
< 60	29	2	27	0.879
≥ 60	36	4	32	
Gender				
Male	31	3	28	1.000
Female	34	3	31	
Histology				
Adenocarcinoma	41	2	39	0.254
Squamous cell carcinoma	24	4	20	
Differentiation				
Moderate/High	16	4	12	0.044*
Low	49	2	47	
Tumor size (cm)				
< 5	45	3	42	0.544
≥ 5	20	3	17	
Nodal (N) metastasis				
N0	30	1	29	0.462
N1	13	2	11	
N2	21	3	18	
N3	1	0	1	
TNM stage				
I	17	0	17	0.263
II	12	2	10	
III	36	4	32	
Survival time after surgery				
< 3 year	20	3	17	0.544
≥ 3 year	45	3	42	

*$P < 0.05$.

investigate whether B7-H6 acts as a novel identified prognostic marker in lung cancer patients.

Materials and methods

Patients and tissue specimens

Formalin-fixed, paraffin-embedded tumor tissues and adjacent non-tumor tissues were selected retrospectively from the First Affiliated Hospital of Jiangnan University (the Fourth People's Hospital, Wu Xi, China). All of 130 cases including 65 tumor tissues and 65 adjacent non-tumor tissues underwent surgical resection between January 2008 and December 2009. None of the patients had received radiotherapy and chemotherapy before operation. According to the American Joint Committee on cancer staging system (AJCC) (7th, edition), these patients were classified as 17 cases (stage I); 12 cases (stage II); 36 cases (stage III), and include 31 males and 34 females. These patients were followed-up for 3 years. The median age at diagnosis was 59.94 (range: 28-78) years. The protocol in this study was approved by the ethics committees of the First Affiliated Hospital of Jiangnan University.

Immunohistochemistry

Paraffin-embedded tissues were cut into 4-μm-thick serial sections, IHC was performed by using Envision method, these slides were transferred onto adhesive slides, dried at 65°C for 30 minutes, then dewaxed in xylene and rehydrated through graded ethanol. Antigen retrieval was done at 100°C for 30 minutes in a citrate buffer (10 nmol/L; pH 6.0), then endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide solution for 30 minutes. After washing three times with phosphate buffer saline (PBS) for 5 minutes each, sections were incubated with rabbit anti-human B7-H6 polyclonal antibody (1:50 dilutions, Abcam) at 4°C overnight, a negative control was carried out by replacing the primary antibody with PBS, then sections were incubat-

ed with horseradish peroxide-labeled goat anti-Rabbit second antibody (1:1000 dilutions, Merck & Millipore).

Evaluation of B7-H6 staining

The sections were examined and evaluated independently by two pathologists. B7-H6 protein was quantified using a visual grading according to the extent of staining: 0 (< 5%); 1 (6%-25%); 2 (26%-50%); 3 (51%-75%); 4 (> 75%), and intensity of staining: 0 (no staining); 1 (weak staining, light yellow); 2 (moderate staining, yellowish brown); 3 (strong staining, brown). The sum of score was determined as followed: 0 (negative); 1-4 (weakly positive); 5-8 (moderately positive); 9-12 (strongly positive) [9].

Flow cytometry for NKp30

The specimens dissected from 7 matched patients of lung cancer and adjacent non tumor tissues were cut into 2 mm fragments, these fragments were washed in PBS, and transferred to a conical tube containing 0.05% collagenase at 37°C for 20 minutes, then these specimens were passed with a mesh to provide a single cell suspension. The cell suspension from lung cancer and adjacent non tumor was washed with PBS for 2 times, stained by phycoerythrin-cyanine 5 (PC5) conjugated anti-CD45, fluorescein isothiocyanate (FITC) conjugated anti-CD56 and phycoerythrin (PE) conjugated-NKp30 antibody (Biolegend) in 4°C incubation for 30 minutes. The staining with anti-CD45 PC5 and anti-CD56 FITC was marked as natural kill cells in lung carcinomas and non-tumor tissues; PE-conjugated-NKp30 antibody was used to detect B7-H6 receptor NKP30 expression on the surface of CD45+ and CD56+ cells, then cells were washed by PBS and detected immediately by flow cytometry on fluorescence activated cell sorter (FACS Calibur cytometer, Becton Dickinson, Heidelberg, Germany).

Statistical analysis

Statistical analysis was performed with SPSS 17.0 software. The association between B7-H6 expression and clinic pathologic features was analyzed by using chi-square test or Fisher's exact test. NKp30 expression was analyzed with rank sum test, *P*-values less than 0.05 were considered as being statistically significant.

Results

B7-H6 expression in tumor tissues and non-tumor tissues

In this study, B7-H6 expression was found in the cytoplasm of cells. B7-H6 protein expression was detected in 6/65 (9.23%) lung carcinomas and 4/65 (6.15%) non tumor tissues (**Table 1**). Lung cancer samples were all moderate staining (6/6), strong staining was not detected in all lung cancer specimens. However, adjacent non-tumor tissues were all weak staining (4/4) in cytoplasm of lung tissues, moderate and strong staining were not found in all lung tissues (**Figure 1**).

B7-H6 expression and clinic pathological characteristics and prognosis

B7-H6 expression presented no significant differences between lung cancer tissues and non-tumor tissues. No matter lung carcinomas or adjacent non-tumor tissues, B7-H6 expression difference was not found with regard to patients' age, sex distribution, histological classification, tumor size, distant metastasis. However, B7-H6 immunoreactivity was associated with degree of differentiation ($P = 0.044$) (**Table 2**). Three year survival rate of B7-H6 positive and B7-H6 negative cases provided no significant difference in lung carcinomas and non-tumor tissues (the data of non tumor tissues was not shown).

NKp30 expression in surgical resected specimens

14 surgical resected samples from tumor and matched non-tumor tissues were detected by FACS. The CD45+ and CD56+ cells were regarded as natural kill cells between lung carcinomas and adjacent non-tumor tissues (**Figure 2A**). The B7-H6 receptor NKp30 expression on surface of natural kill cells was detected with PE-conjugated-NKp30 antibody. The median percent of NKp30 expression in lung cancer tissues is 34.24%, while 28.78% in adjacent non-tumor tissues by FACS, no significant difference was found about NKp30 expression between tumor and non-tumor lung tissues (**Figure 2B**).

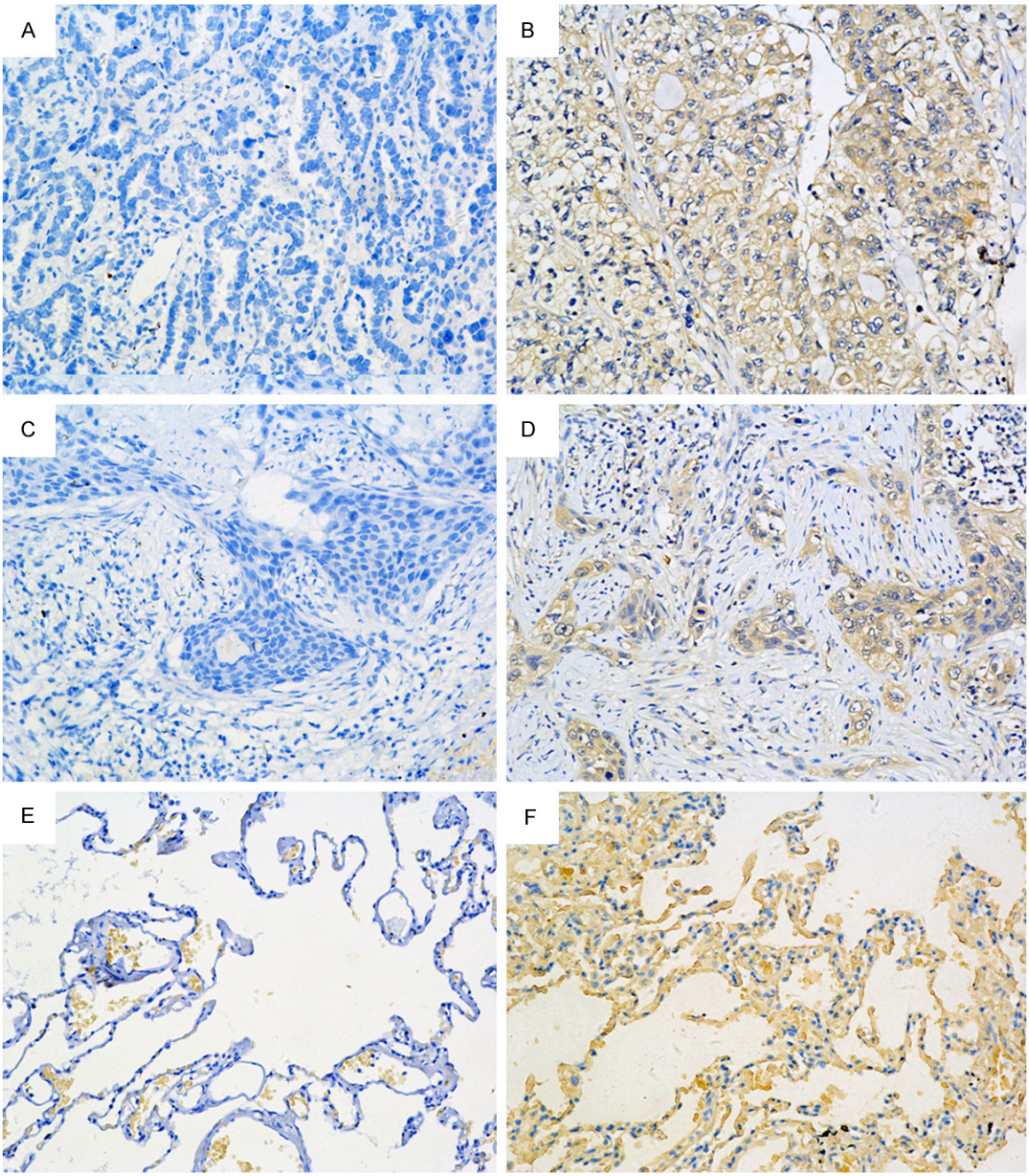

Figure 1. Representative immunohistochemical staining of B7-H6 in non-small lung cancer tissues and adjacent non tumor tissue. B7-H6 negative expression cases (A, C, E): B7-H6 positive expression cases (B, D, F). Adenocarcinoma (A and B); Squamous cell carcinoma (C and D); non-tumor tissue (E and F). Original magnification ×200.

Discussion

The B7 family has been studied in human malignancies. The inhibitory B7 family members interact with their receptors CD28 family to inhibit cytokine secretion, cytotoxicity development and activation of T cells [10, 11]. Many inhibitory B7 family members are expressed in lung cancer and associated with adverse prognosis. The inhibitory B7 family include B7-H1 (PD-L1, CD274), B7-DC (PD-L2, CD273), B7-H2 (ICOSL, CD275), B7-H3 (CD276, B7RP-2), B7-H4 (VTCN1, B7X, B7S1), and newly identified B7-H6 [12]. B7-H1 expression was found in 53.2% of lung cancer tissues, the patients with B7-H1 high expression presented disadvantageous

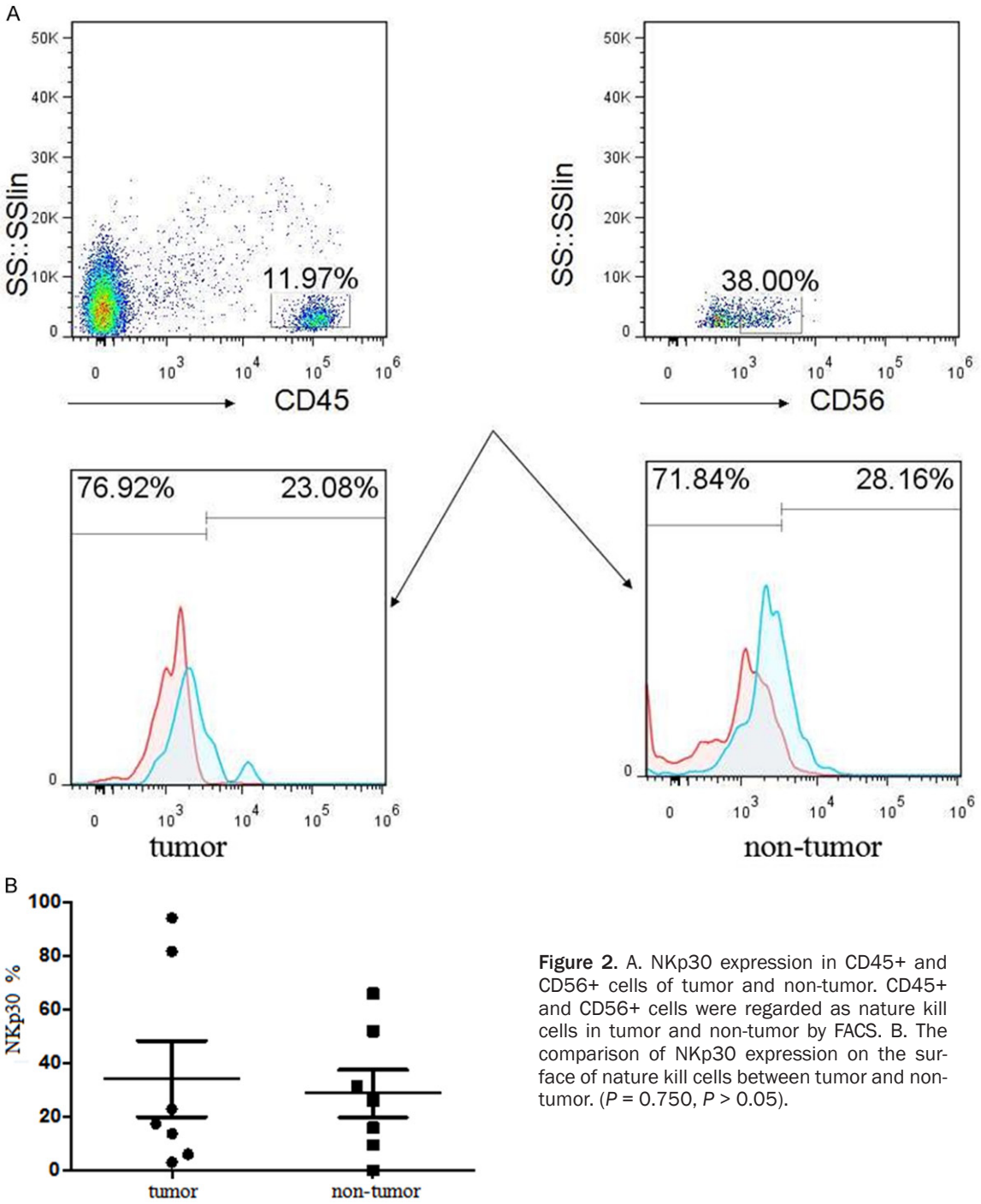

Figure 2. A. NKp30 expression in CD45+ and CD56+ cells of tumor and non-tumor. CD45+ and CD56+ cells were regarded as nature kill cells in tumor and non-tumor by FACS. B. The comparison of NKp30 expression on the surface of nature kill cells between tumor and non-tumor. ($P = 0.750$, $P > 0.05$).

clinical outcome with low infiltration of tumor infiltrating lymphocyte (TIL) [13, 14]. However, the clinical significance of B7-H2 is unclear in lung cancer. B7-H3 and B7-H4 inhibitory molecules are found on the surface and in the cytoplasm of lung cancer, and have significant relevance with lymph node metastasis [15]. The soluble B7-H3 level was significantly associated with tumor size, TNM stage, lymph node metastasis and distant metastasis in peripheral blood of lung cancer patients [16]. B7-H3 expression was an independent prognostic risk factor in NSCLC patients [17]. Similarly, the B7-H4 positive tumor associated macrophages (TAM) were correlated with tumor size, lymph node metastasis and TNM stage of lung cancer [18]. B7-H4 was an independent prognostic indicator, and patients with high level of B7-H4

expression had short median overall survival time and adverse distant metastasis [9]. However, there are no data about clinical significance of B7-H6 expression in lung cancer.

In this study, we provide the first investigation about the relationship of prognostic and clinical value of B7-H6 protein in lung carcinomas and adjacent non tumor tissues. All of lung carcinomas and adjacent non-tumor tissues were stained for B7-H6. The B7-H6 expression had no obvious difference between tumor tissues and non-tumor tissues. Meanwhile, the B7-H6 expression did not reveal significant relevance with clinical pathological features, such as age, sex, tumor size, histological classification, lymphocyte node metastasis, TNM stage and distant metastasis in lung cancer patients, but B7-H6 positive expression significantly correlated with degree of differentiation ($P = 0.044$). This result is similar with study in gastric carcinoma [19]. Three year survival rate after operation presented no significant difference of B7-H6 expression in lung carcinomas. Similarly, the B7-H6 receptor NKp30 expression did not reveal any significant difference between lung carcinomas and adjacent non tumor tissues. Above, it seems that B7-H6 and its receptor NKp30 have no essential clinical meanings in lung carcinomas patients.

Altogether, our investigations suggest that B7-H6 has a limited clinical value as prognostic marker for lung cancer. Although the number of lung cancer patients enrolled is small, the results of B7-H6 expression might be different in many other tumors. So, the B7-H6 expression of other tumors would need to be investigated in a great deal of patients.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jianan Huang, Department of Respiratory Medicine, The First Affiliated Hospital of Soochow University, 188 Shizi Street, Suzhou 215006, China. Tel: 8613506218900; E-mail: huang_jian_an@163.com

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