

Original Article

The expressions and significance of B7-H3 and CTLA-4 in the clinical stages of non-small-cell lung cancer

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Abstract: The aim of this study was to analyze the expressions and significance of B7-H3 and CTLA-4 in the clinical stages of non-small-cell lung cancer (NSCLC). Seventy patients with NSCLC who underwent surgical resection or biopsy between January 2016 and February 2018 were enrolled. Among them, 30 were cases of paracancerous tissues and were assigned to the control group (CON). These cases were analyzed using immunochemical methods. Of the 70 cases, 48 were of adenocarcinoma, 19 were of squamous cell carcinoma, and 3 were of adenosquamous carcinoma. The expression rates of B7-H3 and CTLA-4 in the observation group (OBS) were 64.2% and 57.1% respectively, and those in the CON group were 6.7% and 0%, respectively ($\chi^2=27.988, 28.571, P<0.001$). The expression levels of B7-H3 and CTLA-4 in patients with poor differentiation, in stages III-IV, or with lymph node metastasis were significantly higher than those in patients with good-to-moderate differentiation, in stages I-II, and without lymph node metastasis ($P<0.05$). There was a positive correlation between the expressions of B7-H3 and CTLA-4 in the OBS group ($r=0.74, P<0.05$). B7-H3 and CTLA-4 are highly expressed and positively correlated with each other in NSCLC patients and are also closely related to clinical stages.

Keywords: NSCLC, B7-H3, CTLA-4, histopathology, clinical stages

Introduction

Lung cancer shows the highest morbidity and mortality among all kinds of tumors. Non-small-cell lung cancer is the most common type of lung cancer, accounting for 85% of the total number of lung cancers, and the 5-year survival rate is no more than 15% [1]. Advanced lung cancer treatment has four phases, namely surgery, radiotherapy/chemotherapy, targeted therapy, and immunization. Due to the limitations of targeted therapy and long-term drug resistance, the development of specific targeted tumor immunotherapy that has gradually evolved from first generation non-specific immunity and other such breakthroughs in immunotherapy [2] has brought new hope to cancer patients.

Tumor immunity mainly refers to cellular immunity, in which activated T cells play an important role in the surveillance and clearance of tumor cells. Many immune checkpoint proteins are

expressed abnormally in tumors, including lung cancer, breast cancer, gastric cancer, and esophageal cancer [3]. T lymphocytes that infiltrate the tumor tissues overexpress the negative regulatory molecule PD-1, which can inhibit the cellular immune response of the body and allow the tumor to escape the surveillance and clearance by the immune system. Antibodies such as anti-PD-1/PD-L1 (programmed cell death 1 and ligands) have been effectively applied to the treatment of various tumors, including lung cancer [4-7]. However, the micro-environment of tumor immunity is very complex, and a variety of co-stimulatory molecules are involved in the microenvironmental changes of tumors. Unlike PD-1, which is widely expressed in peripheral tissues and tumor tissues, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) inhibits the T cells during the early stages of the immune cycle in lymph nodes. CTLA-4 is mainly expressed in activated CD4+ and CD8+ T cells and affects the activation of T cells by competitively binding to

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Table 1. Clinical characteristics of the patients

Clinical pathological features	Groups	Number of cases (%)
Gender	Male	40 (57.1%)
	Female	30 (42.9%)
Age	≤64 years	36 (51.4%)
	>64 years	34 (48.6%)
Smoking status	Non-smoker	36 (51.4%)
	smoker	34 (48.6%)
The maximum diameter of the tumor (cm)	≤3.5 cm	37 (52.9%)
	>3.5 cm	33 (47.1%)
T stage	1-2 stage	53 (75.7%)
	3-4 stage	17 (24.3%)
Clinical stage	I-II stage	27 (38.6%)
	III-IV stage	43 (61.4%)
Lymph node metastasis	N0	29 (41.4%)
	N1+N2+N3	41 (58.6%)
Differentiation	Medium-high differentiation	42 (60%)
	Low differentiation	28 (40%)
Distant metastasis	M0	48 (68.6%)
	M1	22 (31.4%)

CD80/86 on the surface of antigen-presenting cells (APC) [8]. The other pathway by which the proliferative capacity of the regulatory T cells (regular cells) can be enhanced is by negative regulation; CTLA-4 and T-reg can activate each other and co-inhibit the activation signals of T cells [9, 10].

The B7 family molecules, including B7-1 (CD80), B7-2 (CD86), and B7-3, are important regulators of tumor immune response. Studies have shown that CD28 is the receptor for B7-1, and CTLA-4 is the receptor for B7-2 and B7-3, but the B7-H3 molecule can inhibit the T cell immune response by binding to the corresponding receptor on the surface of the target cells, thus performing a negative regulatory function. It is abnormally expressed in cancer tissues and either not expressed or under-expressed in normal tissues [11, 12].

The correlation between the expression of PD-1 and CTLA-4 in lung cancer, as well as the correlation between the expression of PD-1 and B7-H3 have been reported. However, studies regarding the correlation between the expression of CTLA-4 and B7-H3 and the relationship between these two checkpoint proteins and the progression and metastasis of tumors are rare. In this study, CTLA-4 and B7-H3 were selected as research indicators in order to explore their

relationship with the clinical stages, metastasis, and pathological features of the tumors. Our aim was to provide a preliminary theory for drug selection against NSCLC.

Materials and methods

General information

A total of 70 paraffin-embedded tissue specimens of NSCLC obtained between January 2016 and August 2018 were collected from the School of Petroleum Clinical Medicine, Hebei Medical University. These specimens included 48 cases of adenocarcinoma, 19 cases of squamous cell carcinoma and 3 cases of adenocarcinoma. 30 samples of paraneoplastic tissues were used as the control group (CON). The patients included 40 males (57.1%) and 30 females (42.9%), with ages ranging between 34 and 87 (63.99±9.92) years, and the median age was 64 years. The diameters of the tumors were between 1 and 11 cm, with a median of 3.5 cm. There were 41 cases of lymph node metastasis and 29 cases of non-lymph node metastasis. TNM staging: 53 cases in T1-2, 17 cases in T3-4, and 19, 8, 21, and 22 cases in clinical stages I-IV, respectively (**Table 1**). This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from

the Ethics Committee of Hebei Medical University. Written informed consent was obtained from all participants.

Inclusion criteria: The paraffin tissue specimens of NSCLC obtained via radical resection or biopsy between January 2016 and August 2018 were used; none of the patients enrolled received treatment-related interventions before surgery or needle biopsy. The complete medical data for all patients were available.

Exclusion criteria: Patients who had undergone preoperative chemotherapy, radiation therapy, biological therapy, targeting therapy, or other anti-tumor treatments; patients with other primary tumors, tuberculosis or connective tissue diseases.

Immunohistochemistry

All the specimens were fixed using 10% formalin, embedded in paraffin, serially sliced into slices with 5- μ m in thickness, placed in a 60°C oven (Qingdao Haier) for 2 hours, and subsequently underwent dewaxing, hydration, antigen retrieval, and enzyme incubation. Then, primary antibodies (rabbit anti-human B7-H3 polyclonal antibody and mouse anti-human CTLA-4 monoclonal antibody, ORIGENE, USA) were added, followed by color development, re-staining, dehydration, transparency, and sealing.

Result determination: The staining results were observed and judged by two qualified pathologists under a lighted microscope (DSY2000, US UOP).

The control group was treated with PBS instead of primary antibodies as the negative control.

Determination of results. Immunohistochemical semi-quantitative analysis: each sample was randomly selected and five 400x fields of view were used for scoring the staining intensity and percentage of positive cells in each field: 1. standards of positive staining intensity: 0 points - no staining, 1 point - light yellow, 2 points - brown yellow, 3 points - tan. 2. percentage of positive cells, namely the average number of positive cells in the 5 fields of the tumor cells/tumor infiltrating lymphocytes: 0 to 5% was recorded as 0 points, 6% to 25% was recorded as 1 point, 26% to 50% was recorded as 2 points, 51% to 75% was recorded as 3 points, and >75% was recorded as 4 points. 3. The

product of the above two items was defined as the positive grade: 0: negative (-), 1 to 4 points: weakly positive (+), 5 to 8 points: moderately positive (++) , and 9 to 12 points: strongly positive (+++); <1 point was defined as negative, and ≥ 1 point was defined as positive.

The surgery results were based on *TNM staging of Lung Cancer*, 8th edition, reported by the International Union Against Cancer (UICC).

Statistical analysis

SPSS 21.00 was used for the data analysis and processing. The count data were compared using the χ^2 test or Fisher's exact probability method; the correlation between B7-H3 and CTLA-4 was analyzed using the Spearman rank correlation, and logistic regression was used for the comparison of multi-variates, with $P < 0.05$ being considered statistically significant.

Results

Expressions of B7-H3 and CTLA-4

Immunohistochemistry showed that B7-H3 was mainly expressed in the cytoplasm and cell membranes of the tumor cells, and its positive expression rate in tumor cells was 64.2%, which is significantly higher than the rate in the paraneoplastic tissues (6.67%) ($\chi^2=27.988$, $P < 0.05$). Among these, the cases with weak, positive, and strong positive expression were 17, 14, and 14 cases, respectively (**Figure 1** and **Table 2**).

CTLA-4 was mainly expressed in the cytoplasm and cell membranes of the tumor infiltrating lymphocytes. The positive expression rate in the tumor cells was 57.1%, and the rate in the paraneoplastic tissues was 0. This difference was statistically significant ($\chi^2=28.571$, $P < 0.05$) (**Figure 2** and **Table 2**).

The relationship between the expression levels of B7-H3 and CTLA-4 and clinicopathological features (Table 3)

The relationship between the expression levels of B7-H3 and CTLA-4 and gender: The positive expression rates of B7-H3 in the male and female patients were 67.5% and 60.0%, respectively, but the difference was not statistically significant ($\chi^2=0.420$, $P=0.616$). The positive expression rates of CTLA-4 in the male and female patients were 60.0% and 53.3%,

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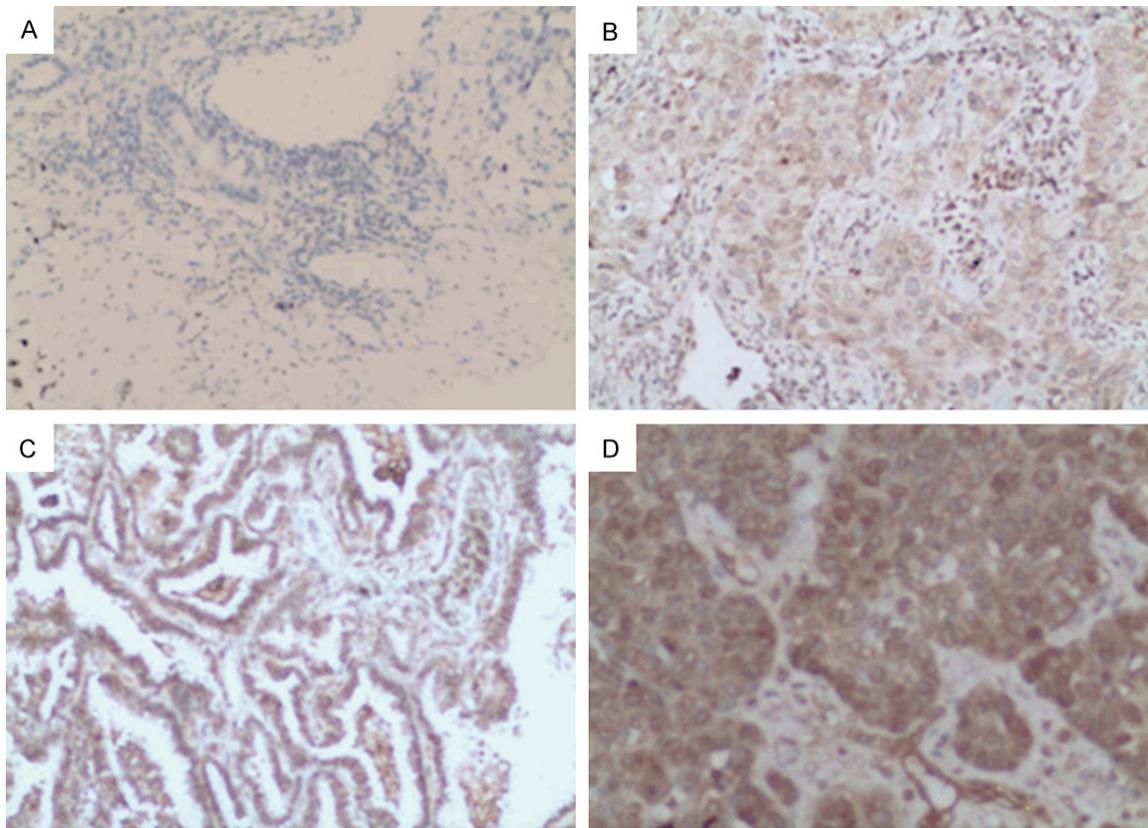


Figure 1. The expression of the B7-H3 protein in NSCLC. A: Negative control (40×); B: Weak positive expression (40×). C: Positive expression (40×); D: Strongly positive expression (40×).

Table 2. Positive expression rates of B7-H3 and CTLA-42 in different tissue samples

	B7-H3		CTLA-4	
	NSCLC	Paraneoplastic tissue	NSCLC	Paraneoplastic tissue
N	70	30	70	30
-	25	28	30	30
+	17	2	19	0
++	14	0	11	0
+++	14	0	10	0
Positive rate (%)	64.2	6.67	57.1	0
X ²	27.988		28.571	
P	<0.001		<0.001	

respectively, but the difference was not statistically significant ($\chi^2=0.311$, $P=0.631$).

The relationship between B7-H3/CTLA-4 and age

The positive expression rate of B7-H3 in the ≤ 64 years age group was 69.4%, and the rate

in the >64 years age group was 58.8% ($\chi^2=0.859$, $P=0.456$). The positive expression rate of CTLA-4 was 58.3% in the ≤ 64 years age group and 55.9% in the >64 years age group. This difference was not statistically significant ($\chi^2=0.043$, $P=1.000$).

The relationship between B7-H3/CTLA-4 and the maximum tumor diameter

The maximum tumor diameters were between 1 and 11 cm, with a median of 3.5 cm. The positive expression rate of B7-H3 in the patients with a maximal tumor diameter ≤ 3.5 cm was 56.8%, which was lower than the rate in the patients with a maximal tumor diameter >3.5 cm (72.7%), but the difference was not statistically significant ($\chi^2=1.938$, $P=0.214$). The positive expression rate of CTLA-4 in the patients with a maximal diameter ≤ 3.5 cm was 54.1%, which was lower

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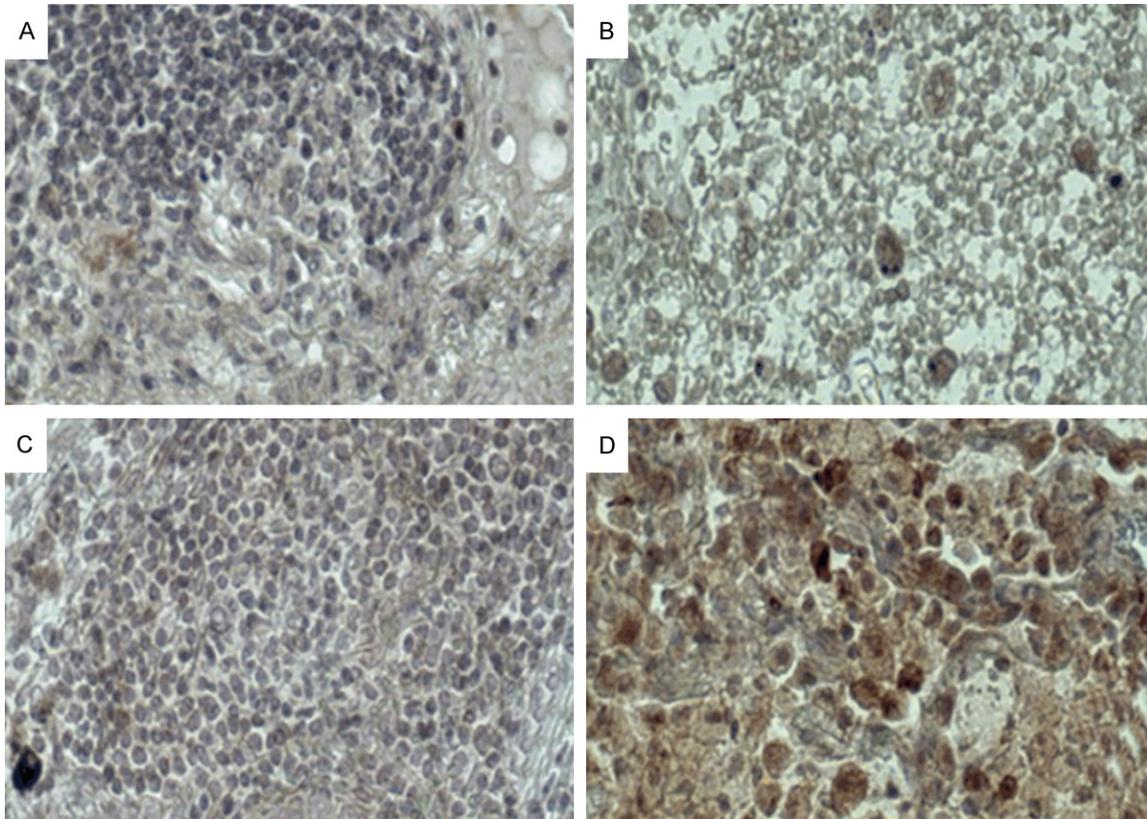


Figure 2. The expression of the CTLA-4 protein in NSCLC. A: Negative control (40×); B: Weak positive expression (40×). C: Positive expression (40×); D: Strongly positive expression (40×).

than the rate in the patients with a maximal tumor diameter >3.5 cm (60.6%), but the difference was not statistically significant ($\chi^2=0.306$, $P=0.634$).

The relationship between B7-H3/CTLA-4 and smoking

The positive expression rate of B7-H3 in the smoking group was 77.8% (28/36), and the rate in the non-smoking group was 50.0%. This difference was statistically significant ($\chi^2=5.877$, $P=0.024$). The positive expression rate of CTLA-4 in the smoking group was 66.7%, and the rate in the non-smoking group was 47.1%, but the difference was not statistically significant ($\chi^2=2.745$, $P=0.147$).

The relationship between B7-H3/CTLA-4 and T staging

The positive expression rate of B7-H3 in stages T1-2 of NSCLC was 55.6%, and the rate in stages T3-4 was 88.2%. This difference was statistically significant ($\chi^2=5.609$, $P=0.021$). The po-

sitive expression rate of CTLA-4 in stages T1-2 of NSCLC was 52.8%, and the rate in stages T3-4 was 70.6%, but the difference was not statistically significant ($\chi^2=1.657$, $P=0.264$).

The relationship between B7-H3/CTLA-4 and clinical stage

The positive expression rates of B7-H3 and CTLA-4 in the patients in stages I-II were 33.3% and 22.2%, respectively and the rate in the patients in stages III-IV were 83.7% and 79.1%, respectively. This difference was statistically significant ($\chi^2=18.341$, 21.886 , $P<0.001$).

The relationship between B7-H3/CTLA-4 and lymph node metastasis

The positive expression rates of B7-H3 and CTLA-4 in the cancer tissues was 37.9%, 27.6% in the non-lymph node metastasis group, and 82.9% and 78.0% in the lymph node metastasis group. The differences were statistically significant ($\chi^2=14.979$, 17.662 , $P<0.001$).

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Table 3. The relationship between the expression levels of B7-H3 and CTLA-4 and the clinicopathological features of NSCLC

	B7-H3				CTLA-4			
	Negative	Positive	X ²	P	Negative	Positive	X ²	P
Gender								
Male	13	27	0.420	0.616	16	24	0.311	0.631
Female	12	18			14	16		
Age								
≤64 year	11	25	0.859	0.456	15	21	0.043	1.000
>64 years	14	20			15	19		
Diameter								
≤3.5 cm	16	21	1.938	0.214	17	20	0.306	0.634
>3.5 cm	9	24			13	20		
Smoking								
Non-smoke	17	17	5.877	0.024	18	16	2.745	0.147
Smoker	8	28			12	24		
T stage								
1-2 stage	23	30	5.609	0.021	25	28	1.657	0.264
3-4 stage	2	15			5	12		
Clinical stage								
I-II stage	18	9	18.341	0.000	21	6	21.886	0.000
III-IV stage	7	36			9	34		
Lymph node metastasis								
N0	18	11	14.979	0.000	21	8	17.662	0.000
N1+N2+N3	7	34			9	21		
Differentiation								
Medium-high differentiation	23	19	16.593	0.000	23	19	6.076	0.016
Low differentiation	2	26			7	21		
Distant metastasis								
M0	21	27	4.295	0.059	4	18	7.977	0.059
M1	4	18			26	22		

The relationship between B7-H3/CTLA-4 and the differentiation degree

The positive expression rates of B7-H3 in the high- and medium-differentiation groups were 45.2% and 45.2%, respectively, which were significantly lower than the rate in the poor-differentiated group (92.9%, 75.0%). The difference was significant ($\chi^2=16.593$, 6.076, $P<0.05$).

The relationship between B7-H3/CTLA-4 and distant metastasis

There was no significant difference between the positive expression of B7-H3 in the NSCLC cases with or without distant metastasis ($\chi^2=4.295$, $P=0.059$). The positive expression rates of CTLA-4 in the distant metastasis group and the non-distant metastasis group were 81.8%

(18/22) and 45.8% (22/48), respectively. This difference was statistically significant ($\chi^2=7.977$, $P=0.008$).

The relationship between B7-H3 and CTLA-4

A spearman rank correlation analysis showed that the expression level of B7-H3 in the NSCLC tissues was positively correlated with the expression level of CTLA-4 ($r=0.74$, $P<0.001$) (Table 4).

Logistic multivariate regression analysis

By setting gender, age, smoking history, maximal tumor diameter, T stage, clinical stage, lymph node metastasis, and distant metastasis as the covariates and the B7-H3 and CTLA-4 expression levels as the dependent variables,

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Table 4. The correlations of B7-H3 and CTLA-4 expressions in NSCLC

	CTLA-4 Negative	B7-H3 Positive	r	P
Negative	23	2	0.74	0.000
Positive	7	38		

the logistic multivariate regression analysis showed that the degree of tumor differentiation and T stage were risk factors for the expression of B7-H3 in NSCLC patients (OR=43.414; 16.267, P=0.003, 0.043). This difference was statistically significant. TNM staging was the risk factor for CTLA-4 in non-small cell lung cancer (OR=6.584; P=0.015), and the difference was statistically significant.

Discussion

Cancer is a multi-factor, multi-gene disease in which cell growth and proliferation are abnormal, and the disease's mechanisms are very complicated. The occurrence and development of tumors are closely related to the body's immunity, especially T cells, that play an important role in anti-tumor immunity. Nivolumab monoclonal antibody, a PD-1 inhibitor that inhibits the T cell differentiation, has become the first FDA-approved treatment for squamous advanced NSCLC. In addition, studies of other immunological sites in NSCLC are becoming increasingly intensive, which all open up new methods for tumor immunotherapy [13, 14]. Most immunological checkpoint inhibitors do not directly attack tumor cells, but rather enhance the anti-tumor immune response through related targeted immunomodulatory pathways, especially pathways that activate specific T lymphocytes.

B7-H3 plays an important role in T cell-mediated cellular immunity. Early studies have shown that B7-H3 stimulates the production of CD4+ and CD8+ T cells that further induce the activation of tumor-specific cytotoxic T lymphocytes, suggesting that B7-H3 is a positive stimulatory molecule [15]. A large number of experiments have proved that B7-H3 has an inhibitory effect on T cells and mediates tumor immune escape [16, 17]. These results indicate that B7-H3 has dual biological characteristics. CTLA-4 is a major factor that inhibits the activation of T cells. A large number of experimental studies also have shown that many regulatory T cells (TReg) infiltrate into the microenvironment of tumors and

can highly express CTLA-4 immunosuppressive molecules [18, 19], thus inhibiting the anti-tumor effects.

The three broad results of this study are: 1) B7-H3 and CTLA-4 are highly expressed in NSCLC and the expression levels in the cases of stage III-IV tumors, with either lymph node metastasis or poor differentiation being significantly higher than those in the cases of stages I-II, without lymph node metastasis, or with high/medium differentiation. The expression level of B7-H3 in patients with a history of smoking and in T3-4 is significantly higher than the level in the patients without a history of smoking and in T1-2. The expression level of CTLA-4 in the distant metastasis group was significantly higher than it was in the non-distant metastasis group. 2) The expressions of B7-H3 and CTLA-4 are positively correlated in NSCLC. 3) TNM staging of NSCLC is an independent risk factor for CTLA-4, and the T staging and differentiation of NSCLC tissues are independent risk factors for B7-H3.

The expression of B7-H3 in NSCLC

A large number of studies have shown that the B7-H3 protein is not expressed or is expressed at extremely low levels in normal tissues and cells while it is highly-expressed in various solid tumor tissues, such as prostate cancer, NSCLC, or renal cell carcinoma [20, 21], and its expression is closely related to the biological characteristics of tumors, tumor progression, and prognosis [6]. Arigamiet et al. [22, 23] found that in studies of breast and pancreatic cancer, the expression level of B7-H3 in tumor tissues is significantly higher than it is in paraneoplastic and normal tissues. The study found that B7-H3 is over-expressed in NSCLC tissues [24]. The results of our study showed that in the 70 NSCLC cases, the positive rate of B7-H3 is 64.2%, but it is only 6.67% in the paraneoplastic tissues. These values are consistent with previous studies, indicating that B7-H3 is an immune escape pathway for tumor cells and can inhibit the apoptosis of tumor-infiltrated lymphocytes (TILs), and that B7-H3 inhibitors can be used as drugs in new treatment methods.

B7-H3 and smoking

Altan et al. and Inamura et al. [25, 26] found that the high expression of B7-H3 is closely related to smoking and is also significantly

associated with the death of lung cancer patients with moderate/severe smoking. In this study, the positive expression rate of B7-H3 in the smoking group is 77.8%, which is higher than the rate in the non-smoking group, indicating that smoking increases the expression of B7-H3 to some extent. B7-H3 and PD-L1 belong to the B7 family. There is some evidence indicating that anti-PD-1/PD-L1 immunotherapy for lung cancer patients who are smokers has achieved good therapeutic effects [27].

B7-H3 and clinicopathological staging

It has been found that B7-H3 is not expressed in normal human lung tissue, but its positive expression rates in lung adenocarcinoma, lung adenocarcinoma with lymph node metastasis, and lung adenocarcinoma tissues with poor differentiation are 68.6%, 71.6% and 83.3%, respectively. The expression rate of B7-H3 in lung adenocarcinoma with poor differentiation is significantly higher than the rate in lung adenocarcinoma with high-middle differentiation, suggesting that the B7-H3 expression level is associated with the differentiation and lymph node metastasis of adenocarcinoma cells. Other scholars have found that the high expression of B7-H3 is associated with tumor grade, lymphatic metastasis, and short-term survival [28, 29]. In this study, the positive expression rates of B7-H3 in NSCLC tissues are also significantly increased in the poor-differentiation group and the lymph node metastasis group, as well as in the stage T3-4 and stage III-IV groups. The multivariate analysis shows that T stage and degree of tissue differentiation can be used as independent risk factors for B7-H3, suggesting that B7-H3 is involved in tumor tissue differentiation and lymphatic metastasis. Kang et al. [30] reported that B7-H3 affects tumor invasion through the JAK3/STAT3/SLUG signaling pathway and promotes tumor cell metastasis by mediating the epithelial-mesenchymal transition [31].

The expression of CTLA-4 in lung cancer

Studies have shown that the expressions of CTLA-4 in the peripheral blood, lymphocytes, and tumor tissues of lung cancer patients are higher than those in normal controls [32, 33]. In this study, CTLA-4 is mainly expressed in the cytoplasm and cell membranes of tumor-infiltrating lymphocytes. The positive expression

rate of CTLA-4 in NSCLC tumor cells is 57.1%, but there is no expression in the paraneoplastic tissues, suggesting that the CTLA-4 expression is closely related to NSCLC.

Current research on CTLA-4 and lung cancer mainly focuses on the impact of the CTLA-4 gene polymorphism on lung cancer, especially the polymorphism of the +49A/G gene which can increase the susceptibility to lung cancer [34, 35]. Soo et al. [36] believe that the expression level of CTLA-4 can be used as a predictor of overall survival, and the higher the expression level, the shorter the overall survival.

CTLA-4 and clinicopathological features

Some scholars have found that the content of CTLA-4 mRNA in the peripheral blood of elderly patients with primary NSCLC is closely related to their clinical TNM staging, tumor cell differentiation, and lymph node metastasis; that is, the more advanced the clinical stage, the lower the degree of differentiation and existence of lymph node metastasis, and the higher the expression of CTLA-4 mRNA. However, there is no significant correlation between the expression of CTLA-4 and the pathological type of the tumor. The results of this study show that the expression levels of CTLA-4 are significantly increased in the poor differentiation group, the stage III-IV group, and in patients with lymph node metastasis/distant metastasis, but the expression is not related to age, sex, smoking history, maximum tumor diameter, or T stage. The conclusions obtained in this study are consistent with those of the previous studies mentioned above. The multivariate analysis of TNM staging in this study can be used as an independent risk factor for CTLA-4. It also indicates that CTLA-4 is more sensitive to NSCLC staging and can be used as a biological indicator to predict the severity of NSCLC.

Analysis of B7-H3 and CTLA-4

The mechanism by which CTLA-4 inhibits the T cell responses involve a shortening of the contact time between T cells and APC, thereby shortening the interaction time between the costimulatory molecule CD28 with its ligand CD80/86. B7-H3 is expressed on APC and can inhibit the activation of T cells. In addition, both B7-H3 and CTLA-4 are associated with regulatory T cells and can inhibit transcription factors

such as NF- κ B and AP-1, indicating that these two co-inhibitory molecules have synergistic effects. In this study, the correlation between the expressions of B7-H3 and CTLA-4 in NSCLC was analyzed using the Spearman rank correlation. The results show that the expressions of B7-H3 and CTLA-4 are positively correlated in NSCLC, indicating that B7-H3 and CTLA-4 are positively correlated. These two co-suppressor molecules participate in the occurrence and development of NSCLC, which is consistent with the above research theory.

In conclusion, the advances in lung cancer immunology provide new directions for the future treatment of tumors. In this study, we found that B7-H3 and CTLA-4 play a role in the microenvironment of NSCLC, and that they are related to the degree of tumor differentiation, clinical stage, and lymph node metastasis, indicating that they may play a synergistic role in the occurrence and development of NSCLC. However, there are some shortcomings in our study. Only 70 cases were included in this study, so a larger sample size is required in order to conduct a more thorough analysis. Further, due to the short observation time, the survival and prognoses of the cases were not investigated.

Disclosure of conflict of interest

None.

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