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Original Article

B7-H3 in combination with regulatory T cell is associated with tumor progression in primary human non-small cell lung cancer

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Abstract: B7-H3 belongs to the co-inhibitory B7 family and plays an important role in the adaptive immune response in regulating T cells. In human malignancies, B7-H3 is reported to be involved in tumor immune evasion. However, the detailed molecular mechanism of B7-H3 in tumor evasion remains unclear, particularly in non-small cell lung cancer (NSCLC). Regulatory T cells (Tregs) are known as a key player in the inhibition of immune mechanisms. The study demonstrated the correlation between B7-H3 on tumor cells and the number of Tregs in the tumor microenvironment in NSCLC. B7-H3 was examined in tumor tissues from 110 patients with NSCLC by immunohistochemical analysis. Forkhead box P3+ (FOXP3+) Tregs in those spencimens were also detected and numbered. Survival curves were drawn using the Kaplan-Meier method and compared by the log-rank test. High B7-H3 expression in tumor cells significantly correlated with male gender, squamous NSCLC, advanced stage and shorter overall survival (OS) (P = 0.035, P = 0.004, P = 0.037, P = 0.014, respectively). Meanwhile, FOXP3 expression in tumor-infiltrating lymphocytes (TILs) was associated with male gender, regional lymph node involvement, advanced stage and worse OS (P = 0.009, P = 0.015, P = 0.014, P = 0.034, respectively). Significant correlation was identified between the expression of B7-H3 and the number of FOXP3+ TILs (P = 0.013). Patients with B7-H3 high/FOXP3 high had poorer OS (P = 0.006), suggesting that B7-H3 and Tregs may play a cooperatively role in tumor immune evasion, leading to poor outcomes for NSCLC patients.

Keywords: B7-H3, regulatory T cell, tumor immune evasion, non-small cell lung cancer

Introduction

Lung cancer is the most common cancer type and has the highest mortality rate across the globe [1]. Non-small cell lung cancer (NSCLC) accounts for 80-85% of all lung cancers [2]. Although gradual improvements have been achieved, 5 year survival rate of patients with NSCLC remains less than 10% in China and 16% in USA. Several mechanisms related to tumor microenvironment have been suggested to result in the immune defects in NSCLC patients, such as a lower number of blood lymphocytes and elevated levels of T-regulatory lymphocytes [3, 4].

B7-H3, a well-known member of the B7 family, is expressed at low levels in several normal lymphoid and peripheral tissues [5]. Previous studies have shown an important function of B7-H3 in regulating T cell mediated immune responses [6]. Recently, high expression of B7-H3 protein has been detected in several tumor cells along with human malignancies of the lymphoma, breast cancer, stomach, prostate, and pancreas, colon, clear cell renal carcinoma and lung carcinoma [7-16]. And increasing immunohistochemical and functional studies have demonstrated a correlation between high expression of B7-H3 and tumor progression and a significant function of B7-H3 in tumor evasion [14-19]. In NSCLC cells, B7-H3 expression was involved in inhibiting T-cell function and inducing the development of monocytes into tumor-associated macrophages [17-19].

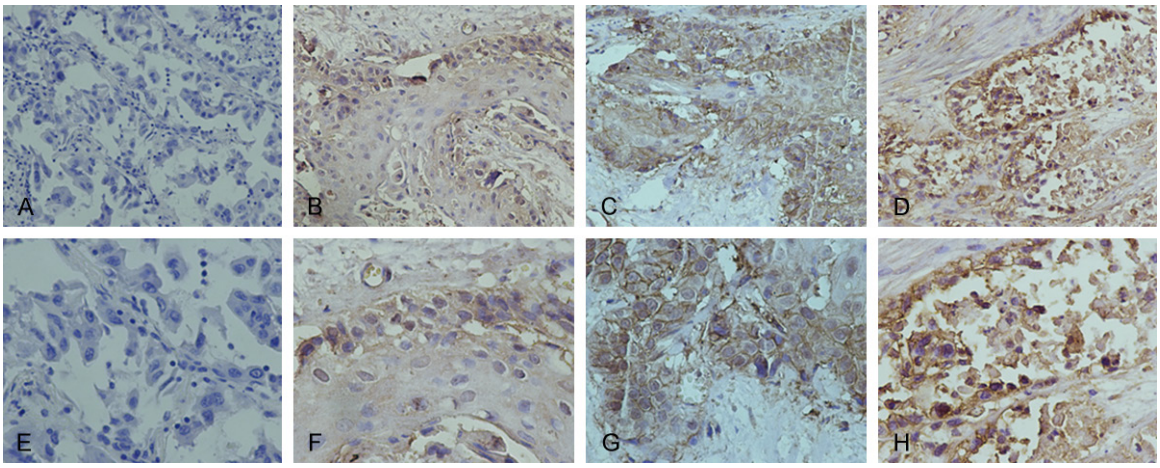

Figure 1. Immunohistochemical analysis of B7-H3 expression in sections from primary NSCLC. B7-H3 expression is shown in both cell membrane and cytoplasm (brown staining). A: Negative expression of B7-H3 (200× magnification); B: Weak expression of B7-H3 (200× magnification); C: Moderate expression of B7-H3 (200× magnification); D: High expression of B7-H3 (200× magnification); E: Negative expression of B7-H3 (400× magnification); F: Weak expression of B7-H3 (400× magnification); G: Moderate expression of B7-H3 (400× magnification); H: High expression of B7-H3 (400× magnification).

T-regulatory lymphocytes (Tregs) have been demonstrated to be involved in the maintenance of immune tolerance through suppressing the actions of cytotoxic lymphocytes [20, 21]. High levels of Tregs have been reported in the tumor specimens and lymph nodes with different types of cancer [22]. Peripheral Tregs (pTregs) were found to be significantly higher in NSCLC patients with advanced stages and poorer recurrence-free survival [23]. Moreover, patients with stage I NSCLC who have a higher proportion of tumor Tregs had a significantly higher risk of recurrence [24]. Forkhead box P3 (FOXP3) is a member of the forkhead/winged-helix family of transcription regulators involved in regulating immune response [25], which plays a crucial role in the generation of CD4+CD25+ Tregs and is identified as the typical marker of CD4+CD25+ Tregs [26]. The prognostic importance of FOXP3 expression in patients with NSCLC has been investigated [23, 24, 27, 28]. In malignancies, FOXP3 was also a strong prognostic factor for distant metastasis-free survival and overall survival [29].

Tumor immune evasion plays an important role in promoting tumor progression and metastasis and requires the interaction of several tumor-derived immune molecules and immune cells. The expression of B7-H3 by tumor cells or FOXP3+ Tregs in tumor stroma can induce inhibition of T-cell proliferation and thereby promote tumor evasion. However, little is currently known about the interplay between B7-H3 and FOXP3+ Tregs in NSCLC. The purpose of this study was to correlate the expression of B7-H3 and the number of Tregs in primary tumors of NSCLC. We focused on the clinicopathological significance of both expressions of B7-H3 in tumor cells and infiltration of Tregs in tumor stroma, and their association with clinical outcome.

Materials and methods

Patients and tissue samples

Participants comprised 110 patients with NSCLC who underwent surgery in the Jinan Central Hospital Affiliated to Shandong University (Shandong, China) between 2006 and 2015. Patients (83 men and 27 women; mean age at diagnosis, 61 years) without any preoperative therapy before surgery were included in the study. Patients were contacted by phone to check upon their health status and the last censor date was on August 10th, 2015. The study was approved by the review board and ethics committee, and all patients gave their written informed consent.

Immunohistochemistry

Sections of 4 μm thickness were cut from paraffin-embedded tissue blocks, mounted on

Table 1. Correlations of B7-H3 and FOXP3 positive cells in TILs with clinicopathological parameters in primary NSCLC tissues

Variables	No. of case	B7-H3 expression			FOXP3 positive cell		
	n	High	Low	*P* value	High	Low	*P* value
Age (yr)							
<60	44	22	22	0.434	24	20	0.049
≥60	66	38	28		48	18	
Gender							
Male	83	50	33	0.035	58	25	0.009
Female	27	10	17		14	13	
Smoking index							
<400	57	31	26	0.972	38	19	0.782
≥400	53	29	24		34	19	
Histology							
Non-squamous NSCLC	65	28	37	0.004	41	24	0.529
Squamous NSCLC	45	32	13		31	14	
Cellular differentiation							
Well	59	29	30	0.222	37	22	0.665
Worse	51	31	20		34	17	
Primary tumor size (cm)							
<5	77	41	36	0.676	53	24	0.255
≥5	33	19	14		19	14	
Regional lymph node involvement							
N0-N1	75	34	41	0.97	44	31	0.015
N2-N3	35	26	9		28	7	
2009 TNM stage groupings							
I	33	15	18	0.037	16	17	0.014
II-III	77	45	22		56	21	

Abbreviations: NSCLC, non-small cell lung cancer; TNM, tumor node metastasis.

silanated slides, and subsequently dewaxed and rehydrated using xylene and graded alcohol washes. Antigen retrieval was carried out by microwaving in Citric Acid buffer (MAX, Fuzhou, China). Sections were blocked with serum-free protein blocker (MAX) for 10 min, followed by the addition of a primary antibody. The following primary antibodies were used in accordance with the instructions from the manufacturer: B7-H3 antibody, 1/200 dilution (Proteintech, Wuhan, China); and FOXP3 antibody, 1/100 dilution (Abcam, Cambridge, UK). After incubation with the primary antibody, slides were washed in two changes of PBS before incubation with labeled polymer horseradish peroxidase rabbit/mouse antibody for 15 min (ElivisionTM plus Polyer HRP, MAX). Negative controls were prepared using normal mouse and rabbit IgG instead of the primary antibody. The sections were scored as positive if the tumor cells showed positive staining in the membrane and cytoplasm. Immunohistochemical assays were performed simultaneously based on immunostaining intensity and area extent by two independent investigators. Each slide was given a score according to the intensity of cytoplasmic staining (-, negative; +, weak; ++, moderate; +++, strong). Depending on the intensity of positive immunoactivity, a final overall score (high or low B7-H3) was established. Tumor samples (B7-H3++; B7-H3+++) were identified as B7-H3 high, while tumor samples (B7-H3-; B7-H3+) were identified as B7-H3 low.

Scoring of FOXP3+ cells in tumor-infiltrating lymphocytes (TILs)

The evaluation of FOXP3+ cells and TILs was performed by two independent observers in a blinded manner. The number of FOXP3+ cells and TILs was counted under light microscope (Olympus, Tokyo, Japan). There were at least 10 independent microscopic fields for the duplicates of each patient sample (400× magnification). Numbers in the fields were cumulated and then averaged to calculate the final number. The ratio of FOXP3+ cells/TILs was calculated for each specimen. We selected the median value as the cut-off for defining TIL subgroups. High and low ratios of FOXP3 were termed FOXP3 high and FOXP3 low, respectively.

Statistical analysis

The X^2 test and the Student two-tailed t test were performed to assess the relationship between expression of B7-H3, FOXP3+ cells

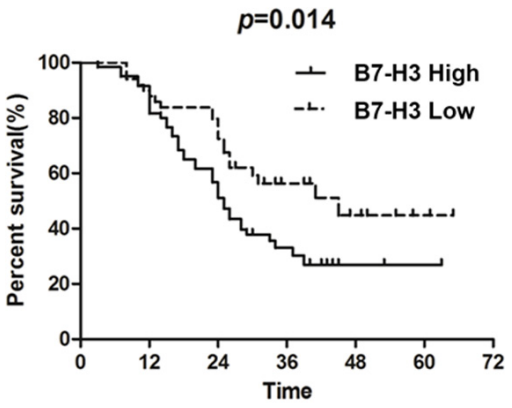

Figure 2. Correlation of OS with B7-H3 expression in NSCLC cells. B7-H3 high was associated with significantly reduced OS in patients with NSCLC.

and clinicopathological characteristics. Actuarial OS rates were calculated by the Kaplan–Meier method and analyzed using the log rank test. *P* values were considered to indicate a statistically significant difference at P<0.05. All analyses were performed using SPSS version 19.0 software (SPSS, Chicago, IL, USA).

Results

High B7-H3 expression of NSCLC cells correlates with poor prognosis of patients

110 tissue sections from NSCLC patients were examined. B7-H3 protein expression was found in the cytoplasm or/and membrane of NSCLC cells at various levels. No expression (B7-H3-) was seen in 11 patients (10.00%; **Figure 1A**), weak expression (B7-H3+) in 39 patients (35.45%; **Figure 1B**), moderate expression (B7-H3++) in 34 patients (30.91%; **Figure 1C**), and strong expression (B7-H3+++) in 26 patients (23.64%; **Figure 1D**). A total of 54.55% of tumor samples (B7-H3++; B7-H3+++) were identified as B7-H3 high, while 45.45% (B7-H3-; B7-H3+), B7-H3 low. High B7-H3 expression in tumor cells correlated significantly with male gender (P = 0.035), squamous NSCLC (P = 0.004) and advanced stage (P = 0.037), respectively. No significant associations were identified between B7-H3 and other clinical pathological parameters (**Table 1**). More importantly, Kaplan-Meier analysis showed B7-H3 high was significantly associated with reduced OS in patients with NSCLC (P = 0.014; **Figure 2**).

Ratio of FOXp3+ cells in TILs of NSCLC tissues correlates with prognosis of patients

Lymphocytes infiltrating in tissue surrounding tumors were abundant (**Figure 3**). We selected the value n = 30 (Median score) as the cut-off for defining TIL subgroups. High and low ratios of FOXP3 in TILs were termed FOXP3 high and FOXP3 low, respectively. High FOXP3 expression in TILs was correlated significantly with male gender, regional lymph node involvement and advanced stage (P = 0.009, P = 0.015, P = 0.014, respectively). No significant relationships were identified between FOXP3+ cell infiltration and other clinical pathological parameters (**Table 1**). Moreover, Kaplan-Meier method showed FOXP3 high was associated with significantly reduced OS in NSCLC patients (P = 0.034; **Figure 4**).

Correlation between B7-H3 expression and tumor-infiltrating FOXP3+ cells

Significant correlation was identified between expression of B7-H3 and the percentage of FOXP3+ TILs. High percentage of tumor-infiltrating FOXP3+ cells was found in B7-H3 high expression sections (P = 0.013; **Figure 5**).

Combined expression of B7-H3 in NSCLC cells and FOXP3+ TILs correlate with poor prognosis of patients

We further categorized patients into four groups according to the expression of B7-H3 and FOXP3 in the same patient: B7-H3 high/FOXP3 high (n = 48); B7-H3 high/FOXP3 low (n = 12); B7-H3 low/FOXP3 high (n = 26); and B7-H3 low/FOXP3 low (n = 24). B7-H3 high/FOXP3 high group was significantly correlated with male gender (P=0.008) and advanced stage (P=0.023) (**Table 2**). Moreover, patients with B7-H3 high/FOXP3 high had shorter overall survival than patients with B7-H3 low/FOXP3 low (P = 0.006; **Figure 6**). However, no matter in the group of B7-H3 high or low, no significant difference in OS was seen between FOXP3 high and low subgroups (P = 0.372, P = 0.214, respectively; Data not shown).

Discussion

Although B7-H3 has been associated with inhibition and evasion of immunity in NSCLC [15, 18, 19], the clinical and functional significance

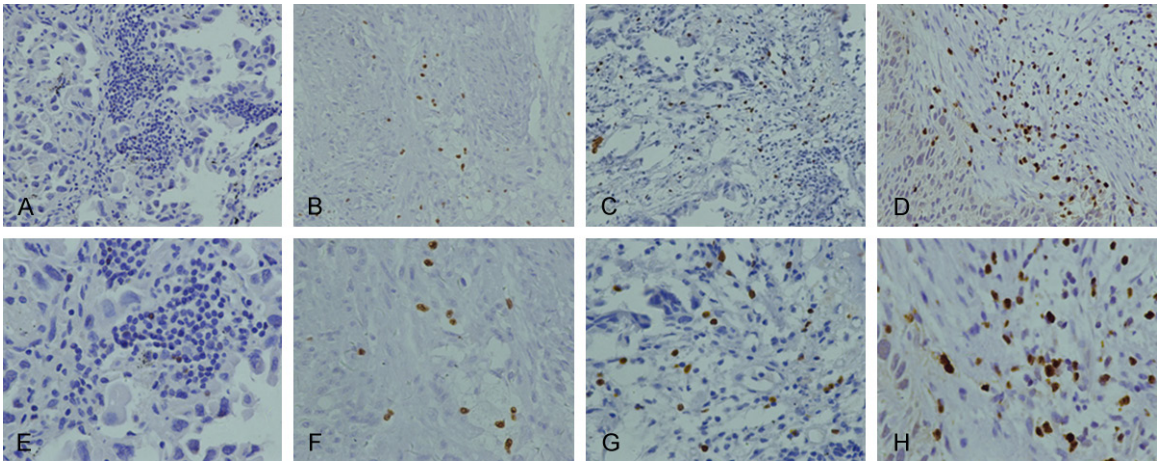

Figure 3. Immunohistochemical detection of FOXP3+ cell in sections from primary NSCLC. The ratio of FOXP3+ cell/TILs was categorized. A: Little expression of FOXP3+ cell (200× magnification); B: Low expression of FOXP3+ cell (200× magnification); C: Moderate expression of FOXP3+ cell (200× magnification); D: High expression of FOXP3+ cell (200× magnification); E: Little expression of FOXP3+ cell (400× magnification); F: Low expression of FOXP3+ cell (400× magnification); G: Moderate expression of FOXP3+ cell (400× magnification); H: High expression of FOXP3+ cell (400× magnification).

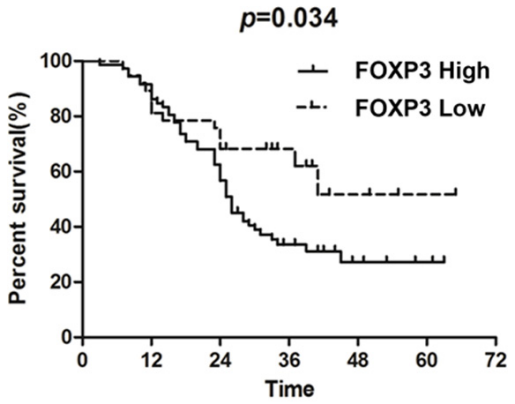

Figure 4. Correlation of OS with FOXP3+ cell in TILs of NSCLC tissues. FOXP3+ high was associated with significantly reduced OS in patients with NSCLC.

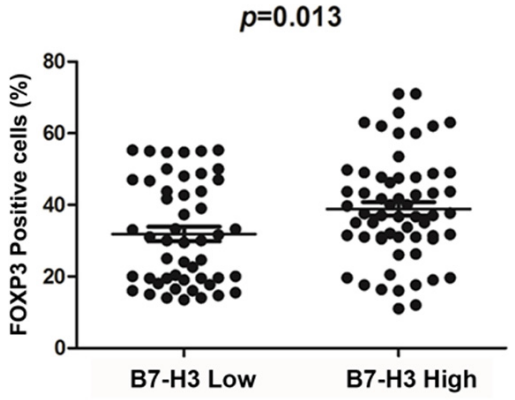

Figure 5. Correlation of B7-H3 expression in NSCLC cancer cells and FOXP3+ cell in TILs. The ratio of FOXP3+ in TILs was higher in B7-H3 high expression group in NSCLC.

of this protein remains unclear. Our study suggested that B7-H3 expression in NSCLC cells was not only related to adverse pathological parameters and shorter overall survival, but also the percentage of FOXP3+ TILs. Moreover, patients with B7-H3 high/FOXP3 high relapsed within a shorter period than patients with B7-H3 low/FOXP3 low.

B7-H3 is an immunoregulatory protein that belongs to the B7 family of T-cell co-regulatory molecules [5]. Human B7-H3 is induced in dendritic cells and monocytes by inflammatory cytokines [30]. Although some reports suggest an opposite function, B7-H3 has been mostly considered as a negative regulator that preferentially downregulates T helper type 1-mediated immune responses [31]. Recently, B7-H3 expression has been found in a variety of human malignancies and often associates with metastasis and poor prognosis [32, 33]. In prostate cancer and colorectal cancers, patients with high levels of B7-H3 expression displayed a worse prognosis [8, 34, 35]. In NSCLC and breast cancer, our previous studies suggested B7-H3 was a factor related to lymph node metastasis [14, 15]. In this study, we also

Table 2. Correlations of B7-H3 and FOXP3 positive cells in TILs with clinicopathological parameters in primary NSCLC tissues

Variables	No. of case	B7-H3 high/ FOXP3 high	B7-H3 low/ FOXP3 high		B7-H3 high/ FOXP3 low		B7-H3 low/ FOXP3 low	
	n	n	n	P1	n	P2	n	P3
Age (yr)								
<60	44	17	7	0.596	5	0.688	15	0.065
≥60	66	31	17		7		11	
Gender								
Male	83	41	18	0.279	9	0.403	15	0.008
Female	27	7	6		3		11	
Smoking index								
<400	57	27	11	0.404	4	0.204	15	0.905
≥400	53	21	13		8		11	
Histology								
Non-squamous NSCLC	65	24	17	0.092	4	0.349	20	0.024
Squamous NSCLC	45	24	7		8		6	
Cellular differentiation								
Well	59	24	13	0.739	5	0.605	17	0.204
Worse	51	24	11		7		9	
Primary tumor size (cm)								
<5	77	35	18	0.676	6	0.127	18	0.737
≥5	33	13	6		6		8	
Regional lymph node involvement								
N0-N1	75	32	12	0.854	9	0.735	22	0.11
N2-N3	35	16	12		3		4	
2009 TNM stage groupings								
I	33	10	6	0.688	5	0.136	12	0.023
II-III	77	38	18		7		14	

Note: P1, *P* value between B7-H3 high /FOXP3 high and B7-H3 low/FOXP3 high; P2, *P* value between B7-H3 high/FOXP3 high and B7-H3 high/FOXP3 low; P3, *P* value between B7-H3 high/FOXP3 high and B7-H3 low/FOXP3 low. Abbreviations: NSCLC, non-small cell lung cancer; TNM, tumor node metastasis.

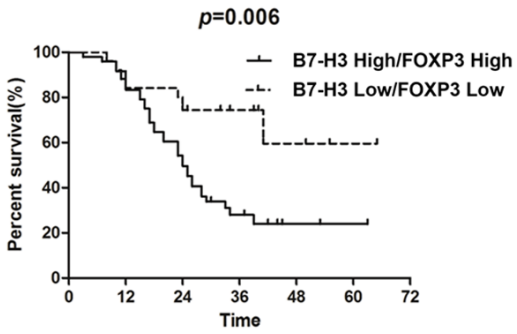

Figure 6. Correlation of OS with B7-H3 high/FOXP3 high expression in NSCLC tissues. Patients with B7-H3 high/FOXP3 high relapsed within a shorter period than patients with B7-H3 low/FOXP3 low.

found high expression of B7-H3 was significantly associated with shortened OS in NSCLC. Further identification and understanding of the B7-H3 signaling pathway and potential receptors may offer new therapeutic strategies for NSCLC.

FOXP3 is a master regulatory gene for lineage commitment or development of CD4+CD25+ Tregs [26]. Moreover, FOXP3 remains the best single marker of Tregs. In cancer patients Tregs have been demonstrated to associate with tumor progression and suppression of antitumor immune response [21, 22]. Increasing numbers of Tregs have been reported in several human cancers, including lung, breast, pancreas, ovarian cancer and some other tumors [36, 37]. Even in stage I NSCLC, tumor with high infiltration of FOXP3+ Tregs has been shown to have a worse prognosis [24]. In this study, high FOXP3 expression in TILs was not only signifi-

cantly correlated with several adverse clinical parametors, but also short OS.

It is reported that B7-H3 plays an important role in tumor associated myeloid-derived suppressor cells (MDSC) mediated Tregs induction. The major pathway of immunosuppression by Tregs in tumor may be through direct cell-to-cell suppression of effector T cells, producing soluble factors, such as immunosuppressive IL-10 and TGF-β, leading to the decrease number of TILs. Our previous study has demonstrated the significant correlation between B7-H3 and the less number of TILs, suggesting the immunosuppressive function of B7-H3 in NSCLC. However, the potential mechanism was not clear. In this study, we identified a significant correlation between B7-H3 and FOXP3+ Tregs in NSCLC. Moreover, patients with both B7-H3 high and Treg high correlated with adverse clinical parameters and showed the worst OS. Taken together, we postulated that B7-H3 might drive the activity of Tregs which could inhibit effective T cells, leading to the inhibition of tumor immunity in NSCLC. Although the expression of B7-H3 and Foxp3 were not correlated in breast cancer these molecules might play a cooperatively role in tumor immune evasion, leading to tumor progression in NSCLC. It would be important in future studies to perform deeper investigations to gain better understanding of the role of the Tregs and B7-H3 in certain particular features of NSCLC.

In summary, this study showed that expression of B7-H3 in NSCLC cells and tumor-infiltrating FOXP3+ Tregs predict worsened prognosis. A significant correlation between B7-H3 and FOXP3+ Tregs was detected and patients with both B7-H3 high and Tregs high showed the worst prognosis. At present, the blockade of B7-H1 known as PD-L1 is under clinical trials worldwide [38]. Combined with the high homology of B7-H3 to B7-H1 and the significant function of B7-H3 in tumor progression, blockade of B7-H3 is supposed to be of high feasibility. Moreover, low doses of several chemotherapeutic drugs and irradiation have been reported to deplete Treg cells to improve the prognosis for cancer patients. Based on the detection of these molecules and the significant correlation between them, we speculated the therapy targeting B7-H3 expression in combination with targeting Tregs might be an important approach for controlling the cancer progression in patients with NSCLC having high expression of both B7-H3 and FOXP3+ Tregs.

Acknowledgements

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Disclosure of conflict of interest

None.

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