# B7-H3 and CD133 expression in non-small cell lung cancer and correlation with clinicopathologic factors and prognosis

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## ABSTRACT

الأهداف: دراسة تأثير ظهور بروتينات B7–H3 وCD133 على العينات المأخوذة من سرطان الرئة ذو الخلايا غير الصغيرة ( NSCLC ) و تأثيرها كذلك على أورام الرئة الحميدة، بالإضافة إلى تقييم العلاقة بين هذين الدليلين الحيويين والخواص الباثولوجية السريرية.

الطريقة: تم إجراء دراسة مقارنة بين مجموعة التحكم والمجموعة المُصابة ( case–control study ) في المستشفى الثاني التابع لجامعة سوتشو، سوزو، الصين وذلك خلال الفترة من يناير 2006م إلى ديسمبر 2008م. شملت المجموعة المُصابة 102 عينة مأخوذة من المرضى المصابين بسرطان الرئة ذو الخلايا غير الصغيرة بعد إجراء عملية جراحية صدرية لهم، فيما شملت مجموعة التحكم 25 عينة من الأنسجة المجاورة غير السرطانية، و24 عينة من الأنسجة المُصابة بالتهابات حميدة. خضعت 126 عينة لتلوين اختبار المناعة الكيميائية ( immunohistochemistry test ) وذلك باستخدام برنامج ( Image–Pro Plus ) وتم قياس عدد الخلايا في كل مقطع.

النتائج: ظهر بروتين B7–H3 في 71 عينة، فيما ظهر CD133 في 51 عينة وذلك من أصل 102 عينة مُصابة بسرطان الرئة ذو الخلايا غير الصغيرة، وكان ظهورهما في هذه العينات أعلى من الأنسجة المصابة بأورام حميدة ( p<0.001 )، والأنسجة المجاورة غير السرطانية ( p<0.001 ). لقد كان ظهور بروتين B7–H3 في الأنسجة المصابة بالسرطان الحرشي ( SCC ) أعلى بكثير من ظهوره في الأنسجة المصابة بالسرطان الغدي ( P=0.048 )، وكان ظهور بروتين CD133 في الأنسجة المصابة بسرطان الرئة ذو الخلايا الكبيرة أعلى من ظهوره في الأنسجة المصابة بالسرطان الحرشفي ( P=0.023 ). لقد كان عدد الخلايا اللمفاوية الارتشاحية ( TILs ) في مجموعة الأنسجة التي ظهر فيها B7–H3 أقل من المجموعة التي لم يظهر فيها هذا البروتين ( P=0.026 )، وكان عدد هذه الخلايا في الأنسجة التي ظهر فيها CD133 أقل بكثير من المجموعة التي لم يظهر فيها هذا البروتين ( P=0.029 ). أشارت النتائج إلى ارتباط بروتين CD133 بدرجة تمايز الخلايا السرطانية، غير أن العلاقة بين –B7 H3 و CD133 كانت عكسية. أظهر تحليل كوكس الإحصائي الانحداري بأن العينات التي ظهر فيها CD133 والعينات التي لم يظهر فيها B7–H3 كانت مرتبطة مع تدني نسبة النجاة من مرض سرطان الرئة ذو الخلايا غير الصغيرة.

خاتمة: تشير النتائج بأن B7–H3 و CD133 يقومان بتحفيز الموت الخلوي المبرمج للخلايا اللمفاوية الارتشاحية في الأنسجة المصابة بسرطان الرئة ذو الخلايا غير الصغيرة، كما أنهما يساعدان على نجاة الخلايا السرطانية من الرقابة المناعية، غير أن كلاً منهما لا يعد عاملاً مستقلاً يزيد من خطر الإصابة بسرطان الرئة ذو الخلايا غير الصغيرة.

**Objectives:** To detect the expression of B7-H3 and CD133 in human non-small cell lung cancer (NSCLC) specimens and lung benign lesions, and to evaluate the correlation between the 2 biomarkers and clinicopathologic features.

**Methods:** This is a case-control study of 102 tissue specimens collected from NSCLC participants undergoing thoracic surgery in the Second Affiliated Hospital of Soochow University, Suzhou, China, between January 2006 and December 2008. From the 102 patients, 25 adjacent non-cancer samples were verified pathologically as normal tissue (positive group), and 24 benign inflammatory lesion tissues were used as control (negative group). Specimens from 126 participants were stained immunohistochemically using Image-Pro Plus software, and the cell number was measured in each section.

**Results:** Of the 102 specimens, 71 expressed B7-H3, and 51 expressed CD133, higher than that in benign lesions ($p<0.001$) or non-cancer tissues ($p<0.001$). B7-H3 expression in squamous cell carcinoma (SCC) was significantly higher than those in adenocarcinoma ($p=0.048$), while CD133 expression in large cell lung carcinoma was higher than that in SCC ($p=0.023$). The mean number of tumor-infiltrating lymphocytes (TILs) in the B7-H3-positive group was lower than that in the B7-H3-negative group ($p=0.026$). The mean TILs in the CD133-positive group was significantly lower than that in CD133-negative group ($p=0.029$). We found that CD133 was related to tumor cell differentiation degree and CD133 expression was negatively correlated with B7-H3 expression. The CD133 positive or B7-H3 negative was associated with poor prognosis of NSCLC patients by Cox regression analysis.

**Conclusions:** Both CD133 and B7-H3 might induce apoptosis of TILs in NSCLC and tumor evading host immune surveillance. Either CD133 or B7-H3 might be an independent risk factor of NSCLC participants.

Lung cancer, the leading cause of cancer death in both men and women will cause one billion deaths in this century worldwide.[1] Despite major advances in surgical techniques, chemotherapy and radiotherapy of lung cancer, treatment outcomes for lung cancer are still to be considered dismal.[2] Non-small cell lung cancer (NSCLC), nearly 80% of lung cancer, is refractory to conventional therapeutic methods, and the prognosis of patients remains poor. Conventional therapy seems to have reached a plateau, and difficult to obtain better therapeutic effect in advanced NSCLC. Based on this point, clinical investigation of novel target points and treatment strategies are mandatory in advanced NSCLC.[3] The B7-H3, a new member of the B7 family, has been cloned and named by Chapoval et al in 2001.[4] In humans, B7-H3 mRNA expression can be detected in numerous lympho- or non-lympho- organs, but lower expression in brain, skeletal muscle, kidney, and lung, and not yet in the circumference blood lymphocyte. In addition, B7-H3 mRNA also expresses in epidermis tumor cell lines including G361, HeLa S3, K562, A549, SW480, but not in Molt-4 and Raji lymph cellularity cell lines.[4,5] The B7-H3 protein expression in normal tissue seems to be limited.[4,6] However, protein expression on human dendritic cells and monocytes has only been shown in response to treatment with phorbol 12-myristate 13-acetate and ionomycin.[4] The higher B7-H3 protein expression has been investigated in pathologic tissue,[7-10] and stronger staining intensity is observed in tumor tissue, and associated with disease spread and poor outcome.[11,12] The B7-H3 works as a positive stimulator of immune responses,[7-9,13] and plays a protective role from natural killer (NK)-mediated lysis by interacting with still unknown inhibitory receptors on NK cells.[13] In contrast, B7-H3-deficient mice develop accelerated forms of induced airway inflammation and experimental autoimmune encephalitis, implicating that B7-H3 acts as an inhibitor of T cells-mediated immunity,[14] and plays an immune-inhibitory role in the presence of strong activating signals.[8,15,16] In human neuroblastoma studies, B7-H3 has been implicated as an inhibitor of NK cell-mediated lysis. Although cognate receptors for B7-H3 have not been elucidated, the putative receptors have now been postulated to explain the ability of B7-H3 to inhibit responses by both T cells and NK cells.[14] The B7-H3 has been reported to be expressed preferentially

in tumor tissues, such as prostate cancer,[11,12,17] clear-cell renal cell carcinoma,[18,19] urothelial cell carcinoma,[20] and NSCLC.[21] Cancer stem cells (CSCs) or tumor initiator cells (TICs) that phenotypically increase diverse cancer cells with less proliferative potential in leukemia or varied solid tumor have become the hotspot of cancer research.[22] The CD133 (or AC133) is a new surface antigen of hematopoietic stem and progenitor cells (HSPCs), and there are 2 isoforms of human CD133 Yu et al[23] cloned, and identified CD133-2 following the cloning and identification of CD133-1. The CD133-2 was predominant in fetal tissues such as liver, skeletal muscle, kidney, and heart, also in adult tissues such as kidney, pancreas, and placenta, furthermore, CD133-2 was also detected in poorly differentiated human cell lines of lung, prostatic, pancreatic, colonic, and breast carcinoma. The CD133-1 was predominant in the fetal brain and skeletal muscle, unlike CD133-2, due to its different expression of CD133 isoforms in different human tumors, providing a useful tool to separate stem cell subpopulation specifically. Expanding evidence highlights the roles of CD133 as a marker of cancer stem cells (CSCs) in various human tumors, because CD133 expressed in leukemic cell,[23] glioma,[24-26] colon carcinoma,[27,28] prostatic carcinoma,[29] hepatoma,[30,31] and pancreatic cancer[32,33] except for normal tissue. These CD133+ tumor cells have capacities of self-renewal, proliferation, and multi-lineage differentiation in vitro, consistent with CSC properties.[34] Many different studies showed that CD133+ cells were associated with the tumor immune escape, chemo resistance, and radio resistance, thus, CD133+ cells may be possible targets for immunotherapy. To investigate the expression and functional relevance of stem cell marker CD133-2, co-stimulatory molecule B7-H3, and clinical significance in NSCLC, we detected B7-H3 and CD133 expression in NSCLC tissues and analyzed the relationship between the expression and clinicopathological variables. In addition, we examined the association between B7-H3 and CD133 expression on tumor cells and tumor-infiltrating lymphocytes (TILs).

**Methods.** *Participants' characteristics.* We collected 102 samples (66 males, 36 females; age 36-75 at diagnosis, mean age: 60.51±8.88 years) from NSCLC participants who had undergone surgery and diagnosed pathologically in the Second Affiliated Hospital of Soochow University, Suzhou, China, between January 2006 and December 2008. The date of operation was recorded as the beginning point of the observation. The date of the last follow-up was March 2010. Four of the participants were lost follow-up. Among them, there were 48 cases of squamous cell carcinoma (SCC), 49 adenocarcinoma, and 5 large cell lung carcinoma.

We used the American Joint Committee on Cancer Staging Manual[35] for surgical and pathological staging. Eleven were stage Ia, 19 stage Ib, 4 stage IIa, 16 stage IIb, 39 stage IIIa, 6 stage IIIb, and 7 in stage IV. There were 25 adjacent non-cancer samples, which were verified pathologically as normal tissue, and 24 benign inflammatory lesion tissues as control. The following exclusion criteria were applied: participants that tend to have other diseases, and no follow-up. This study has been approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University.

*Antibodies and immunohistochemistry.* The anti-B7-H3 mAb was prepared and kept in the laboratory.[36] The anti-CD133 mAb was purchased from EHSY Company (EHSY, Shanghai, China). Goat anti-mouse IgG-PE and mouse IgG were all purchased from Immunotech Company (Immunotech, Marseilles, France). Immunohistochemical staining was performed using the Dako ElivisionTM (Dako Inc, Carpiterry, CA, USA) according to the manufacturer's instructions.

*B7-H3, CD133, and CD45 expression.* Staining were quantified using the Software Image-Pro Plus Version 5.0.2 (Media Cybernetics, Bethesda, USA). B7-H3 and CD133 expression in NSCLC and adjacent non-cancer and benign pneumatic disease tissues were measured in each section under ×400 magnification at least 5 fields in randomly selected tumor areas. The intensity of the positive cells was graded semi-quantitatively according to the positive cell percentage: 0 = focal expression in <10%; + = focal expression in 10-40%; ++ = 40-80%, and +++ = >80%.[32] Data were expressed as mean ± SD.

We collected CD45+ cells in both malignant and benign tissues to examine whether B7-H3 and CD133 expression were associated with infiltration of tumor-infiltrating lymphocytes. Consecutive slides from the same tumor, stained for CD45, were super-imposed on the B7-H3 or CD133-stained slide. Using histological landmarks, the corresponding B7-H3 or CD133 positive and negative areas were placed on slides.[37] When we removed the B7-H3 or CD133-stained section, we took photographs microscopically at ×400 magnification at least 5 fields in randomly selected B7-H3 expression tumor areas and we detected CD45+ cells using the Software.

*Statistical analysis.* The association between B7-H3, CD133 expression and TILs, or pathological variables were analyzed statistically using Student's independent-samples t test, paired samples t test, and Fisher's exact test, as appropriate. Data analysis was carried out using Statistical Package For Social Sciences Version 13.0 (SPSS, Chicago, IL, USA). The survival analysis was estimated by the Kaplan-Meier method, and Cox regression was used to evaluate the associations of B7-H3 and CD133 with survival time. A value of $p<0.05$ was considered statistically significant, and all tests were 2-tailed.

**Results.** *B7-H3, CD133 expression in human NSCLC specimens, and lung benign lesions and adjacent non-cancer tissues.* Among 102 surgically resected specimens of NSCLC; B7-H3 or CD133 expression was demonstrated in the cell membrane, cytoplasm, or both, in a focal or scattered pattern microscopically (**Figures 1 & 2**). The B7-H3-positive expression rate was higher compared to the adjacent non-cancer ($p<0.001$) and benign lesion samples ($p<0.001$). CD133-positive expression rate was higher than benign lesions ($p<0.001$) or non-cancer tissues ($p<0.001$) (**Table 1**). But, we could
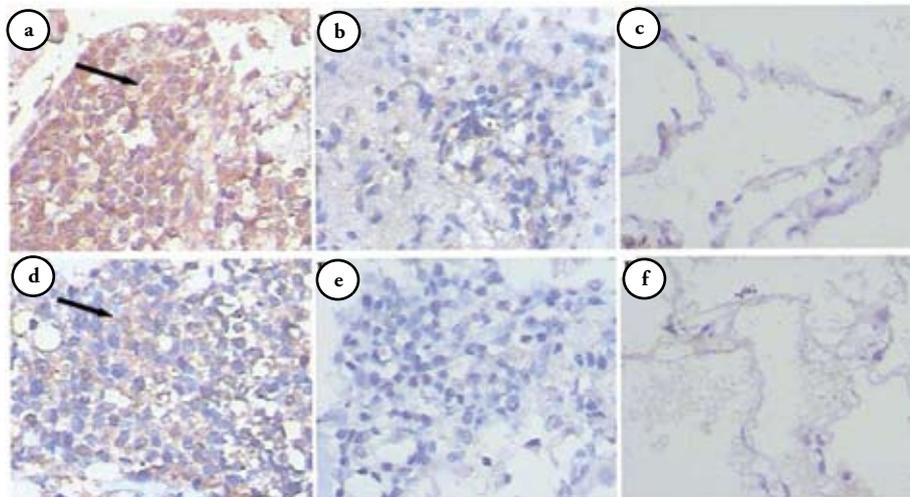


**Figure 1** - B7-H3 or CD133 expression in non-small cell lung cancer (NSCLC), adjacent non-cancer and benign lesion samples immunohistochemically: **a)** B7-H3 & **b)** CD133 expressed (arrows) in the cell membrane, cytoplasm, or both, in a focal or scattered pattern among NSCLC specimens microscopically. The expression in NSCLC tissue was higher than that in adjacent non-cancer **c & d)** and **e & f)** benign lesion samples (×400).

not find B7-H3 or CD133 expression in infiltrating lymphoid cells. CD133 expression was negative related to B7-H3 expression, but there was no statistical difference (*p*=0.21) **Table 1**.

*Relationship between B7-H3, CD133 expression, and clinical pathologic characters.* The percentage of B7-H3 expression in SCC was higher than adenocarcinoma (*p*=0.048). The percentage of CD133 expression in large cell lung carcinoma was higher than in squamous carcinoma (*p*=0.023). The expression of CD133 is correlated with the cell differentiation level, part of the poorly differentiated NSCLC specimens (21 in 51) are CD133 positive, higher than in well differentiated NSCLC specimens. The difference was statistically significant, *p*=0.02. Otherwise, there is no such difference between cell differentiation level and expression of B7-H3 in NSCLC, this is similar to the results of the study of Sun et al.[20] No relationship was found between the expression of B7-H3 or CD133 and gender, age, smoking history, histology, and TNM staging (*p*>0.05) **Table 2**.

*Correlation between B7-H3, CD133, and TILs.* In 102 NSCLC specimens, we assessed the relationship between the expression of B7-H3 or CD133 and TILs. We could not find cases, which contained separate positive- or negative- region in the same sections. The mean number of TILs in B7-H3-positive group was 24±1.90, lower than that in B7-H3-negative group, 40±6.74 (*p*=0.026). The mean TILs in CD133-positive group was 24±2.11, and it was significantly lower than that in CD133-negative group, 35±4.52 (*p*=0.029). **Table 2**.

*Clinical and pathologic characteristics, and participant outcome.* Survival curves were plotted with the method of Kaplan Meier. The statistical difference of survival between B7-H3 positive and negative groups was compared using the log-rank test (*p*=0.020). Kaplan-Meier survival curves for patients with CD133-positive and CD133-negative tumors in NSCLC. There was a significant difference between patients with tumors CD133 expression positive and negative group (*p*=0.006). The results of univariate and multivariate analyses were related to patient prognosis. Univariate
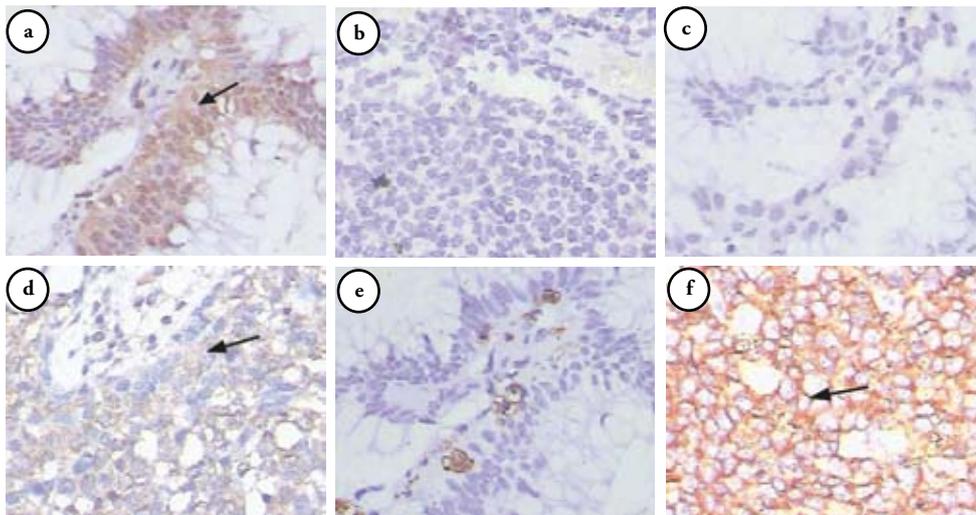


**Figure 2** - Representative immunohistochemical staining in B7-H3, CD133 and CD45 macroscopically: the expression of **a**) B7-H3-positive (arrow), **b**) B7-H3-negative, **c**) CD133-positive (arrow), and **d**) CD133-negative slides in non-small cell lung cancer. In the consecutive tumor sections, tumor-infiltrating lymphocytes were stained by anti-CD45 mAb (**e** & **f**, arrow) in the same section (×400).

**Table 1** - The expression of B7-H3 and CD133 in NSCLC, adjacent non-cancer and benign lesion tissue.

| Features | B7-H3 expression | | *P*-value | CD133 expression | | *P*-value |
|---|---|---|---|---|---|---|
| | Positive n (%) | Negative n (%) | | Positive n (%) | Negative n (%) | |
| NSCLC (n=102) | 71 (69.6) | 31 (30.4) | 0.000000005 | 51 (50.0) | 51 (50.0) | 0.000003 |
| Adjacent non-cancer tissue (n=25) | 3 (12.0) | 22 (88.0) | | 1 (4.0) | 24 (96.0) | |
| Benign lesion (n=24) | 5 (20.8) | 19 (79.2) | | 3 (12.5) | 21 (87.5) | |

Comparison of NSCLC and adjacent non-cancer tissue, *p*=0.0000002 (B7-H3), *p*=0.000008 (CD133)
Comparison of NSCLC and inflammatory pseudotumor *p*=0.00002 (B7-H3), *p*=0.001 (CD133)
Comparison of adjacent non-cancer tissue and inflammatory pseudotumor *p*=0.46 (B7-H3), *p*=0.28 (CD133)

**Table 2** - Relationship between B7-H3 and CD133 expression and pathological features of non-small cell lung cancer (NSCLC).

| Pathological features | Total cases | B7-H3 expression | | | CD133 expression | | |
|---|---|---|---|---|---|---|---|
| | | Positive | Negative | *P*-value | Positive | Negative | *P*-value |
| *Gender* | | | | 0.08* | | | 1.00* |
| Male | 66 | 50 | 16 | | 33 | 33 | |
| Female | 36 | 21 | 15 | | 18 | 18 | |
| Age | | | | 0.36* | | | 1.00# |
| ≤65 | 70 | 51 | 19 | | 35 | 35 | |
| >65 | 32 | 20 | 12 | | 16 | 16 | |
| *Smoking history* | | | | 1.00* | | | 0.55* |
| Positive | 48 | 33 | 15 | | 22 | 26 | |
| Negative | 54 | 38 | 16 | | 29 | 25 | |
| *Histology* | | | | >0.05† | | | <0.05† |
| Squamous cell carcinoma | 48 | 38 | 10 | | 21 | 27 | |
| Adenocarcinoma | 49 | 29 | 20 | | 25 | 24 | |
| Large cell lung carcinoma | 5 | 4 | 1 | | 5 | 0 | |
| *Differentiation* | | | | 0.10* | | | 0.02* |
| Well | 72 | 54 | 18 | | 30 | 42 | |
| Not well | 30 | 17 | 13 | | 21 | 9 | |
| *Pathological T factor* | | | | 0.35* | | | 1.00* |
| T1+T2 | 31 | 24 | 7 | | 16 | 15 | |
| T3+T4 | 71 | 47 | 24 | | 35 | 36 | |
| *Pathological N factor* | | | | 0.50* | | | 0.29* |
| N0 | 34 | 22 | 12 | | 14 | 20 | |
| N1+N2+N3 | 68 | 49 | 19 | | 37 | 31 | |
| *Pathological M factor* | | | | 0.10* | | | 1.00* |
| M0 | 95 | 64 | 31 | | 48 | 47 | |
| M1 | 7 | 7 | 0 | | 3 | 4 | |
| TILs | | 24±1.90 | 40±6.74 | 0.026# | 24±2.11 | 35±4.52 | 0.029# |

*Fisher's exact test, †cross-tabs analysis, #students t test, TILs - tumor-infiltrating lymphocytes

**Table 3** - The univariate and multivariate analyses of postoperative survival in non-small cell lung cancer (NSCLC) participants.

| Independent factors | Univariate | Multivariate | Hazard ratio | 95% confidence interval |
|---|---|---|---|---|
| | *P*-value | | | |
| *B7H3 expression* | | | | |
| Negative/positive | 0.02 | <0.001 | 0.108 | 0.038-0.305 |
| *Pathologic M factor* | | | | |
| Negative/positive | <0.001 | <0.001 | 19.06 | 6.365-57.074 |
| *CD133 expression* | | | | |
| Negative/positive | 0.006 | <0.001 | 5.653 | 2.576- 12.404 |

analysis demonstrated that postoperative survival was significantly related to B7-H3 expression, CD133 expression, and metastasis. Multivariate regression analysis found B7-H3 and CD133 expression to be an independent prognostic factor (*p*<0.001) **Table 3**.

**Discussion.** Invasion and metastasis of lung cancer are the leading causes of poor prognosis. Although the mechanisms of the occurrence and development on lung cancer have been explored for many years, the etiology and pathogenesis of tumor is still unknown, and effective treatment methods have still to be fully assessed. Recently, the discovery of CSCs characterized by self-renewal and the potential ability to invasion, metastasis, and recurrence of tumor, provides a new clue for targeted therapy against cancer. The CD133, as a tumor marker of CSCs, has played an important role in the mechanisms of solid tumors. The CD133+ tumor cells can cause resistant to chemotherapy and radiotherapy. Little is known on the immunological mechanism of CD133+ tumor cells. Wu et al[38] found that activation ligands of MHCI or NK cells did not express on CD133+ cells in astrocytoma and glioma, suggesting that tumor cells evade innate immunity and adaptive immune surveillance in this way. The CD133 has been studied in many kinds of tumors, including

gliomas, colon cancer, prostate cancer, liver cancer, melanoma, and breast cancer, but fewer in NSCLC. The B7-H3 mRNA is ubiquitously expressed in a variety of tissues as previous description.[3,16-20] In protein level, B7-H3 expression is inducible on several cell lines.[39] Our previous studies indicated that B7-H3 is expressed in all NSCLC cell lines at both mRNA and membrane protein levels.[36,40-42] The B7-H3 was expressed at high levels in tumors such as prostate cancer,[10,11] gastric cancer,[43] and NSCLC.[20] The results showed that, both B7-H3 and CD133 expressed in NSCLC tissues, higher than the adjacent non-cancer and benign lung lesions tissues significantly. The expression of CD133 is correlated with the cell differentiation level. The lower expression was found in high degree of differentiation, and higher in low degree of differentiation. Similar results were obtained with astrocytomas,[44] glioma,[45] and liver.[31,32] There was no relationship between B7-H3 expression and cell differentiation, consistent with other study.[20] The B7-H3-positive expression in SCC was significantly higher than in adenocarcinoma, and CD133-positive expression in large cell carcinoma was higher compared to SCC, suggesting that detection of B7-H3 expression in tumor tissue might contribute to the histologic differential diagnosis of NSCLC. The study also demonstrated that TILs in B7-H3 or CD133 -positive group was significantly lower than that in B7-H3 or CD133-negative group, suggesting that both B7-H3 and CD133 on tumor cells might inhibit infiltration of the TILs and induce apoptosis of TILs in NSCLC, and it may be a pathway of tumor evading host immune surveillance. We also found that CD133 and B7-H3 expression were related to postoperative prognosis of NSCLC participants. The overall survival rate of CD133 or B7-H3-positive expression group was lower than CD133 or B7-H3-negative group. The overall survival rate of CD133-positive or B7-H3-negative expression group lower than CD133-negative or B7-H3-positive group, and CD133-positive or B7-H3-negative was an independent prognostic risk factor by COX multivariate regression analysis. In addition, CD133 expression was negative, which is related to B7-H3 expression, suggesting that CD133+ tumor cells might evade the immune surveillance by down regulating B7-H3 expression and contribute to the infiltration of lymph nodes and metastasis. We found that murine B7-H3 bound to triggering receptor expressed on myeloid cells (TREM)-like transcript 2 (TLT-2). Hashiguchi et al[46] has shown that TLT-2 is a counter receptor for B7-H3, and the interaction of B7-H3 with TLT-2 on T cell activation.[46] But, a recent study found that there is no evidence of interaction or role for murine TREML2 as a receptor for human or murine B7-H3.[15] The B7-H3 extensive expression was found in pulmonary benign lesion tissue, but the lower level in alveolar epithelium may play an important role in inflammation immune response.

The limitation of this study was the use of a retrospective design, and the relationship between CD133, B7-H3, and mechanism of NSCLC needs prospective study.

In conclusion, we were successful in demonstrating the expression of B7-H3 and CD133 immunohistochemically in resected specimens of NSCLC. Moreover, we found that CD133 was related to tumor cell differentiation degree, and CD133 expression was negatively correlated with B7-H3 expression. Likewise, CD133 positive or B7-H3 negative was an independent risk factor of NSCLC patients. Intervention of B7-H3 and CD133 on gene level in NSCLC cell lines or animal models should be considered in future research.

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