Clinical importance of VEGFC and PD-L1 co-expression in lung adenocarcinoma patients

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Keywords
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Abstract

Background: Vascular endothelial growth factor C (VEGFC), an activator of lymphangiogenesis, is newly identified as an immunomodulator which can regulate the immune system so that tumor cells more easily escape immune surveillance. Evidence has shown programmed cell death-ligand 1 (PD-L1) can also suppress the immune response. Nevertheless, the clinical significance of co-expression of VEGFC and PD-L1 for predicting outcomes in patients with lung adenocarcinoma has not yet been determined.

Methods: A total of 114 patients with lung adenocarcinoma who underwent surgeries at Tianjin Medical University Cancer Institute and Hospital between December 2011 and September 2016 were retrospectively reviewed. Tissue specimens were collected for immunohistochemistry of VEGFC and PD-L1 which were analyzed with an H-score system.

Results: In this study, 57 (50.0%) and 47 (41.2%) patients were classified as VEGFC high expression and PD-L1 high expression. Co-expression was observed in 33 (28.9%) patients. In addition, a positive correlation was found between VEGFC and PD-L1 ($P = 0.0398$, $r = 0.1937$). In a univariate analysis, both progression-free survival (PFS) and overall survival (OS) were significantly worse in the VEGFC high expression group and the PD-L1 high expression group, respectively. Furthermore, VEGFC/PD-L1 co-expression showed a worse OS ($P = 0.03$) and PFS survival ($P = 0.01$) than the other groups.

Conclusions: Taken together, these results indicate that VEGFC/PD-L1 co-expression can forecast both poor OS and PFS in patients with resected lung adenocarcinoma. Co-expression of VEGFC and PD-L1 may serve as a significant prognostic factor for patients with lung adenocarcinoma.

Key points
VEGFC/PD-L1 co-expression forecasts poor survival in patients with resected lung adenocarcinoma. VEGFC/PD-L1 co-expression may be used as a prognostic indicator and provide the theoretical possibility to screen the optimal population with a combination of anti-VEGFC and anti-PD-L1 therapy in the clinical treatment.

Introduction

Lung cancer is recognized as the leading cause of cancer-related death worldwide. Lung adenocarcinoma is the most common subtype in non-small cell lung cancer (NSCLC), which accounts for 80%–85% of all lung cancer patients. As is known to all, targeted therapies for mutant
driver genes have improved clinical outcomes in certain patients. More recently, immunotherapy has emerged as an exciting alternative treatment for patients without an actionable driver mutation. However, the five-year survival rate for lung adenocarcinoma patients still remains unsatisfactory, partly because cancer immunotherapy is not completely effective in eradicating tumor cells because they escape from host immune scrutiny. To improve the efficacy of immunotherapy, there is an urgent need to find ideal immune-associated biomarkers to accurately assess the clinical decision, progression-free survival (PFS) and overall survival (OS) of patients with lung adenocarcinoma.

Vascular endothelial growth factor C (VEGFC), the classical lymphangiogenic factor, which acts mainly in developmental- and disease-associated lymphangiogenesis, has been newly identified as an immunomodulator which can regulate the immune system so that tumor cells more easily escape immune surveillance. Tacconi et al. reported VEGFC could enhance tumor growth via fostering cancer immune escape. Lund et al. demonstrated VEGFC could promote immune tolerance via suppression of CD8+ T cells. In addition, high expression of VEGFC has been reported to be significantly associated with poor prognosis in a variety of malignancies.

Programmed cell death-ligand 1 (PD-L1), which is widely expressed in immune cells, lymphatic endothelial cells, blood endothelial cells, tumor cells and so on, can suppress immune-response. High expression of PD-L1 is associated with an unfavorable survival in patients with lung cancer.

In the present study, we summarize the data of clinical characteristics of 114 cases of lung adenocarcinoma patients, explore the relationship between VEGFC and PD-L1 expression in patients with resected lung adenocarcinoma, and search for their predictive value in future immunotherapy for patients with lung adenocarcinoma.

**Methods**

**Patients**

A total of 114 patients diagnosed with lung adenocarcinoma (52 with wild-type, 48 with EGFR mutations, 10 with KRAS mutations and four patients with ALK mutations) were included in the study. All tumor samples were surgically resected in Tianjin Medical University Cancer Institute and Hospital from December 2011 to September 2016. The Ethics Committee of the Tianjin Medical University Cancer Institute and Hospital (Tianjin, China) approved the use of human tissues for this study (EK2018039). The study conforms to recognized standards of the Declaration of Helsinki and its outcomes will not affect the future management of the patients. Each patient signed an informed consent. Inclusion criteria were as follows: (i) Patients with an exact date of follow-up; (ii) no neoadjuvant treatment had been carried out before surgery; (iii) patients were stage I to stage III (AJCC/UICC TNM Classification and stage groupings). Clinical characteristics of the patients included age, gender, smoking status, gene mutation status, histological subtypes, clinical stage, postoperative treatments, PFS and OS. The clinical follow-up information was obtained from patients’ medical records.

**Immunohistochemical staining**

The 4 μm thick, formalin-fixed, paraffin-embedded tumors of clinical specimens of lung adenocarcinoma were deparaffinized in xylene and rehydrated in a graded series of alcohols, then rinsed three times with PBS. Antigen retrieval was performed in the pressure cooker at 130°C for three minutes, citrate buffer (PH 6.0) was used for VEGFC staining, and EDTA solution (PH 11.0) was used for PD-L1 staining. The slides were then incubated in 3% H2O2 for 15 minutes. For immunohistochemical staining the slides were incubated with primary antibodies against VEGFC (ab135506, Abcam, USA), 1:100, or against PD-L1/CD274 (66 248, Proteintech, USA), 1:1200, at 4°C, overnight. Incubation of secondary antibody and coloration were then carried out by EIVISON plus (kit-9903, MXB, China) and DAB kit (ZL1-9019, ZSGB-BIO, China), respectively. Counterstain was performed with hematoxylin for two minutes. Three clinical pathologists assessed the intensity of the immunostaining on each section independently in a blinded manner. At least 10 fields per specimen were surveyed.

**Immunohistochemical staining analysis**

VEGFC expression and PD-L1 expression in this study were scored with an H-score system (ranging from 0 to 300). Its specific calculation method was the sum of the intensity of staining (0 was negative; 1 was weak positive; 2 was moderate positive; 3 was strong positive) and the percentage of positive tumor cells (0%–100%, with any intensity of positive tumor cell staining). Two clinical pathologists graded the scores of each slide independently in a blinded manner. When considering the VEGFC expression, the cutoff value was set at 100, ie. H-score > 100 was identified as a VEGFC high expression case. According to previous studies, PD-L1 cutoff value was set at 100, ie. H-score >100 was considered to be a PD-L1 high expression case.

**Statistical analysis**

SPSS v.21 (IBM Corp, Armonk, NY, USA) and GraphPad Prism 6 (USA, GraphPad Software) were used for statistical analyses, and survival curve, respectively. Fisher’s exact
A two-tailed P-value <0.05 was considered statistically significant.

Results

Clinicopathological characteristics in patients with lung adenocarcinoma

A total of 114 patients with lung adenocarcinoma were included in our study cohort. The clinicopathological characteristics are shown in Table 1. There were 52 (45.6%) patients who were male, 48 (42.1%) patients were over 60-years-old, and 66 (57.9%) were smokers. At diagnosis, there were 83 (72.8%) stage I/II patients, 76 (66.7%) with acinar adenocarcinoma, and a total of 48 (42.1%) cases and 14 (12.3%) patients with EGFR mutations and other mutations, including KRAS and ALK, respectively. There were 57 (50.0%) and 47 (41.2%) patients with high VEGFC expression and high PD-L1 expression, respectively. There were 42 (36.8%) patients in the VEGFC−& PD-L1− group, 24 (21.1%) patients in the VEGFC+& PD-L1− group, 15 (13.2%) patients in the VEGFC−&PD-L1+ group and 33 (28.9%) patients in the VEGFC+& PD-L1+ group (Table S1). However, no significant correlation was observed for VEGFC or PD-L1 expression and other clinicopathological characteristics such as gender, age, smoking status, stage, histological subtypes, T factor, N factor, or gene status (all P > 0.05) (Table 2).

Analysis of patient survival

In all patients, the VEGFC high expression group exhibited a significantly worse impact on the OS (P = 0.04) (Fig 2a) and PFS (P = 0.004) (Fig 2b). Further investigation was
performed to analyze the correlation between VEGFC expression and survival in different subgroups. First, when considering patients in the wild-type (WT) subgroup, no significant effect was seen on OS in the high VEGFC expression group and the low VEGFC expression group (Fig 2c); however, high VEGFC expression showed a poor PFS ($P = 0.05$) compared to low VEGFC expression (Fig 2d). Second, we found high VEGFC expression was significantly correlated with OS ($P = 0.01$) (Fig 2e) and PFS ($P = 0.01$) (Fig 2f) in all patients with any gene mutations. We then found that high VEGFC expression had no significant effect on OS ($P = 0.42$) (Fig 2g) but had a significant impact on PFS ($P = 0.04$) (Fig 2h) in patients with $EGFR$ mutations. In addition, we found that high VEGFC expression was significantly correlated with poor survival (OS $P = 0.03$) ([Fig 3a] and PFS $P = 0.002$) ([Fig 2b]) in patients with stage I/II, similarly, in stage III with poor OS ($P = 0.02$) (Fig 3c) and PFS ($P = 0.02$) (Fig 3d). Finally, high VEGFC expression was significantly correlated with an adverse OS ($P = 0.003$) (Fig 3e) and PFS ($P = 0.01$) (Fig 3f) in acinar adenocarcinoma, and a poor PFS ($P = 0.01$) (Fig 3h) in nonacinar adenocarcinoma.

Moreover, we found high PD-L1 expression was significantly correlated with shorter OS ($P = 0.03$) (Fig 4a) and PFS ($P = 0.03$) (Fig 4b) when considering all patients in our cohort. Prognostic analysis in relation to PD-L1 expression was then performed in the different subgroups such as VEGFC. First, we found high PD-L1 expression

### Table 2 Relationship between PD-L1/VEGFC expression and the clinical characteristics in 114 patients with lung adenocarcinoma (SEM)

| Characteristics | PD-L1 negative, n (%) | PD-L1 positive, n (%) | P-value | VEGFC negative, n (%) | VEGFC positive, n (%) | P-value |
|----------------|-----------------------|-----------------------|---------|-----------------------|-----------------------|---------|
| Gender         |                       |                       |         |                       |                       |         |
| Male           | 32 (47.8)             | 20 (42.6)             | 0.583   | 28 (49.1)             | 24 (42.1)             | 0.452   |
| Female         | 35 (52.2)             | 27 (57.4)             |         | 29 (50.9)             | 33 (57.9)             |         |
| Age            |                       |                       |         |                       |                       |         |
| <60            | 42 (62.7)             | 24 (51.1)             | 0.216   | 31 (54.4)             | 35 (61.4)             | 0.448   |
| ≥60            | 25 (37.3)             | 23 (48.9)             |         | 26 (45.6)             | 22 (38.6)             |         |
| Smoking history|                       |                       |         |                       |                       |         |
| Yes            | 36 (53.7)             | 30 (63.8)             | 0.282   | 31 (54.4)             | 35 (61.4)             | 0.448   |
| No             | 31 (46.3)             | 17 (36.2)             |         | 26 (45.6)             | 22 (38.6)             |         |
| Stage          |                       |                       |         |                       |                       |         |
| I              | 43 (64.2)             | 26 (55.3)             | 0.401   | 32 (56.1)             | 37 (64.9)             | 0.626   |
| II             | 6 (9.0)               | 8 (17.0)              |         | 8 (14.1)              | 6 (10.5)              |         |
| III            | 18 (26.8)             | 13 (27.7)             |         | 17 (29.8)             | 14 (24.6)             |         |
| Histological types |                   |                       | 0.284   |                       | 0.116                 |         |
| Lepidic predominate |           | 24 (35.8)             |         | 23 (40.3)             | 18 (31.6)             |         |
| Acinar predominate |        | 23 (34.3)             |         | 12 (21.1)             | 22 (38.6)             |         |
| Papillary/micropapillary predominate | | 8 (11.9)              |         | 9 (15.8)              | 12 (21.0)             |         |
| Solid predominate |            | 5 (7.5)               |         | 4 (7.0)               | 2 (3.5)               |         |
| Other          | 7 (10.5)              | 5 (10.6)              |         | 9 (15.8)              | 3 (5.3)               |         |
| T              |                       |                       | 0.200   |                       | 0.624                 |         |
| T1             | 39 (58.2)             | 28 (59.6)             |         | 33 (57.9)             | 34 (59.7)             |         |
| T2             | 24 (35.8)             | 12 (25.5)             |         | 17 (29.8)             | 19 (33.3)             |         |
| T3             | 4 (6.0)               | 7 (14.9)              |         | 7 (12.3)              | 4 (7.0)               |         |
| N              |                       |                       | 0.669   |                       | 0.670                 |         |
| N0             | 48 (71.6)             | 30 (63.8)             |         | 39 (68.4)             | 39 (68.4)             |         |
| N1             | 3 (4.5)               | 3 (6.4)               |         | 4 (7.0)               | 2 (3.5)               |         |
| N2             | 16 (23.9)             | 14 (29.8)             |         | 14 (24.6)             | 16 (28.1)             |         |
| Gene status    |                       |                       | 0.280   |                       | 0.070                 |         |
| WT             | 32 (47.8)             | 20 (42.6)             |         | 26 (45.6)             | 26 (45.6)             |         |
| EGRF           | 23 (34.3)             | 25 (53.2)             |         | 25 (43.9)             | 23 (40.4)             |         |
| Other mutations |             | 12 (17.9)             |         | 6 (10.5)              | 8 (14.0)              |         |
| Postoperative therapy |                           |                       | 0.712   |                       | 0.843                 |         |
| Treatment      | 45 (67.2)             | 30 (63.8)             |         | 37 (64.9)             | 38 (66.7)             |         |
| Nontreatment   | 22 (32.8)             | 17 (36.2)             |         | 20 (35.1)             | 19 (33.3)             |         |
| VEGFC          |                       |                       |         |                       | 0.001***              |         |
| H-score ≤ 100  | 25 (37.3)             | 32 (68.1)             |         | 20 (35.1)             | 19 (33.3)             |         |
| H-score > 100  | 42 (62.7)             | 15 (31.9)             |         |                       |                       |         |

***P=0.001.
exhibited a poor OS ($P = 0.02$) (Fig 4a,c) and PFS ($P = 0.05$) (Fig 4d) when compared with patients in the PD-L1 low expression group in wild-type patients. Second, no significant correlation between high PD-L1 expression and OS ($P = 0.49$) was found, but an obvious relationship was found between high PD-L1 expression and PFS ($P = 0.04$) (Fig 4f) in patients with any gene mutation ($EGFR$, $KRAS$ or $ALK$). Third, we found that high PD-L1 expression had no important influence on OS ($P = 0.78$) (Fig 5a) and PFS ($P = 0.07$) (Fig 5b) in patients...
with EGFR mutations. However, we found high PD-L1 expression had a significant impact on OS (P = 0.002) (Fig 5c) and PFS (P = 0.004) (Fig 5d) in patients with KRAS mutations. Finally, we found high PD-L1 expression was not significantly correlated with adverse survival in patients with clinical stage I/II (Fig 6a,b), and in contrast was significantly correlated with poor OS (P = 0.03) (Fig 6c) and PFS (P = 0.01) (Fig 6d) in stage III. The same results were found in patients with acinar adenocarcinoma wherein high PD-L1 expression was significantly correlated with an adverse PFS (P = 0.02) (Fig 6f), but not to poor OS (P = 0.37) (Fig 6g) or PFS (P = 0.60) (Fig 6h) in patients with nonacinar adenocarcinoma.

Finally, we conducted combinatory analysis of VEGF-C and PD-L1 and found that the VEGF-C+&PD-L1+ group had worse OS (P = 0.03) (Fig 7a) and PFS (P = 0.01) (Fig 5b) when compared to the other three groups (VEGF-C−&PD-L1−, VEGF-C+&PD-L1− or VEGF-C−&PD-L1+). Additionally, there were no clinical features associated with VEGF-C/PD-L1 co-expression (Table S1 and S2).

In our univariate analysis on all lung adenocarcinoma patients, six clinicopathological characteristics were considered to be adverse prognostic factors for PFS: advanced T factor and N factor (>T2; >N0; all P < 0.001); clinical stage III (HR = 10.661 [95% CI 5.394–21.071], P < 0.0001); high VEGF-C expression (HR = 0.370 [95% CI 0.182–0.375], P = 0.004); high PD-L1 expression (HR = 1.979 [95% CI 1.030–3.800], P = 0.037), and VEGF/PD-L1 co-expression (HR = 2.749 [95% CI 1.410–5.361], P = 0.002). These factors were also determined as poor prognostic factors for OS. In our multivariate analysis, high expression of VEGF-C was an unfavorable prognostic factor for PFS (HR = 2.816 [95% CI 1.058–7.495], P = 0.038), and stage III was an adverse factor for both OS (HR = 3.516 [95% CI 1.278–9.679], P = 0.015) and PFS (HR = 8.884 [95% CI 3.287–24.015], P < 0.0001) (Table 3).

**Discussion**

Emerging evidence indicates that VEGF-C can modulate the immune system to facilitate tumor cells to more easily escape immune surveillance.5–7,21,22 In addition, PD-L1 can
also promote tumor cells to escape from host immune attack.\textsuperscript{23,24} To the best of our knowledge, the present research is the first study which exposes VEGFC and PD-L1 expression in patients with lung adenocarcinoma and evaluates the relationship between their expression and prognosis of lung adenocarcinoma.

First, we found high VEGFC expression was significantly associated with poor survival in patients with lung adenocarcinoma, which is concordant with the results of other studies.\textsuperscript{25,26} In our study, further analysis showed that in the gene mutations subgroup (patients with one of any three mutated genes) and stage I/II, stage III, acinar and nonacinar, there was a significant association between high expression of VEGFC and poor PFS.

Similar to VEGFC expression, we discovered PD-L1 high expression was closely related to poor survival in all patients. When analyzed in the subgroups, there was a significant association between PD-L1 expression and PFS in patients with any gene mutation wherein mutant EGFR accounted for 48/62 (77.4%), mutant KRAS accounted for 10/62 (16.1%) and mutant ALK accounted for 4/62 (6.5%). Further analysis showed that the KRAS mutation subgroup (16.1%) caused the statistical significance in the gene

Figure 6 Kaplan-Meier curves show (a, c, e and g) OS and (b, d, f and h) PFS of different subgroups (clinical stage III, III acinar or nonacinar adenocarcinoma, respectively) with high and low expression of PD-L1. (a) (-----) PD-L1\textsuperscript{low} (N = 50, eight events), and (-----) PD-L1\textsuperscript{high} (N = 34, seven events); (b) (-----) PD-L1\textsuperscript{low} (N = 50, six events), and (-----) PD-L1\textsuperscript{high} (N = 34, eight events); (c) (-----) PD-L1\textsuperscript{low} (N = 17, 10 events), and (-----) PD-L1\textsuperscript{high} (N = 13, 11 events); (d) (-----) PD-L1\textsuperscript{low} (N = 17, 11 events), and (-----) PD-L1\textsuperscript{high} (N = 13, 12 events); (e) (-----) PD-L1\textsuperscript{low} (N = 46, 13 events), and (-----) PD-L1\textsuperscript{high} (N = 30, 11 events); (f) (-----) PD-L1\textsuperscript{low} (N = 46, 11 events), and (-----) PD-L1\textsuperscript{high} (N = 30, 14 events); (g) (-----) PD-L1\textsuperscript{low} (N = 21, five events), and (-----) PD-L1\textsuperscript{high} (N = 17, five events); (h) (-----) PD-L1\textsuperscript{low} (N = 21, six events), and (-----) PD-L1\textsuperscript{high} (N = 17, six events).

Figure 7 Kaplan-Meier curves show (a) OS (-----) VEGFC\textsuperscript{+} & PDL1\textsuperscript{+} (N = 33, 14 events), (-----) VEGFC\textsuperscript{−} & PDL1\textsuperscript{−} (N = 42, 11 events), (-----) VEGFC\textsuperscript{+} & PDL1\textsuperscript{−} (N = 16, four events), and (-----) VEGFC\textsuperscript{−} & PDL1\textsuperscript{−} (N = 23, six events) and (b) PFS in patients with VEGFC\textsuperscript{−} & PDL1\textsuperscript{−}, VEGFC\textsuperscript{+} & PDL1\textsuperscript{−}, VEGFC\textsuperscript{−} & PDL1\textsuperscript{−} and VEGFC\textsuperscript{+} & PDL1\textsuperscript{−} expression (-----) VEGFC\textsuperscript{+} & PDL1\textsuperscript{+} (N = 33, 17 events), (-----) VEGFC\textsuperscript{−} & PDL1\textsuperscript{−} (N = 42, eight events), (-----) VEGFC\textsuperscript{−} & PDL1\textsuperscript{−} (N = 16, three events), and (-----) VEGFC\textsuperscript{+} & PDL1\textsuperscript{−} (N = 23, nine events).
Table 3 Univariate and multivariate cox analysis of factors for progression-free survival and overall survival in patients with lung adenocarcinoma (SEM)

| Variable                         | Overall survival | Progression-free survival |
|----------------------------------|------------------|---------------------------|
|                                  | HR 95% CI        | P-value                   | HR 95% CI          | P-value          |
| Gender (female vs. male)         | 1.407 0.723–2.740| 0.313                     | 1.157 0.607–2.206  | 0.657 0.0001     |
| Age (<60 vs. ≥60)                | 1.596 0.819–3.108| 0.166                     | 1.227 0.642–2.344  | 0.535 0.0001     |
| Smoking history (no vs. yes)     | 1.121 0.576–2.183| 0.737                     | 0.958 0.496–1.850  | 0.899 0.0001     |
| T factor (T ≤2 vs. T >2)         | 5.196 2.419–11.161| <0.0001****               | 5.322 2.466–11.487 | <0.0001****     |
| N factor (N0 vs. >N0)            | 3.052 1.560–5.969 | 0.001**                   | 3.733 2.774–10.405 | <0.0001****     |
| Gene mutations (wild-type vs. EGFR) | 0.958 0.472–1.948 | 0.907                    | 1.237 0.590–2.040  | 0.910 0.0001     |
| Histological subtypes (acinar vs. nonacinar) | 1.037 0.507–2.123 | 0.920 | 0.914 0.458–1.821  | 0.797 0.0001     |
| Stage (I vs. II)                | 4.935 2.532–9.656 | <0.0001****              | 10.661 5.394–21.071 | <0.0001****     |
| VEGFC expression (≤100 vs. >100) | 0.497 0.251–0.983 | 0.041*                    | 0.370 0.182–0.753  | 0.004***         |
| PD-L1 expression (≤100 vs. >100) | 2.038 1.035–4.014 | 0.036*                    | 1.979 1.030–3.800  | 0.037*           |
| Co-expression                   | 2.761 1.360–5.605 | 0.004***                  | 2.749 1.410–5.361  | 0.002***         |

*P=0.05; **P=0.01; ***P=0.001; ****P=0.0001.

**Table 3** Univariate and multivariate cox analysis of factors for progression-free survival and overall survival in patients with lung adenocarcinoma (SEM)

There are several limitations to this study. First, it was retrospective and conducted in our hospital with a small pool of patient samples. Second, it should be emphasized that this was an initial and immature study. Third, the underlying mechanisms need to be highlighted in future investigations.
In conclusion, both high VEGFC and PD-L1 expression indicate a poor prognosis in lung adenocarcinoma patients, and VEGFC is positively correlated with PD-L1. Furthermore, co-expression of VEGFC and PD-L1 led to a significantly worse prognosis among all four types (VEGFC+& PD-L1+, VEGFC+& PD-L1−, VEGFC−& PD-L1+ and VEGFC−& PD-L1−). In the future, VEGFC and PD-L1 co-expression may therefore be used as a prognostic indicator for the clinical outcome. In addition, our study also provides the theoretical possibility to screen the optimal population with a combination of anti-VEGFC and anti-PD-L1 therapy in lung adenocarcinoma.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships which have, or could be perceived to have, influenced the work reported in this article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

**Figure S1** Scatter diagram showing the correlation of VEGFC expression and PD-L1 expression based on the results of H-score.

**Table S1** Correlation of expression of VEGFC and/or PD-L1 and the clinical characteristics in 114 patients with lung adenocarcinoma (SEM).

**Table S2** Correlation of co-expression of VEGFC and PD-L1 and the clinical characteristics in 114 patients with lung adenocarcinoma (SEM).