Original Article

Expression of AKT and p-AKT protein in lung adenocarcinoma and its correlation with PD-L1 protein and prognosis

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Contributions: (I) Conception and design: ZY Hu, XL Li; (II) Administrative support: None; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: None; (V) Data analysis and interpretation: ZY Hu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Background: The PI3K/AKT/mTOR signaling pathway were significantly associated with EGFR mutation in lung adenocarcinoma (LUAD), but its correlation with PD-L1 protein and prognosis are not clear. The aim of this study was to evaluate the expression of AKT and phosphorylated AKT (p-AKT) in LUAD and its correlation with programmed death ligand-1 (PD-L1); and to analyze the factors affecting LUAD prognosis.

Methods: The expression of AKT, p-AKT, and PD-L1 was examined using immunohistochemistry in LUAD tissues from 110 patients who underwent surgical treatment.

Results: AKT protein expression was examined in 64.5% (71/110) of the LUAD samples, and p-AKT protein expression was examined in 44.5% (49/110) of the LUAD samples. The positive rate of PD-L1 at TC1/2/3 was 38.2% (42/110). AKT and p-AKT expression was significantly associated with epidermal growth factor receptor (EGFR) mutation (P=0.016, P=0.014 respectively). Pearson's correlation analysis indicated a negative correlation of p-AKT with PD-L1 protein (P=0.022). Out of the 62 patients with EGFR mutation, the expression of PD-L1 was negatively correlated with that of p-AKT protein (P=0.032). The expressions of AKT and p-AKT were not associated with prognosis. Multivariate analysis showed that tumor-node-metastasis (TNM) stage (P=0.013) and differentiation (P=0.046) were independent prognostic factors for overall survival.

Conclusions: PI3K/AKT/mTOR in the downstream pathway of EGFR may negatively regulate the expression of PD-L1, which may partly explain why patients with EGFR mutation respond poorly to PD-1/PD-L1 inhibitors.

Keywords: Lung adenocarcinoma (LUAD); AKT; p-AKT; PD-L1; protein expression; prognosis

Submitted Jul 19, 2020. Accepted for publication Sep 08, 2020.
doi: 10.21037/atm-20-5865
View this article at: http://dx.doi.org/10.21037/atm-20-5865

Introduction

Lung adenocarcinoma (LUAD) is the major pathological type of non-small cell lung cancer (NSCLC), with evidence indicating that its global incidence rate is on the rise (1). Despite advances in diagnostic and therapeutic techniques, the 5-year survival rate of LUAD remains unsatisfactory (2). Thus, there is an urgent need for further understanding the molecular mechanisms of LUAD and discovering new molecular targets for treatment. As one of the important signal pathways downstream of epidermal growth factor receptor (EGFR), the PI3K/AKT/mTOR signal pathway has been implicated in a large number of human malignancies including lung cancer, and are involved in tumor angiogenesis, cell proliferation, invasion, and migration. AKT, a serine/threonine protein kinase, is activated in response to activation by many different growth factors, including epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and others. P-AKT activates downstream signal factors such as mTOR, p7056k, and 4E-BP1, and transmits survival signals (3).

Therapy based on targeting immune checkpoints, especially anti-programmed death-1 and programmed death ligand-1 (anti-PD-1/PD-L1) monoclonal antibody monotherapy, has made significant progress in the treatment of advanced lung cancer. High expression of PD-L1 expression clearly predicts a better chance of benefit from PD-1/PD-L1 inhibitors, although a minority of PD-L1-negative patients will still benefit from these agents (4,5). One recent study suggested that patients with EGFR mutations had lower PD-L1 expression (6), while another previous study (7) showed that the presence of EGFR mutant-type is associated with low PD-L1 expression, and the response rate to PD-1/PD-L1 inhibitors is lower than that of wild-type patients. Therefore, it is essential to clarify the PD-L1 expression regulation mechanism in order to obtain more clinical benefits. PI3K/AKT/mTOR pathway, one of the important signal pathways downstream of EGFR, serves as a convergence point for activation of many of the oncogenes involved in NSCLC. Previous studies have suggested that PI3K/AKT/mTOR pathway may be responsible for regulating PD-L1 expression. NSCLC cell lines with KRAS, EGFR, BRAF, ALK, or RET mutations have been found to express high levels of PD-L1, and this may be linked to a high level of PI3K/AKT/mTOR pathway activation (8). Another previous study showed that activation of the PI3K/AKT/mTOR pathway increased PD-L1 expression in NSCLC (9). A recent study showed that ALK translocation upregulated PD-L1 expression by activating AKT pathways (10). However, in the study by Chen *et al.* (11), no clear correlation was found between PD-L1 protein and PI3K/AKT/mTOR pathway in NSCLC. The above research results are conflicting, and more studies are needed to further to clarify the relationship between the PI3K/AKT/mTOR pathway and PD-L1. Therefore, in our study, AKT and p-AKT protein were examined by immunohistochemistry, and its correlation with PD-L1 protein was investigated to elucidate the underlying correlation.

We present the following article in accordance with the REMARK reporting checklist (available at http://dx.doi.org/10.21037/atm-20-5865).

Methods

LUAD specimens

Primary LUAD tissues were collected from 110 patients after curative surgical resection which included mediastinal lymph node dissection at the Liaoning Cancer Hospital and Institute from June 2014 through July 2015. The paraffin tissue blocks of the lung adenocarcinoma patients were stored at room temperature at 4 degrees. The patients were included in the study if the following criteria were fulfilled: (I) the patients with newly diagnosed lung adenocarcinoma cancer who were treated by surgery; (II) the patients who did not receive neoadjuvant therapy before surgery; (III) the patients who underwent EGFR gene mutation test (ARMS test). Exclusion criteria: (I) the patients with incomplete histological and clinical medical records; (II) the patients with neoadjuvant therapy before surgery; (III) the patients with no EGFR gene mutation testing; (IV) the patients with other malignant tumor treatments in the past. Patients included 44 males and 66 females. The average age at diagnosis was 58.6 years (range, 34–78 years). Tumor-node-metastasis (TNM) staging revealed stage I in 58 cases, stage II in 16 cases, and stage III in 36 cases. Lymph node metastasis was observed in 47 cases, while the other 63 cases showed no lymph node metastasis. The tumor diameter was ≤3 cm in 85 cases and >3 cm in 25 cases. Poor, moderate, and high differentiation was observed in 28, 44, and 38 cases, respectively. Meanwhile, 62 patients harbored EGFR mutation, while the remaining 48 patients were EGFR wild type. The follow-up period ranged from 5.3 to 56.7 months, with a median time of 46.5 months. At the end of follow-up, 18 patients (16.4%) were lost to

Table 1 Clinicopathological characteristics of patients with lung adenocarcinoma

Characteristic	Number	%
Age		
<60	56	50.9
≥60	54	49.1
Gender		
Male	44	40.0
Female	66	60.0
Smoking status		
Yes	37	33.6
No	73	66.4
Stage		
I	58	52.7
II	16	14.5
III	36	32.7
Tumor size (cm)		
≤3	85	77.3
>3	25	22.7
Tumor differentiation		
Poorly differentiated	28	25.5
Moderately differentiated	44	40.0
Well differentiated	38	34.5
Lymph node metastasis		
Yes	47	42.7
No	63	57.3
EGFR status		
Mutant	62	56.4
Wild type	48	43.6
PD-L1 expression		
Positive	42	38.2%
Negative	68	61.8%

EGFR, epidermal growth factor receptor.

follow up and were treated as censored data. This study was approved by the Ethics Committee of Liaoning Cancer Hospital and Institute (Shenyang, China). Informed consent for the experiments were signed by all participants before the study. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The general clinical characteristics of the patients are presented in *Table 1*.

Immunohistochemistry (IHC)

IHC staining for AKT, p-AKT, and PD-L1 proteins were performed using the streptavidin-peroxidase method (SP method). Briefly, paraffin-fixed slides were delayed in xylene and dehydrated through graded alcohols. Antigen retrieval was carried out by boiling in a pressure cooker in a citric acid buffer (pH 6.0) at 120 ℃ for 2 min and 30 s. Rabbit anti-AKT polyclonal antibody (1:100, AF6261, Affinity Biosciences, USA), rabbit anti-phospho-AKT (Ser473) polyclonal antibody (1:100, AF0908, Affinity Biosciences, USA), and rabbit anti-PD-L1 monoclonal antibody (SP142, Gene Tech Company Limited, China) were incubated on sections for 1 h at room temperature. Sections were then incubated with the appropriate secondary antibodies at room temperature for 20 min. After sufficient phosphate-buffered saline (PBS) rinses, diaminobenzidine was used as chromogen, and the sections were counterstained with hematoxylin. The slides were counterstained with hematoxylin, and then dehydrated and coverslipped.

Evaluation of immunohistochemical staining

All slides were blindly interpreted by two independent observers. Prostate cancer sections with high Gleason score and high levels of AKT and p-AKT served as positive controls (12). Sections were immunostained with the omission of the primary antibody as a negative control. The scoring for AKT expression level was determined in the following fashion (13): absence of staining or <10% tumor cell staining was scored as negative (–); tumor cell staining of ≥10% and <50% was scored as positive (+); and tumor cell staining of ≥50% was scored as strongly positive (++). The scoring for p-AKT (14) was as follows: negative (–), no staining; 1+, weak, homogeneous cytoplasmic staining without a granular staining pattern; 2+ and 3+, strong granular cytoplasmic staining with the 2+ indicating staining in <20% of tumor cells and 3+ indicating staining in >20% of tumor cells. We regarded (–) and (1+) as negative expression, while (2+) and (3+) represented positive expression. Placental specimens with PD-L1 expression were used as a positive control. We scored tumor cells expressing PD-L1 as a percentage of total tumor cells. TC1/2/3 was defined as PD-L1 expression on 1% or more

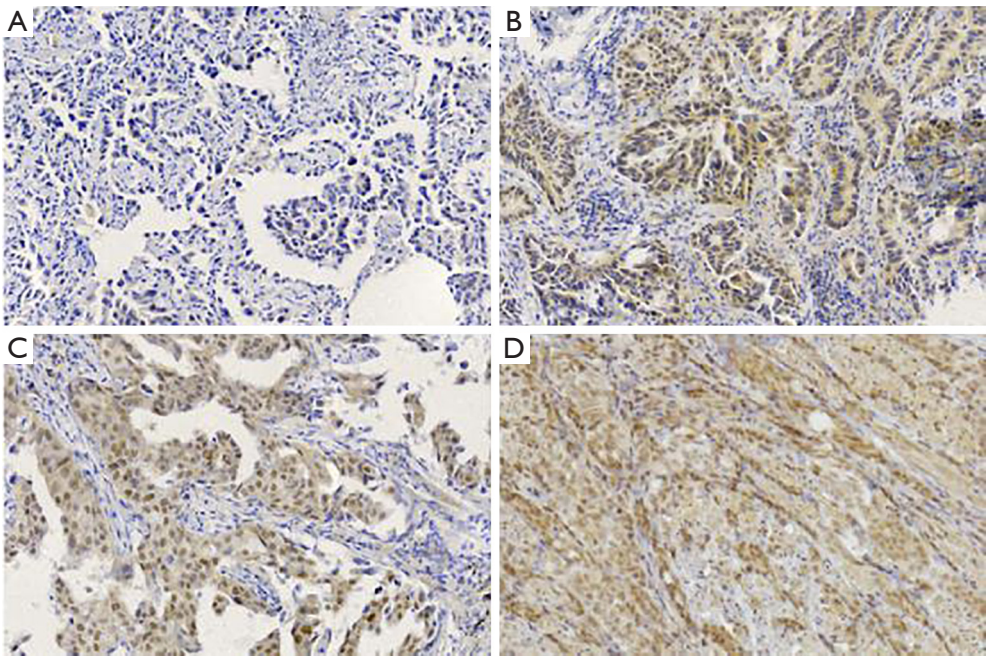

Figure 1 AKT expression in (A) lung adenocarcinoma, (B) lung adenocarcinoma cytoplasm, (C) lung adenocarcinoma nuclei, and (D) prostate cancer. The illustrated sections were interpreted as positive (B, C, and D) or negative (A) for AKT expression. Magnification, ×200.

of tumor cells, TC2/3 was defined as PD-L1 expression in 5% of tumor cells, TC3 was defined as PD-L1 expression in 50% or more of tumor cells, and TC0 was defined as PD-L1 expression in fewer than 1% of tumor cells. We regarded TC0 as a negative expression, while TC1/2/3 represented positive expression (15).

Statistical analysis

The experimental data were analyzed by SPSS 20.0 software (IBM, Armonk, NY, USA). The relationships between clinical parameters and expression levels of AKT and p-AKT were analyzed by the χ^2 test or Fisher's exact test. The correlation between AKT, p-AKT, and PD-L1 were analyzed by Pearson's correlation analysis. Kaplan-Meier survival curves and the log-rank test were used to analyze overall survival. A cox regression model was utilized to analyze the relationships between the independent risk factors and patient prognosis. Statistical significance was set at $P<0.05$.

Results

Expression of AKT, p-AKT, and PD-L1

AKT protein expression was localized mainly in the cytoplasm and was observed in 64.5% (71/110 cases) of the LUAD samples (*Figure 1*). The p-AKT protein was expressed in the cytoplasm or nucleus, and was detected in 44.5% (49/110 cases) of the tumor samples (*Figure 2*). IHC analysis showed that PD-L1 staining was mainly concentrated in the LUAD cytoplasm and cell membrane (*Figure 3*). Among the 110 patients, the positive rate of LUAD cells for PD-L1 at TC1/2/3 (positive percentage of tumor cells greater than or equal to 1%) was 38.2% (42/110 cases), PD-L1 in TC2/3 (positive rate of tumor cells greater than or equal to 5%) was 34.5% (38/110 cases), and PD-L1 in TC3 (positive percentage of tumor cells greater than or equal to 50%) was 14.5% (16/110 cases).

Correlation of AKT and p-AKT expression with protein and clinical pathology characteristics

In the current research, 62 patients harbored the EGFR-sensitive mutation while the remaining 48 cases were EGFR wild type. The expression of AKT and p-AKT protein was correlated with EGFR mutation (P=0.016, P=0.014, respectively), regardless of sex, age, lymph node metastasis, degree of differentiation, or TNM stage (*Table 2*).

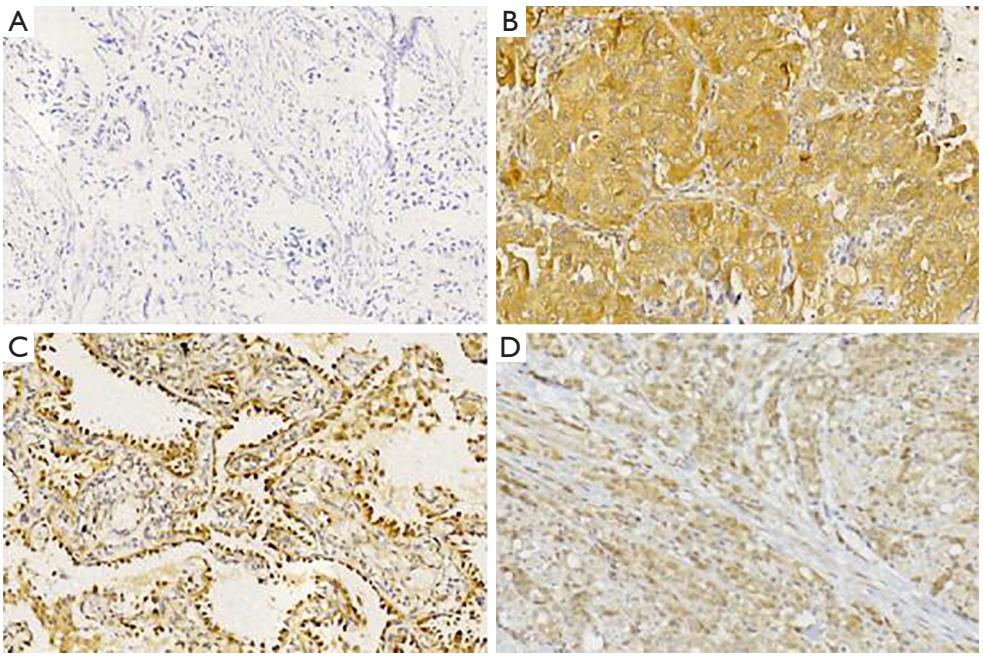

Figure 2 p-AKT expression in (A) lung adenocarcinoma, (B) lung adenocarcinoma cytoplasm, (C) lung adenocarcinoma nuclei, and (D) prostate cancer. The illustrated sections were interpreted as positive (B, C, and D) or negative (A) for p-AKT expression. Magnification, ×200. p-AKT, phosphorylated AKT.

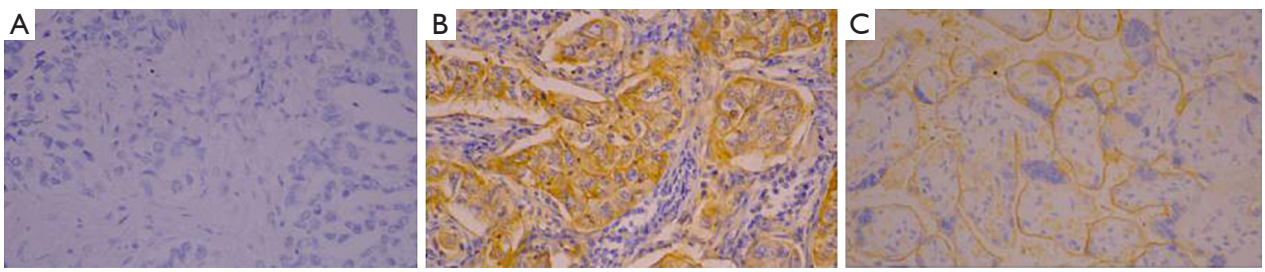

Figure 3 PD-L1 expression in (A) lung adenocarcinoma, (B) lung adenocarcinoma cytoplasm, and (C) placental specimens. The illustrated sections were interpreted as positive (B and C) or negative (A) for PD-L1 expression. Magnification, ×400.

The correlation between AKT, p-AKT, and PD-L1 expression

Pearson's correlation analysis showed that the expression of AKT was not correlated with that of PD-L1 protein (r=–0.025, P>0.05). A negative correlation was found between p-AKT and PD-L1 protein expression in LUAD (r=–0.128, P=0.022). Out of the 62 patients with EGFR mutation, the expression of PD-L1 was negatively correlated with that of p-AKT protein (r=–0.272, P=0.032) (*Figure 4*).

Survival analysis

For the 110 patients with LUAD, median overall survival was not reached. The 3-year overall survival rate for the 110 patients was 82.3%. Univariate analysis indicated that TNM stage (P=0.000), lymph node metastasis (P=0.000), tumor differentiation (P=0.048), and tumor diameter (P=0.000) were significantly associated with poor overall survival (*Figure 5*). There were no significant differences in AKT (P=0.458), p-AKT (P=0.612), gender (P=0.794), age (P=0.123), smoking (P=0.427), and EGFR gene status

Table 2 Correlation between AKT and p-AKT expression and clinicopathological factors

Characteristics	AKT expression			p-AKT expression		
	Positive (%)	Negative (%)	P	Positive (%)	Negative (%)	P
Age			0.392			0.258
<60	34 (60.7)	22 (39.3)		22 (39.3)	34 (60.7)	
≥60	37 (68.5)	17 (31.5)		27 (50.0)	27 (50.0)	
Gender			0.329			0.814
Male	26 (59.1)	18 (40.9)		19 (43.2)	25 (56.8)	
Female	45 (68.2)	21 (31.8)		30 (45.5)	36 (54.5)	
Smoking status			0.102			0.538
Yes	20 (54.1)	17 (45.9)		18 (48.6)	19 (51.4)	
No	51 (69.9)	22 (30.1)		31 (42.5)	42 (57.5)	
Stage			0.266			0.091
I	35 (60.3)	23 (39.7)		31 (53.4)	27 (46.6)	
II	9 (56.2)	7 (43.8)		4 (25.0)	12 (75.0)	
III	27 (75.0)	9 (25.0)		14 (38.9)	22 (61.1)	
Tumor size (cm)			0.589			0.151
≤3	56 (65.9)	29 (34.1)		41 (48.2)	44 (51.8)	
>3	15 (60.0)	10 (40.0)		8 (32.0)	17 (68.0)	
Degree of differentiation			0.151			0.976
Low	19 (67.9)	9 (32.1)		12 (42.9)	16 (57.1)	
Middle	32 (72.7)	12 (27.3)		20 (45.5)	24 (54.5)	
High	20 (52.6)	18 (47.4)		17 (44.7)	21 (55.3)	
Lymph node metastasis			0.283			0.056
Yes	33 (70.2)	14 (29.8)		16 (34.0)	31 (66.0)	
No	38 (60.3)	25 (39.7)		33 (52.4)	30 (47.6)	
EGFR status			0.016*			0.014*
Mutant	46 (74.2)	16 (25.8)		34 (54.8)	28 (45.2)	
Wild type	25 (52.1)	23 (47.9)		15 (31.3)	33 (68.7)	

*, P<0.05. p-AKT, phosphorylated AKT; EGFR, epidermal growth factor receptor.

(P=0.470). By multivariate analysis, TNM stage (P=0.013) and degree of differentiation (P=0.046) were revealed to be independent prognostic factors for overall survival.

Discussion

The investigation of molecular mechanisms underlying lung cancer development and progression is vital. A growing body of evidence indicates that AKT is a cytosolic signal transduction protein that figures prominently in mechanisms of carcinogenesis and chemoresistance. The present study revealed that the positive staining of AKT and p-AKT was observed predominantly in the cytoplasm and occasionally in the nucleus. AKT is overexpressed in various types of human cancer such as lung cancer, breast cancer, and prostate cancer (12,13,16,17). Previous studies have reported positive rates of AKT between 52.1% and 93.3% (13,18,19) and positive rates of p-AKT between 33.3% and

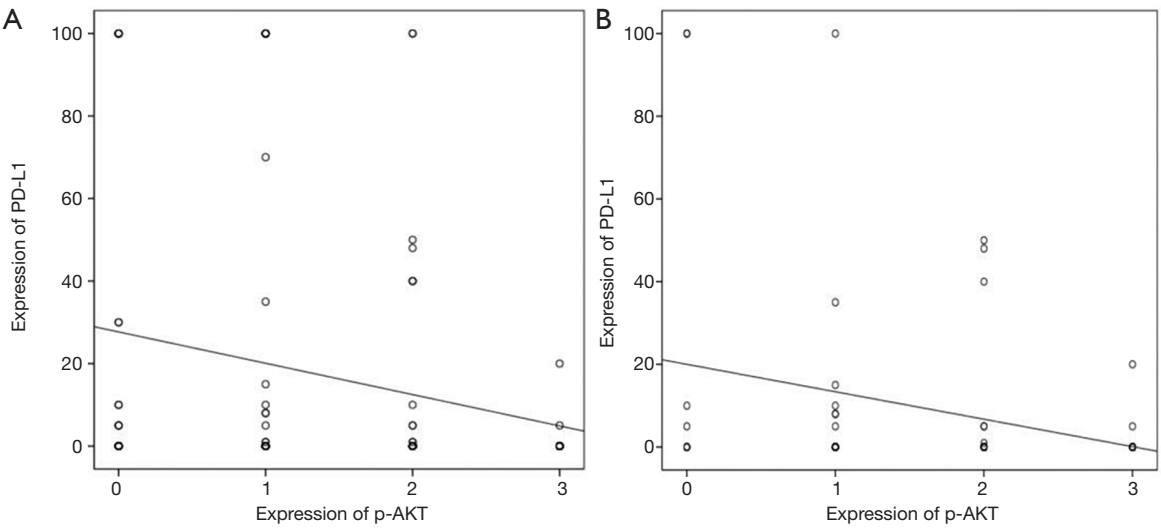

Figure 4 Correlation of PD-L1 and p-AKT expression in: lung adenocarcinoma (A), lung adenocarcinoma with the EGFR mutation (B). p-AKT, phosphorylated AKT; EGFR, epidermal growth factor receptor.

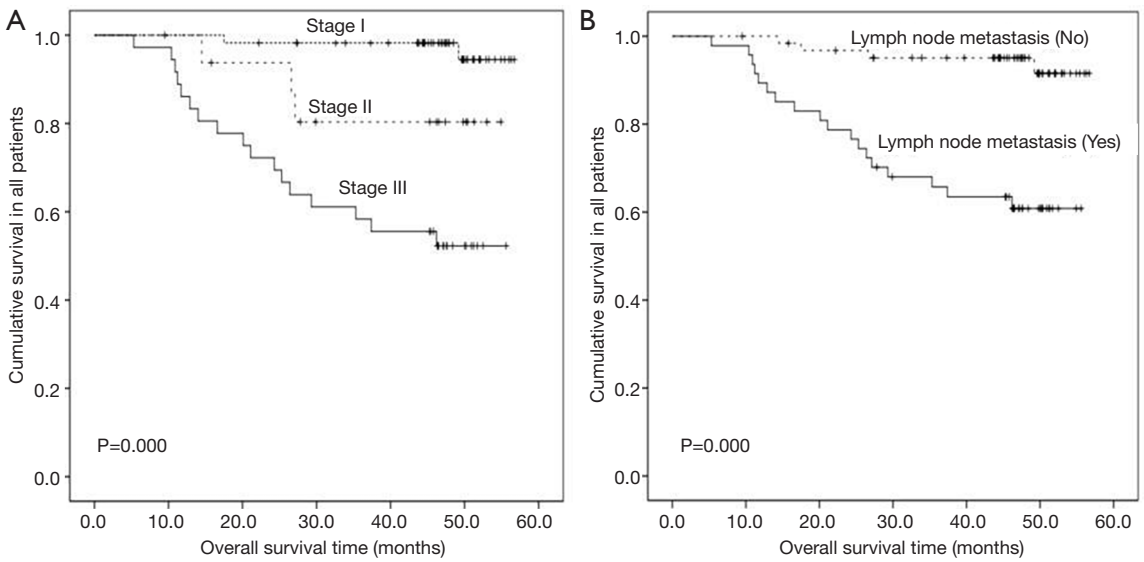

Figure 5 Kaplan-Meier survival curves for overall survival rate and the results of the log-rank test.

87.5% (13,20-22). Our data revealed that AKT and p-AKT were highly expressed in LUAD tissues, which is consistent with previous reports. Moreover, AKT and p-AKT were significantly associated with EGFR mutation, which is in line with the results of Sordella *et al.* (23) and Zhao *et al.* (16). This indicates that EGFR mutation can activate the downstream PI3K/AKT/mTOR signaling pathway, and lead to the overexpression of AKT and p-AKT, thereby promoting the development of lung cancer. Previous studies (16,21,22) have found that the expression of p-AKT in NSCLC is closely related to poor differentiation and lymph node metastasis, and it is believed that the activation of AKT plays an important role in the development of lung cancer. Other studies, however, have observed the opposite effect. Mukohara *et al.* (24) found that the expression of p-AKT was associated with high differentiation and early stage, but not lymph node metastasis. In addition, Tsao *et al.* (25) showed that p-AKT was highly expressed in

NSCLC tissues and premalignant bronchial epithelial tissues, but was barely expressed in normal cells, suggesting that AKT activation is involved in the precancerous lesion process of bronchial epithelial cells, which precipitates the occurrence of NSCLC. In the present study, no correlation between AKT and p-AKT expression and stage, degree of differentiation, and lymph node metastasis was found. The variability in pathological types and clinicopathological features studied may be the cause of these inconsistent conclusions.

There is still controversy concerning the significance of AKT-positive expression for the prognosis of NSCLC. Most studies suggest that the overactivation of AKT predicts a poor prognosis for NSCLC (20,21,26,27), but Shah *et al.* (28) and Cappuzzo *et al.* (29) believe that patients with high p-AKT expression have good prognosis. In addition, some studies have concluded that p-AKT-positive expression is not associated with the prognosis of NSCLC (14,30,31). Our results suggested that the positive expression of AKT and p-AKT protein in LUAD was not associated with prognosis. Many factors can contribute to conflicting research results, such as a heterogeneity in the clinical and pathological features of the study subjects, the number of selected samples, experimental methods, experimental reagents, and interpretation criteria.

Research into PD-1/PD-L1 pathway is currently the fastest growing and most mature of the studies into immunological checkpoint pathways being conducted, and this research has figured prominently in tumor immunotherapy. The importance of PD-L1 is reflected by the antitumor activity observed when PD-L1 blocking antibodies are used. This cell surface protein is upregulated in many epithelial tumors such as breast cancer (32), colon cancer (33), and NSCLC (34). Our study showed that the positive rate of PD-L1 expression in LUAD cells was 38.2%, which was consistent with a previous study (35). However, the regulation mechanism of PD-L1 expression is unclear, the multiple mechanisms may be involved which includes the status of underlying transcriptional and signaling networks. The published study showed that EGFR mutation was correlated with lack of T-cell infiltration, decreased PD-L1 expression and lower TMB, which were predictive biomarkers for PD-1 blockade immunotherapy (6), however, the molecular mechanism of PD-L1 mediated by EGFR in NSCLC was not illustrated in this paper. Another report showed that PD-L1 expression was regulated by ERK, AKT, and STAT3 signaling pathways in NSCLC with ALK translocation (10). Our study found that LUAD with EGFR mutation had high expression of p-AKT and low level of PD-L1 protein, and the expression of p-AKT protein negatively correlated with PD-L1. This finding differs from that of previous study, which found that PI3K/AKT/mTOR increases the expression of PD-L1 (8). This difference is likely due to the heterogeneity of the study populations. According to the results of our study, we speculated that mutated EGFR activated the downstream PI3K/AKT/mTOR signal pathway, upregulated the expression of p-AKT protein and then negatively regulated the expression of PD-L1, which to some extent explains why NSCLC patients with positive EGFR-sensitive mutation respond poorly to PD-1/PD-L1 inhibitors. Conversely, we speculate that PD-L1 expression may be upregulated after EGFR-TKI treatment, and post-TKI treatment patients may benefit from PD-1/PD-L1 inhibitors. This potential for clinical benefit supports efforts to study the mechanisms that regulate PD-L1 expression and therapeutic interventions to increase PD-L1 levels. The components of PI3K/AKT/mTOR signaling pathway are complex, and there are many cascade-effect proteins in the pathway, which are involved in the physiological and pathological process of cells. In this study, only the correlation between AKT, p-AKT, and PD-L1 was analyzed, while the correlations between other signal transduction proteins and PD-L1 are not yet clear, and should be further studied.

Our study has several limitations. The subjects of this cohort were patients with stage I–III LUAD after radical operation, with stage I–II patients accounting for a relatively high proportion; there was also a lack of stage IV patients. The sample size of this study was relatively small, consequently, these results required further validation in a large cohort. But it showed a trend that LUAD patients with EGFR mutation had lower PD-L1 expression, which was consistent with previous studies (6,7). In addition, we only analyzed the relationship between AKT, p-AKT, and PD-L1 in the archive wax block at the protein level. In the follow-up, we can explore the correlation of p-AKT and PD-L1 in LUAD at the cellular level and gene level to clarify the underlying regulatory mechanism.

In conclusion, the present study demonstrated that AKT and p-AKT were overexpressed and positively correlated with EGFR mutation in LUAD tissues. A negative correlation was established between p-AKT and PD-L1 protein, which was also found in LUAD cases with the EGFR mutation. These data suggest that EGFR mutation activates the downstream PI3K/AKT/mTOR signaling

pathway, and then decreases the expression of PD-L1. This partly explains why patients with EGFR mutation respond poorly to PD-1/PD-L1 inhibitors. Post–EGFR-TKI-treatment patients may thus benefit from PD-1/PD-L1 inhibitors, as their PD-L1 expression may be upregulated. Despite these promising findings, a large number of clinical studies are still required to further verify our conclusions.

Acknowledgments

The authors gratefully acknowledge the time and energy contributed by the participants.
Funding: None.

Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at http://dx.doi.org/10.21037/atm-20-5865

Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-5865

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-5865). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The present study was approved by the Ethics Review Committee of the Cancer Hospital of China Medical University (Shenyang, China). Written informed consent was obtained from all patients included within the present study. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

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Cite this article as: Hu ZY, Huang WY, Zhang L, Huang B, Chen SC, Li XL. Expression of AKT and p-AKT protein in lung adenocarcinoma and its correlation with PD-L1 protein and prognosis. Ann Transl Med 2020;8(18):1172. doi: 10.21037/atm-20-5865

