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# Programmed death-ligand 1 expression associated with molecular characteristics in surgically resected lung adenocarcinoma

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## Abstract

**Background:** Several clinical trials have shown that immune treatment focus on programmed death-1 and programmed death-ligand 1 (PD-L1) yields a good clinical efficacy in advanced non-small cell lung cancer (NSCLC). We investigated whether the PD-L1 expression was related to clinicopathologic and molecular characteristics in patients with surgically resected NSCLC.

**Methods:** Between December 2008 and 2013, formalin-fixed, paraffin-embedded samples were obtained from patients with lung adenocarcinoma at Zhejiang Cancer Hospital. RT-PCR was used to analyze *EGFR*, *KRAS*, *NRAS*, *PIK3CA*, *BRAF*, *HER2* mutations and *ALK*, *ROS1*, *RET* fusion genes. The PD-L1 expression was evaluated by immunohistochemistry and staining of 5 % or more was scored as positive expression. Survival analysis was evaluated using the Kaplan-Meier method. Multivariate regression was performed using the Cox proportional hazards model.

**Results:** Mutations were detected in 76.6 % of the 385 patients tested: *EGFR* mutation (n = 205, 53.2 %), followed by *EML4-ALK* rearrangement (n = 18, 4.7 %), *KRAS* (n = 16, 4.2 %), *HER2* (n = 9, 2.3 %), *ROS1* rearrangement (n = 8, 2.1 %), *PIK3CA* (n = 6, 1.6 %), *RET* rearrangement (n = 6, 1.6 %), *BRAF* (n = 2, 0.5 %), and *NRAS* mutations (n = 1, 0.2 %). Twenty-four (6.2 %) patients carried coexisting mutations. PD-L1 expression was detected in 48.3 % (186/385) of all the patients. PD-L1 positive patients more frequently carried coexisting mutations (18/24, 75 %), followed by single-gene (145/271, 53.5 %) and pan-negative mutations (23/90, 25.6 %). PD-L1 expression decreased disease-free survival (DFS) in univariate analysis ( $P = 0.014$ ). Multivariate analysis revealed that PD-L1 expression was not an independent risk factor for poor DFS and overall survival (OS) ( $P = 0.22$  and  $0.37$ , respectively).

**Conclusions:** PD-L1 overexpression is more frequently observed in oncogene-mediated lung adenocarcinoma, especially with coexisting mutation subtypes. PD-L1 expression is not a prognostic factor in surgically resected lung adenocarcinoma patients.

**Keywords:** Non-small cell lung cancer, Programmed cell deathligand 1, Lung adenocarcinoma, Gene mutation, Coexisting mutations, Prognosis

## Background

Lung cancer is the leading cause of cancer-related death in China [1]. The standard treatment of lung cancer, especially non-small cell lung cancer (NSCLC) comprises platinum-based chemotherapy and driver gene-based targeted therapy, which resulted in extended survival

and increased the quality of life in NSCLC patients [2–7]. However, drug resistance is a major challenge in most patients [8]. The median survival time in advanced NSCLC is no more than 2 years because of limited treatments available excluding chemotherapy and targeted therapy [9, 10].

Blockade of immune checkpoints in cancer with monoclonal antibodies has recently emerged as a promising approach to the treatment of solid tumors. Programmed death 1 (PD1), which belongs to the CD28 family of

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proteins, is a T cell surface receptor that regulates T cell activation and proliferation. Its ligand, programmed death-ligand 1 (PD-L1), is frequently expressed in many types of carcinomas [11–14]. Recent clinical trials found that inhibition of the PD-L1-PD1 interaction using specific antibodies resulted in promising antitumor efficacy in patients with various carcinomas [15, 16]. PD-L1 overexpression in NSCLC was reported ranging from 19 to 100 % [17–19]. Although several studies elucidated the association between common driver genes and PD-L1 expression in NSCLC, the results remain controversial and the prognostic value of PD-L1 expression is unclear [20].

This study focused on patients with completely resected lung adenocarcinoma and evaluated the association of PD-L1 expression with clinicopathologic parameters and driver genes, as well as its prognosis value in Chinese patients.

## Patients and methods

### Patients

A total of 385 adenocarcinoma patients underwent resection between December 2008 and 2013 in Zhejiang Cancer Hospital. Histological typing was determined according to the 2004 World Health Organization classification [21]. Tumor-node-metastasis (TNM) staging was based on the 7th edition of the lung cancer staging system. The recurrence or metastases were confirmed using chest CT, brain MRI, and bone scan as well as ultrasound and/or CT of the abdomen. The exclusion criteria included: (1) preoperative chemotherapy or radiation therapy, (2) death from other diseases unrelated to NSCLC. *The Ethics Committee of Zhejiang Cancer Hospital approved this study and written informed consent was obtained from each participant.*

### Immunohistochemical analysis of PD-L1 expression

Immunohistochemical (IHC) staining of PD-L1 expression was performed on 4–6  $\mu$ m thick formalin-fixed, paraffin-embedded tissue. The concentration of rabbit primary antibody that reacts to PD-L1 The concentration of rabbit primary antibody that reacts to PD-L1 (**Proteintech Group Inc., Chicago, IL, USA**, Catalog number: 66248-1-Ig) was 1:100 in Dako antibody diluent; slides were incubated with this antibody overnight at 4 °C. Then, the slides were incubated with Ventana Omni Mapanti-rabbit secondary antibody for 60 min. AVentana Chromo MapKit was used for antibody detection, and then the slides were counterstained with hematoxylin. Next, the slides were dehydrated and cover slipped as per normal laboratory protocol. Two independent pathologists (Wei Wu and Guoping Cheng) assessed the expressions.

PD-L1 immunostaining results were classified into two groups based on the degree and intensity of staining: (1)

negative, when staining was absent or detected in <5 % of the cells; and (2) positive, when membranous staining was present in  $\geq$ 5 % of the cells.

We used another antibody (5H1, Cell Signaling Technology, Beverly, MA, USA) to confirmed the PD-L1 expression in 102 patients. The PD-L1 immunostaining criterion is same with the former antibody.

### Gene analysis

Genomic DNA or RNA was extracted from tumor tissues according to standard protocols (RNeasy Mini Kit, and QiAamp DNA Mini Kit, Qiagen, Hilden, Germany). Briefly, the isolated RNA samples were used for reverse transcription into cDNA using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany). Either genomic DNA or cDNA was used for PCR amplification and sequencing. EGFR, HER2, KRAS, NRAS, BRAF, and PIK3CA were PCR amplified using genomic DNA. Cycle sequencing of the purified PCR products was carried out with PCR primers using the commercially available ADx Mutation Detection Kits (Amory, Xiamen, China).

The ALK, ROS1, and RET fusion mRNA was detected by PCR with fusion gene detection kit (Amory, Xiamen, China). In brief, total RNA was extracted with Qiagen-RNeasy FFPE Kit. The mRNA was reverse-transcribed to cDNA at 42 °C for 1 h.  $\beta$ -actin was used as the internal control. The RT-PCR conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 95 °C for 25 s, 64 °C for 20 s, and 72 °C for 20 s to ensure the specificity; and 31 cycles at 93 °C for 25 s, 60 °C for 35 s, 72 °C for 20 s were performed for data collection and sensitivity analysis. All of the positive genes including mutations or fusions were confirmed with Sanger sequencing. All the experiments were performed according to the user manual as described previously [22].

### Statistical analysis

The Chi squared test was used to evaluate the relationships between different driver genes and PD-L1 expression. Survival curves of pathologically confirmed samples were calculated using the Kaplan–Meier method until death or last follow-up. Multivariate analysis was performed using the Cox regression model. Statistical analysis was performed with the SPSS 18 software (Chicago, IL, USA).  $P < 0.05$  was considered statistically significant. The median follow-up was 54 months (6.5–65) and the last follow-up date was July 31, 2015.

## Results

### Patient characteristics

Patients' clinical profile is presented in Table 1. One hundred and ninety-eight patients (51.4 %) were male with a

median age of 59 years. One hundred and fifty (39.0 %) patients were former or current smokers. Pathologic stage I was seen in 121 patients, stage II in 79 patients, and stage III in 185 Patients.

### Gene analysis results

All the patients were analyzed for *EGFR*, *KRAS*, *NRAS*, *PIK3CA*, *BRAF* and *HER2* mutations and *ALK*, *ROS1*, *RET* fusion genes. This analysis included *EGFR* mutations (n = 205, 53.2 %), followed by *EML4-ALK* rearrangements (n = 18, 4.7 %), *KRAS* (n = 16, 4.2 %), *HER2* (n = 9, 2.3 %), *ROS1* (n = 8, 2.1 %), *PIK3CA* (n = 6, 1.6 %), *RET* (n = 6, 1.6 %), *BRAF* (n = 2, 0.5 %), and *NRAS* (n = 1, 0.2 %), and 24 coexisting mutations (6.2 %).

**Table 1 Demographic characteristics of the study population (n = 385)**

|                       | Number |
|-----------------------|--------|
| Gender                |        |
| Male                  | 198    |
| Female                | 187    |
| Age                   |        |
| Range                 | 28–79  |
| Median                | 59     |
| <60                   | 207    |
| ≥60                   | 178    |
| Smoking status        |        |
| Never                 | 235    |
| Former/current        | 150    |
| Stage                 |        |
| I                     | 121    |
| II                    | 79     |
| III                   | 185    |
| PD-L1 expression      |        |
| Yes                   | 186    |
| No                    | 199    |
| Gene alteration       |        |
| EGFR                  | 205    |
| ALK                   | 18     |
| KRAS                  | 16     |
| HER2                  | 9      |
| ROS1                  | 8      |
| PIK3CA                | 6      |
| RET                   | 6      |
| BRAF                  | 2      |
| NRAS                  | 1      |
| Concurrent alteration | 24     |
| Pan-negative          | 90     |
| Adjuvant treatment    |        |
| Yes                   | 269    |
| No                    | 116    |

All the nine genes were negative in 90 patients, defined as pan-negative. The details of coexisting mutations are listed in Table 2.

### PD-L1 expression correlated with driver genes

The PD-L1 membrane expression was detected in 186 of the 385 lung adenocarcinoma patients (48.3 %) (Figs. 1, 2). The relationships between clinical parameters or gene characteristics and PD-L1 expression are shown in Table 3. PD-L1 expression was not significantly associated with any clinicopathologic parameters. Patients with PD-L1 positive expression more frequently presented with coexisting mutations (18/24, 75 %), followed by single-gene mutation (145/271, 53.5 %) and pan-negative (23/90, 25.6 %) genes. Differences in PD-L1 expression were found among the coexisting mutations, single-gene mutations and pan-negative genes ( $P < 0.001$ ).

Another antibody (5H1, Cell Signaling Technology, Beverly, MA, USA) was used in 102 patients to detect the PD-L1 expression. The same trend of PD-L1 expression difference existed in patients with different gene abnormality. The PD-L1 positive patients was more frequently carried coexisting mutations (5/8, 62.5 %), followed by single-gene positive (32/66, 48.5 %) and pan-negative mutations (10/28, 25.6 %)( $P = 0.337$ ).

### Survival analysis

The median DFS and OS were 48.3 and 58.1 months, respectively. Patients with positive PD-L1 expression had shorter DFS than those with negative PD-L1 expression (38.0 vs. 50.4 months,  $P = 0.014$ ) (Fig. 3), but the OS between the two groups showed no significant difference (52.9 vs. 68.2 months,  $P = 0.069$ ) (Fig. 4; Table 4).

In univariate analysis, early stage (stage I and II versus III) and PD-L1 expression negative were significantly risk factors for tumor recurrence or metastasis (Figs. 3, 4), while only early stage was a favorable prognostic factor of OS (Table 4).

In multivariate analysis, only early stage suggested lower risk for DFS, while PD-L1 expression was not correlated with recurrence or metastasis. Early stage was an independent and favorable prognostic factor for OS (Table 5).

### Discussion

This study shows that PD-L1 is overexpressed in 48.3 % (186/385) of lung adenocarcinoma patients and this overexpression is more frequently seen in patients with coexisting mutations, but less frequently in patients with pan-negative genes. The PD-L1 overexpression is not a prognostic factor for overall survival. To the best of our knowledge, this is the first study with the largest number of patients correlating the nine common driver genes in lung adenocarcinoma and PD-L1 expression.

**Table 2 Clinical characteristics and PD-L1 expression in concurrent gene alteration patients**

| Case | Gender | Age | Stage | Smoking | Gene type   | PD-L1 expression | OS (month) |
|------|--------|-----|-------|---------|-------------|------------------|------------|
| 1    | Male   | 43  | IB    | Yes     | EGFR+PIK3CA | Yes              | 67+        |
| 2    | Female | 51  | IIIA  | No      | RET+PIK3CA  | Yes              | 42         |
| 3    | Female | 58  | IIIA  | No      | EGFR+ALK    | Yes              | 34         |
| 4    | Male   | 74  | IA    | No      | EGFR+PIK3CA | No               | 66+        |
| 5    | Male   | 60  | IIIA  | Yes     | KRAS+ALK    | Yes              | 35+        |
| 6    | Female | 60  | IB    | No      | EGFR+RET-M2 | Yes              | 54         |
| 7    | Female | 60  | IA    | No      | EGFR+PIK3CA | Yes              | 36+        |
| 8    | Male   | 64  | IIA   | No      | RET+PIK3CA  | No               | 55         |
| 9    | Male   | 69  | IB    | No      | KRAS6+HER2  | Yes              | 43+        |
| 10   | Male   | 45  | IIIA  | Yes     | KRAS+PIK3CA | Yes              | 25         |
| 11   | Female | 64  | IIB   | Yes     | EGFR+HER2   | No               | 46+        |
| 12   | Female | 75  | IIIA  | No      | EGFR+PIK3CA | No               | 24+        |
| 13   | Female | 69  | IIA   | No      | KRAS+PIK3CA | Yes              | 36         |
| 14   | Female | 49  | IB    | No      | EGFR+HER2   | Yes              | 48+        |
| 15   | Female | 55  | IIB   | Yes     | ROS1+HER2   | Yes              | 37+        |
| 16   | Male   | 62  | IB    | Yes     | EGFR+ALK    | No               | 46         |
| 17   | Male   | 55  | IIIA  | No      | EGFR+PIK3CA | Yes              | 39         |
| 18   | Female | 68  | IB    | No      | EGFR+PIK3CA | Yes              | 58+        |
| 19   | Female | 76  | IB    | No      | ALK+RET-M16 | No               | 28         |
| 20   | Male   | 43  | IB    | No      | EGFR+PIK3CA | Yes              | 55+        |
| 21   | Male   | 59  | IIIA  | Yes     | KRAS+PIK3CA | Yes              | 18+        |
| 22   | Female | 61  | IB    | No      | EGFR+PIK3CA | Yes              | 66+        |
| 23   | Female | 68  | IIA   | No      | EGFR+HER2   | Yes              | 45         |
| 24   | Male   | 62  | IIIA  | Yes     | KRAS+HER2   | Yes              | 16         |

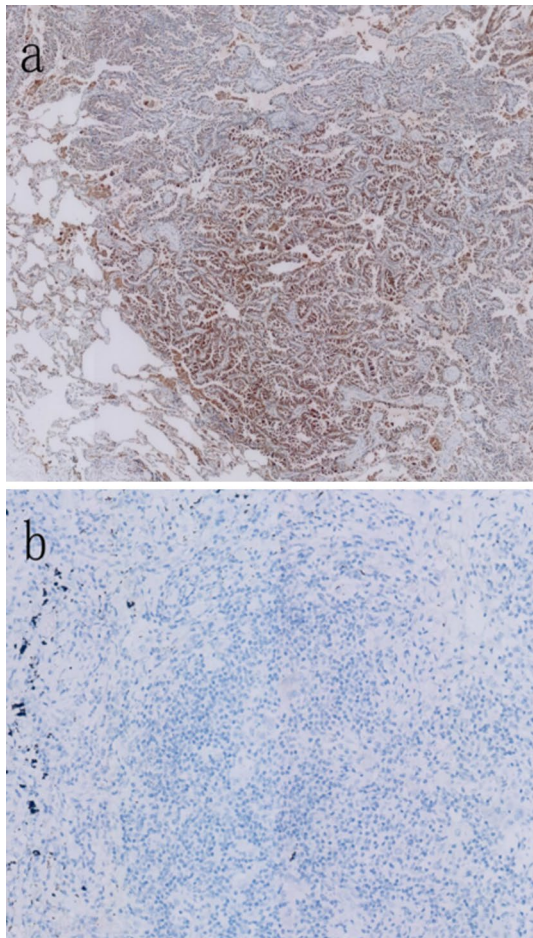
Several studies have reported the association between *PD-L1* expression and driver genes [23, 24]. The results of the correlation were controversial. Azuma et al. [14] observed that *PD-L1* positive status was significantly associated with *EGFR* mutations. Mu et al. observed no significant correlation between *PD-L1* expression and *EGFR/ALK* status in stage I NSCLC patients [25]. Similarly, Zhang et al. found that no association between *PD-L1* expression and *EGFR* status in lung adenocarcinoma [25]. Therefore, the role of inhibition of PD-1/*PD-L1* pathway and driver genes based on the results of existing studies is inconclusive, due to several reasons. First, most of the samples in previous studies were relatively small. Second, most of the studies focused on *EGFR* mutations or *ALK* rearrangements, and other driver genes were not well investigated. Last but not least, racial differences may play an important role in the controversial results.

In the present study, *PD-L1* overexpression was more frequent in patients with coexisting mutations than in pan-negative patients. One explanation is that the genetic differences affected epigenetics, which may alter the expression of tumor-associated self-antigens, which in turn, affected tumor antigenicity. Increased number of driver genes reflects a higher level of neoantigens, which

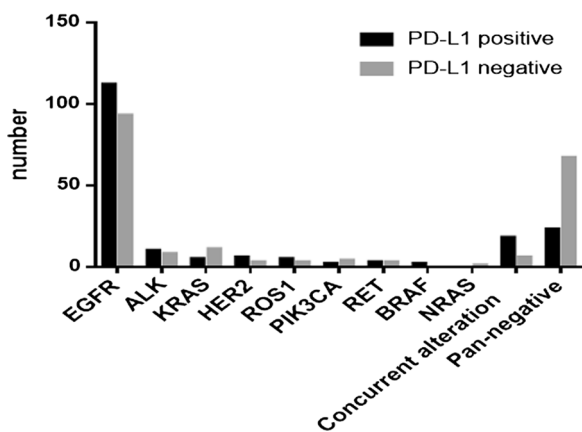
alters the immune microenvironment and increases the *PD-L1* expression [26].

Because of heterogeneity of tumors, the efficacy of chemotherapy or molecular targeted treatment is relatively limited, combination treatment with different anti-cancer mechanisms drugs hold much potential in this area. Previous studies demonstrated that *EGFR* and *ALK* genes could induce *PD-L1* expression to facilitate evasion of the host anti-tumour immune response, suggesting an active role for these genes in remodelling the immune microenvironment [27, 28]. In this way, combination of PD-1/*PD-L1* blockade with targeted inhibitor or other drugs may be a promising therapeutic strategy to increase the duration of treatment response and delay development of drug resistance.

The role of *PD-L1* in predicting the prognosis of NSCLC was controversial in previous studies [20]. Some studies found that negative *PD-L1* expression led to superior OS in NSCLC patients compared with positive *PD-L1* expression [14, 29], while Yang et al. [30] concluded that *PD-L1* expression had no significant correlation with OS. In the present cohort, we found no association between the *PD-L1* expression and overall survival in NSCLC patients. However, *PD-L1* expression



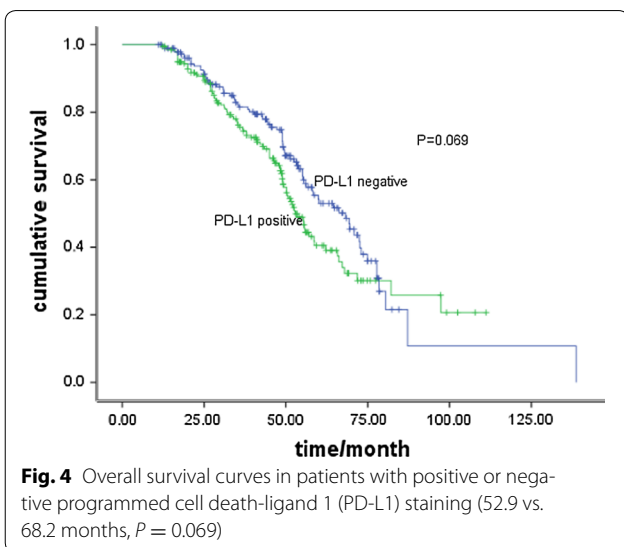
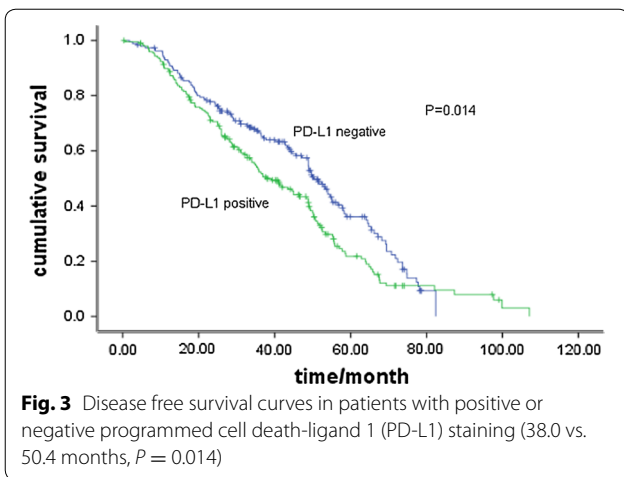
**Fig. 1** **a** Positive programmed cell death-ligand 1 (PD-L1) immunohistochemical staining in a patient with adenocarcinoma. **b** Negative PD-L1 immunohistochemical staining in another patient with adenocarcinoma



**Fig. 2** Relationship between PD-L1 expression and driver genes

**Table 3** Clinical characteristics comparison between PD-L1 positive and negative expression in NSCLC patients

| Variables             | PD-L1 positive (n = 186) | PD-L1 negative (n = 199) | P     |
|-----------------------|--------------------------|--------------------------|-------|
| Gender                |                          |                          | 0.07  |
| Male                  | 87                       | 111                      |       |
| Female                | 99                       | 88                       |       |
| Age                   |                          |                          | 0.68  |
| <60                   | 102                      | 105                      |       |
| ≥60                   | 84                       | 94                       |       |
| Smoking status        |                          |                          | 0.12  |
| Never                 | 121                      | 114                      |       |
| Former/current        | 65                       | 85                       |       |
| Pathologic stage      |                          |                          | 0.09  |
| I + II                | 105                      | 95                       |       |
| III                   | 81                       | 104                      |       |
| EGFR                  |                          |                          | 0.008 |
| Yes                   | 112                      | 93                       |       |
| No                    | 74                       | 106                      |       |
| ALK                   |                          |                          | 0.53  |
| Yes                   | 10                       | 8                        |       |
| No                    | 176                      | 191                      |       |
| KRAS                  |                          |                          | 0.16  |
| Yes                   | 5                        | 11                       |       |
| No                    | 181                      | 188                      |       |
| HER2                  |                          |                          | 0.44  |
| Yes                   | 6                        | 3                        |       |
| No                    | 180                      | 196                      |       |
| ROS1                  |                          |                          | 0.65  |
| Yes                   | 5                        | 3                        |       |
| No                    | 181                      | 196                      |       |
| PIK3CA                |                          |                          | 0.74  |
| Yes                   | 2                        | 4                        |       |
| No                    | 184                      | 195                      |       |
| RET                   |                          |                          | 0.74  |
| Yes                   | 3                        | 3                        |       |
| No                    | 183                      | 196                      |       |
| BRAF                  |                          |                          | 0.45  |
| Yes                   | 2                        | 0                        |       |
| No                    | 184                      | 199                      |       |
| NRAS                  |                          |                          | 0.97  |
| Yes                   | 0                        | 1                        |       |
| No                    | 186                      | 198                      |       |
| Concurrent alteration |                          |                          | 0.01  |
| Yes                   | 18                       | 6                        |       |
| No                    | 168                      | 193                      |       |
| Pan-negative          |                          |                          | <0.01 |
| Yes                   | 23                       | 67                       |       |
| No                    | 163                      | 132                      |       |



was related to shorter DFS. The results may contribute to the treatment after recurrence or metastasis.

Our study limitations are as follows. One major limitation was its retrospective nature. Second, only 24 patients with coexisting mutations were included, and the small sample size may influence the results of the current study. Third, different antibodies were used in different anti-PD-1 or PD-L1 therapies in clinical trials currently. The choice of antibody and the threshold for positivity might influence the results of different studies. Only one antibody and 5 % threshold were used in the present study. Different anti-PD-L1 antibodies may need to be validated in the same sample in future studies.

**Table 4 Univariate analysis for disease-free survival and overall survival**

| Variables          | Median DFS | P      | Median OS | P      |
|--------------------|------------|--------|-----------|--------|
| Gender             |            | 0.74   |           | 0.44   |
| Male               | 44.6       |        | 55.6      |        |
| Female             | 48.9       |        | 59.9      |        |
| Age                |            | 0.23   |           | 0.39   |
| <60                | 49.3       |        | 59.3      |        |
| ≥60                | 42.9       |        | 55.2      |        |
| Smoking status     |            | 0.16   |           | 0.59   |
| Never              | 49.0       |        | 58.6      |        |
| Former/current     | 41.3       |        | 56.0      |        |
| Pathologic stage   |            | <0.001 |           | <0.001 |
| I + II             | 52.5       |        | 66.2      |        |
| III                | 30.2       |        | 45.0      |        |
| Adjuvant treatment |            | 0.54   |           | 0.76   |
| Yes                | 49.7       |        | 59.2      |        |
| No                 | 46.5       |        | 56.5      |        |
| Driver genes       |            | 0.23   |           | 0.24   |
| Positive           | 48.9       |        | 58.7      |        |
| Negative           | 42.0       |        | 50.4      |        |
| PD-L1 expression   |            | 0.014  |           | 0.069  |
| Yes                | 38.0       |        | 52.9      |        |
| No                 | 50.4       |        | 62.0      |        |

**Table 5 Multivariate survival analysis for disease-free survival and overall survival**

| Variables                                | DFS  |           |      | OS   |           |      |
|--|------|-----------|------|------|-----------|------|
|  | HR   | 95 % CI   | P    | HR   | 95 % CI   | P    |
| Smoking status (smokers vs. non-smokers) | 0.84 | 0.66–1.07 | 0.16 | 1.12 | 0.81–1.53 | 0.48 |
| Stage (III vs. I + II)                   | 1.71 | 1.32–2.21 | 0.00 | 1.16 | 0.84–1.58 | 0.00 |
| PD-L1 expression (positive vs. negative) | 1.17 | 0.91–1.51 | 0.22 | 1.79 | 1.30–2.46 | 0.37 |

**Conclusions**

In conclusion, we demonstrated the expression of PD-L1 in over 48 % of lung adenocarcinoma patients and the expression was associated with coexisting driver genes. PD-L1 expression is not associated with overall survival in patients with completely resected NSCLC.

**Abbreviations**

PD-1: programmed death-1; NSCLC: non-small cell lung cancer; DFS: disease free survival; OS: overall survival.

**Authors' contributions**

CG carried out the molecular genetic studies and carried out the immunoassays. ZY and SZ participated in the design of the study and performed the statistical analysis. ZY and YX conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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None declared.

**Competing interests**

The authors declare that they have no competing interests.

**Availability of data**

All of the histology slides have been scanned. It is available if the reader or reviewers required.

**Ethics approval**

The Ethics Committee of Zhejiang Cancer Hospital approved this study and written informed consent was obtained from each participant.

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