Immunolocalization of CD80 and CD86 in Non-Small Cell Lung Carcinoma: CD80 as a Potent Prognostic Factor

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It has been demonstrated that tumor cells express programed cell death protein 1 (PD-L1) to escape T lymphocytes that express programed cell protein 1 (PD-1), and PD-1/PD-L1 immune checkpoint inhibitors have been regarded in lung cancer patients. CD80 and CD86 are members of B7 superfamily which regulates T lymphocyte activation and tolerance. However, immunolocalization of CD80 and CD86 has not been examined in the lung carcinoma tissues and their clinical significance remains unknown. Therefore, to clarify clinical significance of CD80 and CD86, we immunolocalized these in 75 non-small cell lung carcinomas (NSCLC) in this study. Immunoreactivities of CD80 and CD86 were mainly detected in tumor-infiltrating macrophages. Immunohistochemical CD80 status was high in 56% of NSCLC, and it was positively associated with stage, pathological T factor, distant metastasis, histological type and PD-L1 status. Moreover, multivariate analysis turned out that the CD80 status was an independent worse prognostic factor. CD86 status was high in 53% of the cases, but it was not significantly associated with any clinicopathological parameters. These findings suggest that CD80 is a potent worse prognostic factor possibly in association with escape from immune attack in NSCLC.

Key words: CD80, CD86, immunohistochemistry, lung cancer, PD-L1

I. Introduction

Lung cancer is one of the most common fatal malignancies in worldwide [24], and the incidence is increasing. Histologically, lung carcinoma is subclassified into small (approximately 20%) and non-small cell lung carcinoma (NSCLC). NSCLC accounts for approximately 80% of lung carcinomas and is composed of heterogenous groups including adenocarcinoma and squamous cell carcinoma. NSCLC generally responds poorly to chemotherapy compared to small cell carcinoma [32], and various molecular targeted therapeutic agents have been developed [22]. Recently, it has been demonstrated that tumor cells express programed cell death protein 1 (PD-L1) to escape T lymphocytes that express programed cell protein 1 (PD-1) [17], and PD-1/PD-L1 immune checkpoint inhibitors have been regarded as a promising therapeutic strategy for lung cancer patients [5].

B7-1 (CD80) and B7-2 (CD86) are members of B7 superfamily which regulates T cell activation and tolerance [18], as well as PD-L1. CD80/86 molecules on the surface...
of antigen presenting cells bind to cytotoxic T cell antigen 4 (CTLA-4) on the surface of T cells, with much higher affinity to CD28, and suppress T cell activation [29]. CD80 also binds PD-L1 and inhibits T cell responses [1]. Therefore, it is suggestive that CD80/86 molecules play important roles in the regulation of immune microenvironment in lung carcinoma tissues. Expression of CD80 and/or CD86 molecules has been reported in hematologic malignancies [9] and several solid tumors such as glioma [3], gastric carcinoma [14] and pancreatic carcinoma [27]. However, immunolocalization of CD80 and CD86 has not been examined the lung carcinoma to the best of our knowledge. Therefore, in this study, we performed immunohistochemistry for CD80 and CD86 as well as PD-L1 in 75 NSCLC to clarify their clinicopathological significance.

II. Materials and Methods

Patients and tissues

75 specimens of primary NSCLC were obtained from Japanese patients (age range; 43–90 years) who underwent surgical or endoscopic treatment. These cases were obtained from 2016 to 2018 from Fukushima Medical University Aizu Medical Center (Aizuwakamatsu, Japan), and the specimens were fixed in 10% formalin and embedded in paraffin wax. Among the 75 patients, 28 patients received adjuvant chemotherapy after the surgical or endoscopic treatment. The intratumoral mononuclear infiltration was histologically evaluated as low (no areas or scattered small foci) or high (scattered large foci, numerous large or broad areas with pertinent changes) according to a previous report [25]. Clinical outcome of the patients was evaluated by overall survival, which was defined as the time from surgery or endoscopy to death. The mean follow-up time was 983 days (range; 21–1,504 days) in this study. The research protocol was approved by the Ethics Committee at the Fukushima Medical University.

Immunohistochemistry

Rabbit monoclonal antibodies for CD80 (ab269587, clone EPR1157(2)), CD86 (ab134120, clone EP1158-37) and PD-L1 (SP142) were purchased from Abcam (Cambridge, UK), Abcam and Roche Diagnostics Japan (Tokyo, Japan), respectively. Immunostaining for CD80, CD86 and PD-L1 antibodies was automatically performed using Ventana Benchmark XT platform (Roche Diagnostics Japan), Bond III platform (Leica Biosystems Japan, Tokyo, Japan) and Ventana Benchmark XT platform (Table 1).

Table 1. Procedures of automatic immunostaining for CD80, CD86 and PD-L1 in this study

| Platform                  | CD80                  | CD86                  | PD-L1                  |
|---------------------------|-----------------------|-----------------------|------------------------|
| Primary antibody (clone)  | EPR1157(2)            | EP1158-37             | SP142                  |
| Detection kit             | OptiView DAB IHC Detection Kit | BOND Polymer Refine Detection | OptiView DAB IHC Detection Kit and OptiView Amplification Kit |
| Antigen retrieval         | CC1a for 64 min       | BOND Epitope Retrieval Solution 2b for 20 min | CC1a for 48 min       |
| Dilution of primary antibody | 1:500                | 1:100                 | Diluted antibody       |
| Reaction time to primary antibody | 32 min               | 15 min                | 16 min                |
| Reaction time to detection kit | OptiView DAB for 8 min | DAB solution for 10 min | OptiView DAB for 8 min |
|                          | OptiView Peroxidase Inhibitor for 4 min | Peroxide Block for 5 min | OptiView Peroxidase Inhibitor for 4 min |
|                          | OptiView HQ Universal Linker for 8 min | Post Primary for 8 min | OptiView HQ Universal Linker for 8 min |
|                          | OptiView HRP Multimer for 8 min | Polymer for 8 min | OptiView HRP Multimer for 8 min |
|                          |                       |                       | OptiView Amplifier and OptiView Amplification H2O2 for 8 min |
|                          |                       |                       | OptiView Amplification Multimer for 8 min |

2a, Roche Diagnostics Japan (Tokyo, Japan), 2b, Leica Biosystems Japan (Tokyo, Japan), 2c, Abcam (Cambridge, UK). DAB; 3,3'-diaminobenzidine
Scoring of immunohistochemistry

CD80 and CD86 immunoreactivity was detected in tumor-infiltrating immune cells in stroma adjacent to the carcinoma cells (IC), and the case that had more than 1% positive stromal cells was considered high [6]. PD-L1 was immunolocalized in tumor cells (TC) and IC, and the case that had more than 1% positive cells in each area was
considered high for PD-L1 (TC) and PD-L1 (IC), respectively [6, 20, 26]. Immunoreactivity for CD80, CD86, PD-L1 (TC) and PD-L1 (IC) was further semi-quantitatively evaluated by modified labeling index (LI) system according to a previous report [10]. Briefly, the percentage of immunoreactivity (LI) was categorized as 0 (no expression), 10 (up to 10%), 20 (11–20%) until 100 (91–100%) in this study.

**Table 2. Association between CD80 and clinicopathological parameters in 75 lung carcinomas**

|                      | CD80 status | CD80 LI                  |
|----------------------|-------------|--------------------------|
|                      | high (n = 42) | low (n = 33) | P value | mean ± SEM | P value |
| Age (years)          |              |                        |         |                 |         |
| >70                  | 22           | 19                      | 0.654   | 8.780 ± 1.645 | 0.301  |
| ≤70                  | 20           | 14                      |         | 11.471 ± 2.031|         |
| Gender               |              |                        |         |                 |         |
| Male                 | 28           | 15                      |         | 11.628 ± 1.594 |         |
| Female               | 14           | 18                      | 0.065   | 7.812 ± 2.093 | 0.144  |
| Smoking history      |              |                        |         |                 |         |
| Smoking              | 33           | 19                      |         | 11.923 ± 1.578 |         |
| Non-smoking          | 9            | 14                      | 0.050   | 5.652 ± 1.971 | 0.023  |
| Stage                |              |                        |         |                 |         |
| 0–I                  | 17           | 23                      | **0.012** | 7.000 ± 1.485 | **0.012** |
| II–IV                | 25           | 10                      |         | 13.429 ± 2.047 |         |
| Pathological T factor (pT) |           |                        |         |                 |         |
| pTis-I               | 15           | 22                      |         | 6.757 ± 1.553 | 0.119  |
| pT2-4                | 27           | 11                      | **0.008** | 13.158 ± 1.927 |         |
| Lymph node metastasis|              |                        |         |                 |         |
| Positive             | 11           | 4                       |         | 13.333 ± 3.187 | 0.198  |
| Negative             | 31           | 29                      | 0.131   | 9.167 ± 1.392 |         |
| Distant metastasis   |              |                        |         |                 |         |
| Positive             | 8            | 1                       |         | 14.444 ± 2.940 |         |
| Negative             | 34           | 32                      | **0.034** | 9.394 ± 1.397 | 0.205  |
| Histological type    |              |                        |         |                 |         |
| Adenocarcinoma       | 23           | 28                      |         | 7.059 ± 1.322 |         |
| Squamous cell carcinoma | 16        | 4                       |         | 16.500 ± 2.542 |         |
| Others*              | 3            | 1                       | **0.021** | 15.000 ± 8.660 | **0.003** |
| Mononuclear infiltration |          |                        |         |                 |         |
| high                 | 19           | 8                       |         | 13.333 ± 2.201 |         |
| low                  | 23           | 25                      | 0.060   | 8.125 ± 1.537 | 0.052  |
| CD86 status          |              |                        |         |                 |         |
| high                 | 25           | 15                      |         | 10.750 ± 1.732 |         |
| low                  | 17           | 18                      | 0.225   | 9.143 ± 1.939 | 0.537  |
| PD-L1 (IC) status    |              |                        |         |                 |         |
| high                 | 33           | 8                       | **<0.0001** | 15.122 ± 1.785 | **<0.0001** |
| low                  | 9            | 25                      |         | 3.824 ± 1.195 |         |
| PD-L1 (TC) status    |              |                        |         |                 |         |
| high                 | 10           | 1                       | **0.012** | 20.909 ± 4.146 | **0.0003** |
| low                  | 32           | 32                      |         | 8.125 ± 1.197 |         |

*P*-value < 0.05 was significant (in bold).

*: Others included large cell neuroendocrine carcinoma (n = 2), sarcomatoid carcinoma (n = 1) and carcinosarcoma (n = 1).

**Statistical analysis**

Association between immunohistochemical status of CD80, CD86, PD-L1 (TC) and PD-L1 (IC) and clinicopathological factors were evaluated using Student’s t test or a cross-table using the χ²-test. Over survival curves were generated according to the Kaplan-Meier method, and statistical significance was calculated using the log-rank test. Univariate and multivariate analyses were evaluated using a proportional hazard model (Cox). Significant (P < 0.05) and borderline-significant (0.05 ≤ P < 0.10) values were
examined in the multivariate analyses in this study [30]. The statistical analyses were performed using the JMP Pro 15 software (SAS, Institute, Inc, Japan) in this study.

**Bioinformatic analysis**

In order to confirm prognostic values of CD80, CD86 and PD-L1 immunoreactivity in the lung cancer patients, we used Kaplan-Meir Plotter for lung cancer which is a large online database containing microarray gene expression data and prognosis of the patients (https://kmplot.com/analysis/index.php?p=service&cancer=lung). Briefly, we selected CD80, CD86 and PD-L1 genes from the database and correlated these expressions with overall survival of lung cancer patients (n = 1,925) by Kaplan-Meier plot.

### Table 3. Association between CD86 and clinicopathological parameters in 75 lung carcinomas

| CD86 status | CD86 LI | mean ± SEM | P value |
|-------------|---------|------------|---------|
| high (n = 40) | low (n = 35) | | |
| Age (years) | >70 | 23 | 18 | 9.512 ± 1.911 | 0.598 |
| | ≤70 | 17 | 17 | 7.941 ± 1.677 | 0.547 |
| Gender | Male | 22 | 21 | 10.233 ± 2.010 | 0.662 |
| | Female | 18 | 14 | 6.875 ± 1.304 | 0.199 |
| Smoking history | Smoking | 27 | 25 | 10.192 ± 1.769 | 0.713 |
| | Non-smoking | 13 | 10 | 5.652 ± 1.057 | 0.104 |
| Stage | 0-I | 23 | 17 | 10.250 ± 1.977 | 0.439 |
| | II-IV | 17 | 18 | 7.143 ± 1.565 | 0.231 |
| Pathological T factor (pT) | pTis-I | 19 | 18 | 7.297 ± 1.533 | 0.734 |
| | pT2-4 | 21 | 17 | 10.263 ± 2.048 | 0.252 |
| Lymph node metastasis | Positive | 8 | 7 | 6.667 ± 2.108 | >0.999 |
| | Negative | 32 | 28 | 9.333 ± 1.519 | 0.411 |
| Distant metastasis | Positive | 5 | 4 | 5.556 ± 1.757 | 0.887 |
| | Negative | 35 | 31 | 9.242 ± 1.437 | 0.355 |
| Histological type | Adenocarcinoma | 29 | 22 | 8.431 ± 1.465 | 0.442 |
| | Squamous cell carcinoma | 10 | 10 | 10.500 ± 2.945 | 0.000 |
| Others* | 1 | 3 | 5.000 ± 5.000 | 0.617 |
| Mononuclear infiltration | high | 13 | 14 | 7.778 ± 1.949 | 0.450 |
| | low | 27 | 21 | 9.375 ± 1.695 | 0.555 |
| PD-L1 (IC) status | high | 21 | 20 | 8.293 ± 1.743 | 0.687 |
| | low | 19 | 15 | 9.412 ± 1.932 | 0.668 |
| PD-L1 (TC) status | high | 6 | 5 | 9.091 ± 3.426 | 0.499 |
| | low | 34 | 30 | 8.750 ± 1.400 | 0.926 |

*; Others included large cell neuroendocrine carcinoma (n = 2), sarcomatoid carcinoma (n = 1) and carcinosarcoma (n = 1).

#### III. Results

**CD80, CD86 and PD-L1 immunolocalization in lung carcinoma tissues**

As shown in Fig. 1A, CD80 was immunolocalized in the cytoplasm and membrane of IC in NSCLC. A great majority of the CD80-positive cells was morphologically identified macrophages and CD68 immunoreactivity was also positive (Fig. 1B). In addition, some CD80-positive cells were recognized as CD3-positive T lymphocytes (Fig. 1C) and CD20-positive B lymphocytes. On the other hand, CD80 immunoreactivity was negative in TC (Fig. 1A), non-neoplastic epithelium, and stroma far from TC.

Immunoreactivity of CD86 was also detected in the cytoplasm and membrane of IC (Fig. 1D). The CD86-
positive cells were mainly macrophages, but some T and B lymphocytes were also positive for CD86. CD86 immunoreactivity was negligible in TC, non-neoplastic epithelium and stroma far from TC. Immunoreactivity of PD-L1 was detected in the cytoplasm and membrane of IC (Fig. 1E) and TC (Fig. 1F).

As shown in Table 2, immunohistochemical CD80 status was high in 42 out of 75 NSCLC (56%) and it was positively associated with stage \( (P = 0.012) \), pathological T factor (pT) \( (P = 0.004) \), lymph node metastasis \( (P = 0.005) \), histological grade \( (P = 0.001) \) and PD-L1 (IC) status \( (P < 0.0001) \). Similar tendencies were detected between PD-L1 (IC) LI and clinicopathological factors. While, PD-L1 (TC) status was high in 11 out of 75 NSCLC (15%), and it was significantly correlated with stage \( (P = 0.002) \), pT \( (P = 0.004) \) and histological type \( (P = 0.006) \) (Table 5).

Association between CD80, CD86 and PD-L1 status and clinical outcome of lung cancer patients

As demonstrated in Fig. 2A, CD80 status was significantly associated with adverse clinical outcome of the patients \( (P = 0.015 \text{ using the log-rank test}) \). No significant relationship was detected between CD80 status and effec-

| Table 4. Association between PD-L1 (IC) and clinicopathological parameters in 75 lung carcinomas |
|---------------------------------------------------|------------|------|------------------|
| PD-L1 (IC) status                                | PD-L1 (IC) LI |
| high \((n = 41)\)                              | low \((n = 34)\) | \(P\) value | mean ± SEM | \(P\) value |
| Age (years)                                      |              |      |                  |
| >70                                              | 23          | 18   | 0.785            | 8.293 ± 1.518 | 0.822 |
| ≤70                                              | 18          | 16   |                  | 8.824 ± 1.829 |
| Gender                                           |              |      |                  |
| Male                                             | 28          | 15   | 0.035            | 10.698 ± 1.677 | 0.030 |
| Female                                           | 13          | 19   |                  | 5.625 ± 1.415 |
| Smoking history                                  |              |      |                  |
| Smoking                                          | 32          | 20   |                  | 10.000 ± 1.479 | 0.058 |
| Non-smoking                                      | 9           | 14   | 0.072            | 5.217 ± 1.648 |
| Stage                                            |              |      |                  |
| 0-1                                              | 15          | 25   | 0.001            | 4.750 ± 1.132 | 0.0003 |
| II–IV                                            | 26          | 9    |                  | 12.857 ± 1.904 |
| Pathological T factor (pT)                       |              |      |                  |
| pTis-1                                           | 14          | 23   | 0.004            | 4.054 ± 0.905 | <0.0001 |
| pT2–4                                            | 27          | 11   |                  | 12.895 ± 1.882 |
| Lymph node metastasis                            |              |      |                  |
| Positive                                         | 13          | 2    | 0.005            | 15.333 ± 2.557 | 0.003 |
| Negative                                         | 28          | 32   |                  | 6.833 ± 1.223 |
| Distant metastasis                               |              |      |                  |
| Positive                                         | 7           | 2    |                  | 11.111 ± 3.093 | 0.418 |
| Negative                                         | 34          | 32   | 0.138            | 8.182 ± 1.257 |
| Histological type                                |              |      |                  |
| Adenocarcinoma                                   | 22          | 29   |                  | 5.686 ± 1.094 |
| Squamous cell carcinoma                          | 15          | 5    |                  | 14.500 ± 2.854 |
| Others*                                          | 4           | 0    | 0.009            | 15.000 ± 2.887 | 0.001 |
| Mononuclear infiltration                         |              |      |                  |
| high                                             | 18          | 9    |                  | 11.111 ± 2.222 | 0.097 |
| low                                              | 23          | 25   | 0.117            | 7.083 ± 1.296 |
| PD-L1 (TC) status                                |              |      |                  |
| high                                             | 11          | 0    | 0.001            | 24.545 ± 2.817 | <0.0001 |
| low                                              | 30          | 34   |                  | 5.781 ± 0.913 |

\(P\)-value < 0.05 was significant (in bold).

*; Others included large cell neuroendocrine carcinoma \((n = 2)\), sarcomatoid carcinoma \((n = 1)\) and carcinosarcoma \((n = 1)\).
tiveness of adjuvant chemotherapies in this study. On the other hand, no significant association was detected between CD86 status and overall survival in these patients (Fig. 2B). PD-L1 (IC) status was significantly associated with worse prognosis of the lung cancer patients ($P = 0.046$; Fig. 2C), while PD-L1 (TC) was not significantly ($P = 0.178$) associated with the overall survival in this study (Fig. 2D). When we further examined association between combined CD80/PD-L1 (IC) status and clinical outcome of the patients, high/high group was not significantly associated with worse prognosis compared to low/high ($P = 0.586$) or high/low ($P = 0.845$) group (Fig. 2E).

When we analyzed association between CD80, CD86 and PD-L1 mRNA expression and overall survival of lung cancer patients using Kaplan-Meir Plotter for lung cancer, CD80 ($P = 0.0054$) and PD-L1 (IC) ($P = 0.023$) mRNA expressions were significantly associated with the worse prognosis, but not CD86 ($P = 0.39$), which is consistent with our immunohistochemical results (Fig. 3).

As shown in Table 6, results of univariate analysis of overall survival using Cox showed distant metastasis ($P = 0.002$), lymph node metastasis ($P = 0.008$), stage ($P = 0.019$), pT ($P = 0.028$) and CD80 ($P = 0.043$) status were significant prognostic factors, and PD-L1 (IC) ($P = 0.067$), and mononuclear infiltration ($P = 0.070$) were borderline significant. Following multivariate analysis demonstrated that mononuclear infiltration ($P = 0.008$), CD80 status ($P = 0.041$) and stage ($P = 0.048$) were turned out independent prognostic factors for overall survival of NSCLC. CD80 L1 ($P = 0.006$) was a significant prognostic factor and PD-L1 (IC) ($P = 0.062$) was borderline significant by Cox. When we used these continuous variables instead of CD80 and PD-L1 (IC) statuses in the multivariate analysis as well as distant metastasis, lymph node metastasis, stage, pT and mononuclear infiltration, only CD80 L1 ($P = 0.024$) and mononuclear infiltration ($P = 0.026$) were independent prognostic factors in 75 NSCLC patients.

| Table 5. Association between PD-L1 (TC) and clinicopathological parameters in 75 lung carcinomas |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| PD-L1 (TC) status                               | PD-L1 (TC) LI                                   | PD-L1 (TC) status                               | PD-L1 (TC) LI                                   |
| high ($n = 11$)                                 | low ($n = 64$)                                  | $P$ value                                       | mean $\pm$ SEM                                  | $P$ value                                       |
| Age (years)                                     | $>70$                                           | 4                                               | 37                                              | 0.187                                           | 1.463 $\pm$ 0.746                              | 0.209                                           |
|                                                | $\leq70$                                        | 7                                               | 27                                              |                                                 | 3.235 $\pm$ 1.247                              |                                                 |
| Gender                                          | Male                                            | 8                                               | 35                                              | 0.264                                           | 2.558 $\pm$ 0.886                              | 0.632                                           |
|                                                | Female                                          | 3                                               | 29                                              |                                                 | 1.875 $\pm$ 1.139                              |                                                 |
| Smoking history                                 | Smoking                                         | 9                                               | 43                                              | 0.331                                           | 2.692 $\pm$ 0.916                              | 0.364                                           |
|                                                | Non-smoking                                     | 2                                               | 21                                              |                                                 | 1.304 $\pm$ 0.954                              |                                                 |
| Stage                                           | 0–1                                             | 1                                               | 39                                              | 0.002                                           | 0.250 $\pm$ 0.250                              | 0.002                                           |
|                                                | II–IV                                           | 10                                              | 25                                              |                                                 | 4.571 $\pm$ 1.381                              |                                                 |
| Pathological T factor (pT)                      | pTis-1                                          | 1                                               | 36                                              | 0.004                                           | 0.270 $\pm$ 0.270                              | 0.004                                           |
|                                                | pT2–4                                          | 10                                              | 28                                              |                                                 | 4.211 $\pm$ 1.286                              |                                                 |
| Lymph node metastasis                           | Positive                                        | 4                                               | 11                                              | 0.142                                           | 5.333 $\pm$ 2.557                              | 0.027                                           |
|                                                | Negative                                        | 7                                               | 53                                              |                                                 | 1.500 $\pm$ 0.574                              |                                                 |
| Distant metastasis                              | Positive                                        | 2                                               | 7                                               | 0.495                                           | 4.444 $\pm$ 2.940                              | 0.253                                           |
|                                                | Negative                                        | 9                                               | 57                                              |                                                 | 1.970 $\pm$ 0.690                              |                                                 |
| Histological type                               | Adenocarcinoma                                  | 3                                               | 48                                              | 0.006                                           | 1.373 $\pm$ 0.793                              | 0.147                                           |
|                                                | Squamous cell carcinoma                         | 7                                               | 13                                              |                                                 | 4.500 $\pm$ 1.535                              |                                                 |
|                                                | Others                                          | 1                                               | 3                                               |                                                 | 2.500 $\pm$ 2.500                              |                                                 |
| Mononuclear infiltration                        | high                                            | 5                                               | 22                                              | 0.479                                           | 2.593 $\pm$ 1.144                              | 0.729                                           |
|                                                | low                                             | 6                                               | 42                                              |                                                 | 2.083 $\pm$ 0.891                              |                                                 |

$P$-value $< 0.05$ was significant (in bold).

*; Others included large cell neuroendocrine carcinoma ($n = 2$), sarcomatoid carcinoma ($n = 1$) and carcinosarcoma ($n = 1$).
This is the first study that immunolocalized CD80 and CD86 in lung carcinoma tissues. In this study, CD80 and CD86 immunoreactivities were mainly detected in tumor-infiltrating macrophages in 56% and 53% of NSCLC, respectively. CD80/86 molecules on the surface of antigen-presenting cells bind to CD28 on the surface of T lymphocytes, which leads to the activation and differentiation of lymphocytes. However, CTLA-4 competes with CD28 for binding to ligands on the antigen-presenting cells with a higher affinity and thereby displaces CD28 from association with CD80/86 [29]. The binding of CTLA-4 to CD80/86 leads to the inhibitory reaction-suppression of the immune response by blocking the T-lymphocyte reducing proliferation of T lymphocytes, inhibiting the activity of Treg lymphocytes, and reducing cytokine secretion and consequently, to immunosuppression [19, 28, 31]. In addition, CD80 specially interacted with PD-L1 and inhibited T cell activation [1, 2]. Therefore, it is suggested that aberrant expression of CD80 and CD86 are involved in the immune microenvironment in NSCLC tissues.

Overall survival of 75 NSCLC patients according to CD80, CD86, PD-L1 (IC), PD-L1 (TC) and combined CD80/PD-L1 (IC) status. The solid line shows their high group, and the dashed line shows their low group in Fig. 2A–D. P-values < 0.05 were considered significant and shown in bold.

**IV. Discussion**

This is the first study that immunolocalized CD80 and CD86 in lung carcinoma tissues. In this study, CD80 and CD86 immunoreactivities were mainly detected in tumor-infiltrating macrophages in 56% and 53% of NSCLC, respectively. CD80/86 molecules on the surface of antigen-presenting cells bind to CD28 on the surface of T lymphocytes, which leads to the activation and differentiation of lymphocytes. However, CTLA-4 competes with CD28 for binding to ligands on the antigen-presenting cells with a higher affinity and thereby displaces CD28 from association with CD80/86 [29]. The binding of CTLA-4 to CD80/86 leads to the inhibitory reaction-suppression of the immune response by blocking the T-lymphocyte reducing proliferation of T lymphocytes, inhibiting the activity of Treg lymphocytes, and reducing cytokine secretion and consequently, to immunosuppression [19, 28, 31]. In addition, CD80 specially interacted with PD-L1 and inhibited T cell activation [1, 2]. Therefore, it is suggested that aberrant expression of CD80 and CD86 are involved in the immune microenvironment in NSCLC tissues.
In this study, immunohistochemical CD80 status was significantly associated with stage, pT and distant metastasis in NSCLC. Moreover, CD80 status was significantly associated with the worse prognosis, and it turned out an independent prognostic factor. Limited information is available about clinicopathological significance of CD80 in human carcinomas. Previously, Koyama et al. [14] reported that almost all patients with gastric carcinoma showed high levels of expression of CD80 and CD86 but the CD80+/CD86+ phenotype was abrogated during tumor invasion and tumor finally acquired the CD80/CD86+ phenotype. In addition, Feng et al. [7] reported that CD80 immunoreactivity was a favorable prognostic factor in the gastric adenocarcinoma patients. On the other hand, Wang et al. [27] demonstrated that expression level of several immune-suppressive checkpoint molecules, including CD80 and PD-L1, were associated with poor prognosis in pancreatic adenocarcinoma. Considering that clinicopathological significance of CD86 was not evident in the lung carcinoma in this study, it is suggested that CD80 plays an important role to escape from immune attack possibly through CTLA-4 and/or PD-L1 signaling in the lung carcinoma.

Our present study also revealed that CD80-high/PD-L1-high group was not significantly associated with worse prognosis compared to CD80-high/PD-L1-low or CD80-low/PD-L1-high group (Fig. 2E) and PD-L1 (IC) was not an independent prognostic factor. Therefore, it is possible to speculate that CD80 and PD-L1 (IC) signaling pathways are not necessarily independent in NSCLC.

CD80 status was also significantly associated with PD-L1 (IC) status, PD-L1 (TC) status and mononuclear infiltration in this study. In addition, CD80 immunoreactivity was frequently detected in squamous cell carcinoma, and it was marginally associated with smoking history ($P = 0.050$). Previously, Calles et al. [4] reported that PD-L1 expression was more frequently detected in squamous cell carcinoma than adenocarcinoma and associated with smoking status, which is generally consistent with our present results of PD-L1. CD80 and PD-L1 were upregulated on antigen presenting cells upon activation [12], and these interacted [1, 2]. PD-L1 was induced by common γ-chain cytokines [13], and INF-γ induced both CD80 and PD-L1 expression [15]. Recently, Cai et al. [3] demonstrated that various immune checkpoints, including PD-1, PD-L1, CTLA-4, CD80 and CD86, were significantly higher in the glioma-associated stromal cells, and which was correlated with high-grade gliomas. Therefore, it is suggested both PD-1/PD-L1 and CD80/CTLA-4 pathways are important to regulate immune microenvironment in NSCLC.

Previous studies have shown that PD-L1 expressions in TC and IC are associated with aggressive malignant potential and worse prognosis of NSCLC [21, 23]. In this study, both PD-L1 (TC) and PD-L1 (IC) status was significantly associated with worse prognosis of lung cancer patients, which is in good agreement with these previous reports. Immunohistochemical evaluation of PD-L1 (TC) status is currently used to determine the treatment of PD-1/PD-L1 inhibitors, which is approved by the US Food and Drug Administration [11]. In addition, combined treatment with anti-PD-1/PD-L1 and anti-CTLA-4 is also investigating in several malignant tumors [29], and for instance,
Table 6. Univariate and multivariate analyses of overall survival in 75 lung cancer patients

| Variable                        | Univariate P value | Multivariate P value | Relative risk (95% CI) |
|---------------------------------|--------------------|----------------------|------------------------|
| Distant metastasis (positive/negative) | 0.002†             | 0.566                | 1.683 (0.285–9.934)    |
| Lymph node metastasis (positive/negative) | 0.008†             | 0.572                | 1.733 (0.257–11.680)   |
| Stage (II–IV/0–I)                | 0.019†             | 0.048                | 11.049 (1.018–119.890) |
| Pathological T factor (pT) (pT2-4/pTis-1) | 0.028†             | 0.448                | 2.398 (0.250–23.066)   |
| CD80 status (high/low)           | 0.043†             | 0.041                | 24.306 (1.134–520.841) |
| PD-L1 (IC) status (high/low)     | 0.067†             | 0.817                | 1.307 (0.136–12.584)   |
| Mononuclear infiltration (high/low) | 0.070†             | 0.008                | 0.037 (0.003–0.419)    |
| Gender (Male/Female)             | 0.145              |                      |                        |
| Histological type* (adenocarcinoma/squamous cell carcinoma) | 0.163              |                      |                        |
| PD-L1 (TC) (high/low)            | 0.193              |                      |                        |
| Smoking history (smoking/non-smoking) | 0.458              |                      |                        |
| Patient age (>70/≤70)            | 0.617              |                      |                        |
| CD86 status (high/low)           | 0.661              |                      |                        |

Statistical analysis was evaluated by a proportional hazard model (Cox). P-value < 0.05 and 0.05 ≤ P-value < 0.10 were considered significant and borderline significant, respectively. †: Significant (P < 0.05) and borderline-significant (0.05 ≤ P < 0.10) values were examined in the multivariate analyses in this study. 95% CI, 95% confidence interval.

*: Other histological types (n = 4) rather than adenocarcinoma and squamous cell carcinoma were excluded in this analysis.

A combination with nivolumab (PD-1 inhibitor), ipilimumab (CTLA-4 inhibitor) and chemotherapy seems to be superior first-line immunotherapy for patients with advanced non-small cell lung carcinoma [16]. PD-L1 inhibitor durvalumab blocks PD-L1 binding to CD80 as well as PD-1, and clinical trial to investigate effects of durvalumab with or without tremelimumab (CTLA-4 inhibitor) versus standard chemotherapy is also undergoing in non-small cell lung cancer [8]. Appropriate biomarker for the treatment of anti-CTLA-4 inhibitors is currently unknown, and further examinations are required to clarify the biological functions of CD80 to improve the immunotherapy in NSCLC patients.

In summary, we immunolocalized CD80 and CD86 in 75 NSCLC tissues. CD80 status was high in 56% of lung carcinomas and it was positively associated with stage, pT, distant metastasis, histological type, intratumoral mononuclear infiltration, PD-L1 (IC) status and PD-L1 (TC) status. Moreover, CD80 status was significantly associated with poor prognosis of the patients, and multivariate analysis turned out it as an independent prognostic factor. CD86 status was high in 53% of the cases, but it was not significantly associated with any clinicopathological parameters. These findings suggest that CD80 is a potent worse prognostic factor possibly in association with escape from immune attack in NSCLC.

V. Conflicts of Interest

The authors declare that there are no conflicts of interest.

VI. References

1. Butte, M. J., Keir, M. E., Phamduy, T. B., Sharpe, A. H. and Freeman, G. J. (2007) Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* 27; 111–122.

2. Butte, M. J., Peña-Cruz, V., Kim, M. J., Freeman, G. J. and Sharpe, A. H. (2008) Interaction of human PD-L1 and B7-1. *Mol. Immunol.* 45; 3567–3572.

3. Cai, X., Yuan, F., Zhu, J., Yang, J., Tang, C., Cong, Z., *et al.* (2021) Glioma-Associated Stromal Cells Stimulate Glioma Malignancy by Regulating the Tumor Immune Microenvironment. *Front. Oncol.* 11; 672928.

4. Calles, A., Liao, X., Sholl, L. M., Rodig, S. J., Freeman, G. J., Butaney, M., *et al.* (2015) Expression of PD-1 and Its Ligands, PD-L1 and PD-L2, in Smokers and Never Smokers with KRAS-Mutant Lung Cancer. *J. Thorac. Oncol.* 10; 1726–1735.

5. Chen, L., Cao, M. F., Zhang, X., Dang, W. Q., Xiao, J. F., Liu, Q., *et al.* (2019) The landscape of immune microenvironment in lung adenocarcinoma and squamous cell carcinoma based on PD-L1 expression and tumor-infiltrating lymphocytes. *Cancer Med.* 8; 7207–7218.

6. Fehrenbacher, L., Spira, A., Ballinger, M., Kowanetz, M., Vansteenkiste, J., Mazieres, J., *et al.* (2016) Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial. *Lancet* 387; 1837–1846.

7. Feng, X. Y., Lu, L., Wang, K. F., Zhu, B. Y., Wen, X. Z., Peng, R. Q., *et al.* (2019) Low expression of CD80 predicts for poor prognosis in patients with gastric adenocarcinoma. *Future Oncol.* 15; 473–483.

8. Garon, E. B., Cho, B. C., Reinmuth, N., Lee, K. H., Luft, A., Ahn, M. J., *et al.* (2021) Patient-Reported Outcomes with Durvalumab With or Without Tremelimumab Versus Standard Chemotherapy as First-Line Treatment of Metastatic Non-Small-Cell Lung Cancer (MYSTIC). *Clin. Lung Cancer* 22; 301–312.
