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# Clinical significance of PD-L1-positive cancer-associated fibroblasts in pN0M0 non-small cell lung cancer

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ABSTRACT

*Objectives:* Cancer-associated fibroblasts (CAFs) are a dominant cell type in tumor stroma and support the generation of pro-tumorigenic microenvironment. CAFs have frequent opportunities to interact with immune cells infiltrating the tumor stroma, but the process remains to be determined. In this study, we focused on immune checkpoint mechanism. We also examined the induction of programmed cell death-ligand 1 (PD-L1) on CAFs by immune cell, and the clinical significance of PD-L1-expressed CAFs in non-small cell lung cancer (NSCLC).

*Materials and methods:* CAFs were isolated from human NSCLC tissues, and PD-L1 expression levels in CAFs were analyzed by real-time polymerase chain reaction and flow-cytometry. Following immunohistochemical analysis of PD-L1 in surgically resected pN0M0 NSCLC (n = 125, including 88 invasive adenocarcinomas and 37 squamous cell carcinomas), the correlation of PD-L1-positive CAFs with clinicopathological features was investigated.

*Results:* PD-L1 mRNA and protein expression on CAFs was upregulated by exogenously supplemented interferon-gamma (IFN-γ) and downregulated through the depletion of IFN-γ. PD-L1 expression on CAFs was upregulated by co-culture with activated lymphocytes releasing IFN-γ. Immunohistochemistry revealed that PD-L1-positive CAFs were observed in 31 cases (24.8%). Postoperative relapse-free survival was significantly prolonged in patients with PD-L1-positive CAFs as compared with those with PD-L1-negative CAFs, with 5-year relapse-free probabilities of 84.5% and 66.3%, respectively ($P = 0.031$). Multivariate analysis revealed that PD-L1 expression on CAFs was an independent prognostic factor of longer relapse-free survival after surgery (hazard ratio: 3.225, $P = 0.027$).

*Conclusion:* PD-L1 expression on CAFs is reversibly regulated by environmental stimuli including IFN-γ from activated lymphocytes. In the non-metastatic NSCLC, PD-L1 expression on CAFs suggests the induction of anti-tumor immune responses, contributing to better prognosis after surgery.

## 1. Introduction

Since the development of immune checkpoint inhibitors targeting the programmed cell death 1–programmed cell death-ligand 1 (PD-1–PD-L1) axis, therapeutic advances have been achieved in non-small cell lung cancer (NSCLC) [1–5]. In advanced NSCLC, combination therapy of immune checkpoint inhibitors and platinum-based chemotherapy has yielded improved overall survival compared with platinum-based chemotherapy alone [6–8]. These immune checkpoint inhibitors are believed to prevent PD-1-positive cytotoxic T lymphocytes (CTLs) from lapsing into exhaustion via interplay with PD-L1-positive tumor cells and to preserve the anti-tumor activity of CTLs (reviewed in

*Abbreviations:* CAFs, Cancer-associated fibroblasts; PD-L1, programmed cell death-ligand 1; NSCLC, non-small cell lung cancer; IFN-γ, interferon-gamma; CTLs, cytotoxic T lymphocytes; RFS, relapse-free survival; 5-y RFP, 5-year relapse-free probability; LAK cells, lymphokine-activated killer cells

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Ref. [9]).

In NSCLC, the clinical success of immune checkpoint inhibitors has prompted studies to determine the clinical significance of PD-L1 expression on tumor cells. However, correlations between PD-L1 expression on tumor cells and patient prognosis have generally been inconsistent, with some studies finding that high PD-L1 expression was associated with a poor prognosis [10–12] and other studies reaching the opposite conclusion [13–15]. To further explore the clinical significance of PD-L1 expression in tumor tissues, we need to focus on the PD-L1 status of not only tumor cells but also of other cell types in tumor stroma.

We previously reported that alveolar macrophages constantly express PD-L1 in pulmonary adenocarcinomas [16], while other papers reported that PD-L1 is expressed on some stromal cells [17,18]. Given that multiple cell types including immune cell types and fibroblasts interact in tumor stroma (reviewed in Ref. [19]), PD-L1 on stromal cell types may also have a role in determining the prognosis of patients. A previous paper reported that patients with PD-L1-positive regulatory T cells in their tumor tissue had a better response to anti-PD-1/PD-L1 immunotherapy in NSCLC [17].

Cancer-associated fibroblasts (CAFs) are a dominant cell type in tumor stroma and are considered to generate a pro-tumorigenic tumor microenvironment (reviewed in Ref. [20]). CAFs are also thought to interact with stroma-infiltrating immune cells in the tumor stroma; however, this crosstalk remains to be fully elucidated. We previously reported that PD-L1 expression on NSCLC cells is reversibly regulated by interferon-gamma (IFN-γ) [21]. Given that IFN-γ in tumor tissues is mainly released from activated lymphocytes (reviewed in Ref. [22]), CAFs could be affected by IFN-γ released from these immune cells, resulting in their expression of PD-L1. Few papers describe PD-L1 expression on CAFs [23] and its clinical significance in NSCLC; however, it is of value to clarify the features of NSCLC with PD-L1-positive CAFs.

In the context of interplay of CAFs with immune cells, we sought to illuminate the mechanism of PD-L1 expression on CAFs and examine the clinical significance of PD-L1 expression on CAFs in patients with pN0M0 NSCLC.

Here, we report that PD-L1 expression on CAFs is induced by IFN-γ released from CTLs, and significantly associated with extended relapse-free survival (RFS) after surgery in patients with pN0M0 NSCLC. PD-L1 expression on CAFs indicates the induction of anti-tumor immune responses in the tumor microenvironment and can serve as a biomarker to predict good prognosis following surgery in these patients.

## 2. Materials and methods

### 2.1. Isolation of CAFs from NSCLC tissues

CAFs were isolated from human NSCLC tissues as described elsewhere [24]. Briefly, tumor tissue specimens of approximately 27 $mm^3$ were placed on culture plates and soaked in Dulbecco's modified Eagle's medium (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum. The medium was changed once a week until the tissue was surrounded by adherent fibroblasts. Around 3 weeks later, the fibroblasts were separated from contaminating epithelial and endothelial cells by differential trypsinization with 0.25% trypsin (Supplementary Fig. 1A). To confirm the expression of alpha-smooth muscle actin (αSMA), a marker of CAFs, the cells were harvested, and stained with a FITC-labeled anti-human αSMA antibody (clone: 1A4) (Abcam, Cambridge, UK) or a mouse IgG2a, k isotype control (BD Biosciences) (Supplementary Fig. 1B). Flow cytometric analysis was performed using a BD FACS Calibur, and the data were analyzed using BD CellQuest Pro software (BD Biosciences).

### 2.2. Evaluation of PD-L1 expression levels in CAFs

To examine the effect of IFN-γ on PD-L1 expression, CAFs were cultured in medium containing 0.5 ng/ml recombinant human IFN-γ (PeproTech, Rocky Hill, NJ, USA) for 48 h. The culture medium was then replaced by fresh medium without IFN-γ, and cell culture was continued for an additional 48 h. The cells were then harvested, and stained with a phycoerythirin-labeled anti-human PD-L1 antibody (clone: MIH1) (BD Biosciences, San Jose, CA, USA) or a mouse IgG1, k isotype control (BD Biosciences).

Total RNA was extracted from CAFs using a RNeasy mini kit (Qiagen, Hilden, Germany), and was subjected to reverse transcription using a cloned AMV first-strand cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA). The cDNA was amplified by polymerase chain reaction (PCR) with specific paired primers as follows: *CD274* (*PD-L1*) (forward) 5′-GAGCCTCCAAGCAAATCATC-3′; *CD274* (*PD-L1*) (reverse) 5′-GCAACCAACGGTTTGATCTT-3′; *GAPDH* (forward) 5′-TGGAAGGAC TCATGACCACA-3′; *GAPDH* (reverse) 5′-CCCTGTTGCTGTAGCCAAAT -3′.

To quantify the levels of *CD274* mRNA, cDNA was synthesized from extracted total RNA using Prime script RT master mix (Takara Bio Inc, Kusatsu, Japan). Real time-PCR was performed using TaqMan fast universal PCR master mix and the StepOnePlus real time PCR system (Applied Biosystems, Austin, TX, USA). Probe and primer sets were prepared for human CD274 (Hs00204257_m1) and human *ACTB* (Hs01060665_g1) (Applied Biosystems). Gene expression was normalized against human *ACTB* as an endogenous control and the relative levels were calculated using the ΔΔCt model.

### 2.3. Induction of lymphokine-activated killer cells

Lymphokine-activated killer (LAK) cells were generated from human peripheral blood mononuclear cells (PBMCs). Briefly, PBMCs were stimulated with anti-human CD3 antibody (clone: OKT-3) (Thermo Fisher Scientific, San Jose, CA, USA), and cultured in AIM-V medium (Thermo Fisher Scientific) supplemented with 5% human AB serum (Sigma-Aldrich, St. Louis, MO, USA) and human interleukin-2 for at least 7 days. For activation, the LAK cells were stimulated with 25 μg/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich) for 4 h.

### 2.4. Tumor samples

Tumor samples were obtained from specimens of NSCLC that were surgically resected at the Shiga University of Medical Science Hospital, between January 2008 and December 2014. All cases with invasive pulmonary adenocarcinomas and squamous carcinomas of the lung, with no metastases to regional lymph nodes and distant organs, were included in this study. The data on clinicopathological findings of patients were obtained from their medical records. The study design was approved by the Institutional Ethics Review Board of Shiga University of Medical Science.

### 2.5. Immunohistochemistry

Four-μm thick sections of formalin-fixed paraffin-embedded tissue specimens were stained by standard indirect immunoperoxidase procedures, according to the manufacturer's protocol (Cell Signaling Technology, Danvers, MA, USA). Briefly, each tissue section was deparaffinized in xylene, and rehydrated in ethanol and distilled water. Antigen retrieval was performed by microwave treatment in SignalStain EDTA unmasking solution (Cell Signaling Technology) for 10 min; endogenous peroxidase activity was blocked by treatment with 3% $H_2O_2$ for 10 min. After blocking with 5% normal goat serum in Tris-buffered saline with Tween 20 for 1 h at room temperature, the sections were incubated overnight with anti-human PD-L1 monoclonal antibody (clone: E1L3N, diluted at 1:200; Cell Signaling Technology) at 4 °C. The following day, the sections were incubated with SignalStain boost IHC detection reagent (Cell Signaling Technology), and visualized using the

SignalStain DAB substrate kit (Cell Signaling Technology) for 1 min, followed by counterstaining with hematoxylin.

### 2.6. Evaluation of PD-L1 expression on CAFs

Following the PD-L1 immunohistochemistry, the sections were independently examined by two researchers who did not know the clinicopathological information corresponding to each section. Spindle-shaped cells in tumor stroma were morphologically identified as CAFs, and either membranous or cytoplasmic PD-L1 staining intensity of each CAF was classified into four levels: non-, weakly, moderately, and strongly stained. Weakly, moderately and strongly stained CAFs were defined as PD-L1-positive CAFs. We counted all CAFs in three representative fields under ×200 magnification for a section. When the frequency of PD-L1-positive CAFs in a section was more than 1%, the case was defined as having PD-L1-high CAFs. An intra-class correlation coefficient (ICC) between observers on evaluation of the frequency of PD-L1-positive CAFs (ICC (3,1)) was 0.816.

For the sections following the PD-L1 immunohistochemistry, we semi-quantitatively evaluated the PD-L1 expression intensity of tumor cells as described previously [16]. When the PD-L1 expression score was above 150.0, the PD-L1 tumor proportion score is estimated to be more than 50%. Thus the case with a PD-L1 expression score above 150.0 was defined as having high PD-L1 expression intensity. In addition, we observed tumor-infiltrating lymphocytes in tumor stroma in the sections, and evaluated a correlation of PD-L1-positive CAFs with the infiltration of lymphocytes.

### 2.7. Statistical analysis

Correlations between variables were analyzed using the Fisher's exact test for categorical variables and the Mann-Whitney *U* test for continuous variables. Relapse-free survival (RFS) after surgery was calculated using Kaplan–Meier analysis, and RFS in different groups was compared with the log-rank test. Multivariate analysis of postoperative RFS was performed using the Cox proportional hazards model. *P*-values of less than 0.05 were considered statistically significant. All analyses were performed using SPSS Statistics 22.0 software (IBM, Armonk, NY, USA).

## 3. Results

### 3.1. Effect of IFN-γ on PD-L1 expression on CAFs

Several studies have previously reported that PD-L1 expression in human cancer cell lines was induced by IFN-γ in vitro [23,25]. On the basis of these data, we examined whether PD-L1 expression on CAFs was induced by IFN-γ. Recombinant human IFN-γ was added to the culture medium of CAFs isolated from human NSCLC tissues. Forty-eight hours after supplementation of IFN-γ, flow cytometry demonstrated that PD-L1 expression levels on CAFs were upregulated (Fig. 1A). Then, IFN-γ was depleted from the culture medium of these CAFs, and 48 h later, PD-L1 expression on CAFs was examined by flow cytometry. The data showed that PD-L1 expression that had been induced by IFN-γ was down-regulated to background level (Fig. 1A).

Next, we examined the effect of IFN-γ on the regulation of *CD274* (*PD-L1*) mRNA levels in CAFs. PD-L1 expression was reversibly regulated by IFN-γ (Fig. 1B-C). The data demonstrated that both mRNA and protein levels of PD-L1 expression on CAFs was reversibly regulated by exogenously supplemented IFN-γ.

### 3.2. PD-L1 expression on CAFs by interaction with activated lymphocytes

Next, given that IFN-γ in tumor tissues is mainly released from activated lymphocytes (reviewed in Ref. [22]), we examined PD-L1 expression on CAFs through the interaction with activated lymphocytes. In this study, we used LAK cells as a model cell line for activated lymphocytes. The majority of the LAK cell population was confirmed to be positive for CD8 and possibly secreted IFN-γ after stimulation with PMA and ionomycin (Supplementary Fig. 1C). CAFs were co-cultured with stimulated LAK cells for 24 h, and then PD-L1 expression on the CAFs was examined by flow cytometry. The data showed that PD-L1 expression on CAFs was upregulated by the interaction with IFN-γ-releasing activated lymphocytes (Fig. 1D). These data suggest that the existence of PD-L1-expressing CAFs in tumor tissues might indicate the activation and infiltration of IFN-γ-releasing cytotoxic T lymphocytes in the tumor microenvironment.

### 3.3. Patients' characteristics

To examine PD-L1 expression on CAFs in NSCLC tissues, tumor samples from 125 patients were analyzed by PD-L1 immunohistochemistry (Table 1). Patients included in this study comprised 84 men (67.2%) and 41 women (32.8%) with a median age of 69 years (range, 46–88 years) at surgery. Eighty-four patients (67.2%) were current or former smokers. The pathological subtypes were invasive pulmonary adenocarcinoma ($n = 87$, 69.6%) and squamous cell carcinoma of the lung ($n = 38$, 30.4%). The median tumor diameter was 25 mm (range, 4–127 mm). Fifty-six (44.8%) tumors were tumor cell grade 1, 58 (46.4%) were grade 2, and 11 (8.8%) were grade 3. Invasion of tumor cells to lymphatic vessels and micro blood vessels was observed in 63 cases (50.4%) and 84 cases (67.2%), respectively. Postoperative pathological staging according to the eighth edition of the TMN classification for lung cancer was IA in 74 cases (59.6%), IB in 30 (24.0%), IIA in six (4.8%), IIB in nine (7.2%), IIIA in six (4.8%).The median time of observation was 1977 days (range, 252–3814 days). Thirty-one patients (24.8%) received adjuvant chemotherapy (uracil/tegafur or platinum-based doublet) around one month after surgery.

### 3.4. Correlations between PD-L1 expression on CAFs and clinicopathological factors in pN0M0 NSCLC

Among 125 pN0M0 NSCLC patients, PD-L1-expressed CAFs (Fig. 2A) were observed in 31 cases (24.8%). We analyzed correlations between PD-L1 expression on CAFs and clinicopathological features (Table 2). PD-L1 expression on CAFs was not associated with age ($P = 0.148$), sex ($P = 0.233$), smoking habits ($P = 0.389$), pathological types ($P = 0.520$). Pathologically, PD-L1 expression on CAFs was not associated with tumor cell grade ($P = 0.398$), lymphatic invasion ($P = 0.398$), or microvascular invasion of tumor cells ($P = 0.099$). On the other hand, PD-L1 expression on CAFs was significantly associated with tumor size ($P = 0.026$); however, in patients with pT1N0M0 which means that tumor size is smaller than 3cm, the expression was not associated with tumor size ($P = 0.146$) (data not shown). These data suggest that PD-L1 expression on CAFs is likely to be affected by tumor growth in pN0M0 NSCLC.

In addition, PD-L1 expression on CAFs was significantly associated with PD-L1 expression intensity of tumor cells ($P = 0.020$). These data might support that PD-L1 expression on CAFs is also upregulated by IFN-γ released from cytotoxic T lymphocytes as is done in tumor cells. However, we found no correlation between PD-L1 expression on CAFs and infiltration of lymphocytes in tumor stroma.

### 3.5. Correlation between PD-L1 expression status of CAFs and postoperative relapse-free survival in pN0M0 NSCLC

Next, we examined clinicopathological features that were associated with postoperative RFS in patients with pN0M0 NSCLC. The data revealed that postoperative RFS was not correlated with clinicopathological factors such as age ($P = 0.541$), adjuvant chemotherapy ($P = 0.081$), pathological types ($P = 0.516$), tumor cell grade ($P = 0.636$), lymphatic invasion ($P = 0.136$), microvascular

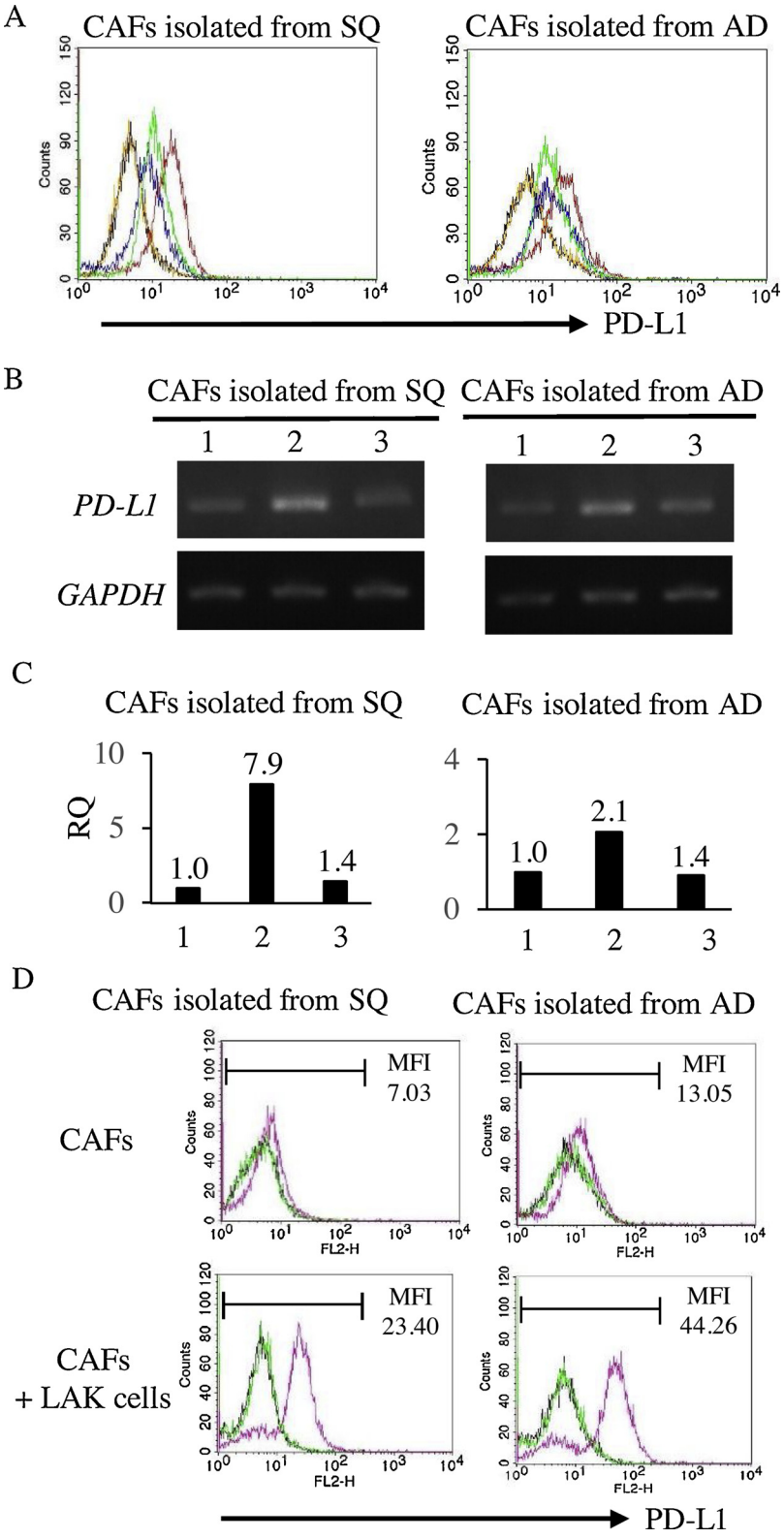


**Fig. 1.** PD-L1 expression on CAFs by IFN-γ stimulation.
A. Flow-cytometric analysis on PD-L1 expression after stimulation with IFN-γ (*black line:* non-staining, *orange line:* isotype control antibody, *blue line:* baseline, *red line:* stimulation with IFN-γ, green line: discontinuation of IFN-γ stimulation).
B. RT-PCR for PD-L1 mRNA in CAFs (*Lane 1:* baseline, *Lane 2:* stimulation with IFN-γ, *Lane 3:* discontinuation of IFN-γ stimulation).
C. Relative quantification (RQ) of PD-L1 mRNA level in CAFs (*1:* baseline, *2:* stimulation with IFN-γ, *3:* discontinuation of IFN-γ stimulation).
D. Flow-cytometric analysis on PD-L1 expression after co-culture with activated LAK cells secreting IFN-γ (*black line:* non-staining, *green line:* isotype control antibody, *red line:* anti-PD-L1 antibody).

invasion ($P = 0.396$), or PD-L1 expression intensity of tumor cells ($P = 0.114$) (Table 3). However, male patients had significantly poor postoperative RFS compared with female patients ($P = 0.015$). In addition, patients with smoking habits and those with tumors larger than 3.0cm in size had significantly poor postoperative RFS compared with their counterparts ($P = 0.012$ and $P = 0.006$, respectively).

Furthermore, we examined the correlation between PD-L1 expression status of CAFs and postoperative RFS. The data revealed that postoperative RFS was significantly longer for patients with PD-L1-positive CAFs than for patients with PD-L1-negatve CAFs ($P = 0.031$, Fig. 2C), with 5-year relapse-free probabilities (5-y RFP) of 84.5% and 66.3%, respectively. In patients with pT1N0M0 NSCLC (n = 74), the postoperative RFS tended to be longer for patients with PD-L1-positive CAFs than for patients with PD-L1-negatve CAFs ($P = 0.083$), with 5-y

**Table 1**
Patients' characteristics (n = 125).

| | |
|---|---|
| Age (y.o.) (median, range) | 69, 46–88 |
| Gender, n (%) | |
| male | 84 (67.2) |
| female | 41 (32.8) |
| Smoking habits, n (%) | |
| never | 41 (32.8) |
| current/formaer | 84 (67.2) |
| Adjuvant chemotherapy, n (%) | 31 (24.8) |
| Pathology, n (%) | |
| adenocarcinoma | 87 (69.6) |
| squamous cell ca. | 38 (30.4) |
| Tumor size (mm) (median, range) | 22, 4–127 |
| Cell grade, n (%) | |
| grade 1 | 56 (44.8) |
| grade 2 | 58 (46.4) |
| grade 3 | 11 (8.8) |
| Pathological stage, n (%) | |
| 1A | 74 (59.6) |
| 1A1 | 5 (4.0) |
| 1A2 | 26 (20.8) |
| 1A3 | 43 (34.4) |
| 1B | 30 (24.0) |
| 2A | 6 (4.8) |
| 2B | 9 (7.2) |
| 3A | 6 (4.8) |
| Invasion to lymphatics, n (%) | 64 (51.2) |
| Invasion to microvessels, n (%) | 83 (66.4) |

**Table 2**
Correalations between PD-L1 expressnon on CAFs and clinicopathological factors (n = 125).

| | PD-L1-positive (n = 31) | PD-L1-negative (n = 94) | *P* |
|---|---|---|---|
| Age (y.o.) (median, range) | 72, 51–83 | 69, 46–88 | 0.148 |
| Gender, n (%) (male/female) | 23 (74.2) / 8 (25.8) | 61 (64.9) / 33 (35.1) | 0.233 |
| male (n = 84) | 23 (27.4) | 61 (72.6) | |
| female (n = 41) | 8 (19.5) | 33 (80.5) | |
| Smoking habits, n (%) | 22 (71.0) | 62 (66.0) | 0.389 |
| Adjuvant chemotherapy | 4 (12.9) | 27 (28.7) | 0.059 |
| Pathology, n (%) (Ad/Sq) | 22 (71.0) / 9 (29.0) | 66 (70.2) / 28 (29.8) | 0.520 |
| Ad (n = 88), n (%) | 22 (25.0) | 66 (75.0) | |
| Sq (n = 37), n (%) | 9 (24.3) | 28 (75.7) | |
| Tumor size (mm) (median, range) | 22, 4–68 | 26, 10–127 | 0.026 |
| Cell grade, n (%) (1/2 + 3) | 15 (48.4) / 16 (51.6) | 41 (43.6) / 53 (56.4) | 0.398 |
| grade 1 (n = 56), n (%) | 15 (26.8) | 41 (73.2) | |
| grade 2 + 3 (n = 69), n (%) | 16 (23.2) | 53 (76.8) | |
| Invasion to lymphatics, n (%) | 17 (54.8) | 47 (50.0) | 0.398 |
| Invasion to microvessels, n (%) | 24 (77.4) | 59 (62.8) | 0.099 |
| PD-L1 H-score of tumor cells (median, range) | 84.2, 0.4–283.5 | 32.6, 0.0–284.2 | 0.020 |

Ad; pulmonary adenocarcinoma, Sq; squamous cell carcinoma of lung, N.S.; not significant,

RFP of 87.9% and 75.9%, respectively (Fig. 2D).

These data demonstrate that high PD-L1 expression on CAFs is significantly associated with better prognosis for patients with pN0M0 NSCLC after radical surgery.

### *3.6. PD-L1 expression status of CAFs to predict postoperative prognosis in pN0M0 NSCLC*

We further explored a biomarker to predict tumor relapse after radical surgery in patients with pN0M0 NSCLC. Multivariate analyses revealed that some clinicopathological factors, such as age ($P = 0.527$), sex ($P = 0.204$), smoking habits ($P = 0.637$), adjuvant chemotherapy ($P = 0.820$), tumor size ($P = 0.085$), microvessel invasion ($P = 0.342$), and PD-L1 expression intensity of tumor cells ($P = 0.161$) did not serve as a significant prognostic factor (Table 4). However, PD-L1 expression on CAFs was revealed to be an independent prognostic factor of higher 5-y RFP (hazard ratio [HR]: 3.225, 95% confidence interval [CI]: 1.144–9.086, $P = 0.027$). In addition, as well as in patients with pT1N0M0 NSCLC, PD-L1 expression on CAFs was revealed to be a positive prognostic factor of higher 5-y RFP (HR: 5.275, 95% CI: 1.118–24.899, $P = 0.036$). Microvessel invasion of tumor cells was also

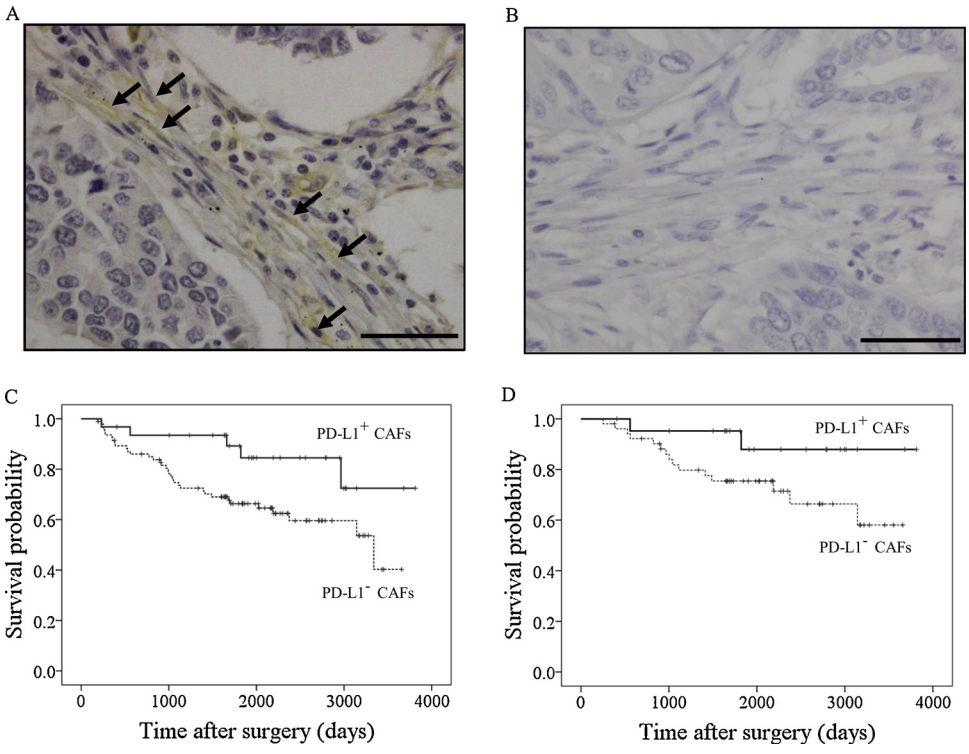


**Fig. 2.** PD-L1 expression on CAFs and relapse-free survival.
A. A case with PD-L1-positive CAFs (*arrows*) (×400, scale bar; 50 μm).
B. A case with PD-L1-negative CAFs (×400, scale bar; 50 μm).
C. Kaplan-Meier analysis on relapse-free survival after surgery in patients with pN0M0 NSCLC (*solid lone*: cases with PD-L1-positive CAFs, *dotted line*: cases with PD-L1-negative CAFs).
D. Kaplan-Meier analysis on relapse-free survival after surgery in patients with pT1N0M0 NSCLC (*solid lone*: cases with PD-L1-positive CAFs, *dotted line*: cases with PD-L1-negative CAFs).

**Table 3**
Univariate analyses on correlations of patients' prognosis and clinicopathological factors.

| Variables | pN0M0 (n = 125) | | | pT1N0M0 (n = 74) | | |
|---|---|---|---|---|---|---|
| | n (%) | 5-y RFP (%) | *P* | n (%) | 5-y RFP (%) | *P* |
| Age, years | | | | | | |
| < 70 | 66 (52.8) | 73.4 | 0.541 | 37 (52.8) | 77.7 | 0.635 |
| ≥70 | 59 (47.2) | 67.6 | | 37 (47.2) | 80.7 | |
| Gender | | | | | | |
| male | 84 (67.2) | 63.8 | 0.015 | 47 (67.2) | 73.7 | 0.132 |
| female | 41 (32.8) | 84.9 | | 27 (32.8) | 88.7 | |
| Smoking | | | | | | |
| never | 41 (32.8) | 85.1 | 0.012 | 26 (32.8) | 88.5 | 0.110 |
| current/former | 84 (67.2) | 63.6 | | 48 (67.2) | 74.0 | |
| Adjuvant chemotherapy | | | | | | |
| not performed | 94 (75.2) | 72.6 | 0.081 | 67 (77.6) | 80.5 | 0.085 |
| performed | 31 (24.8) | 65.8 | | 7 (22.4) | 71.4 | |
| Pathology | | | | | | |
| adenocarcinoma | 87 (69.6) | 74.1 | 0.516 | 52 (69.6) | 81.8 | 0.840 |
| squamous cell ca. | 38 (30.4) | 63.0 | | 22 (30.4) | 72.4 | |
| Tumor size, cm | | | | | | |
| ≤3.0 | 86 (68.8) | 77.3 | 0.006 | | | |
| > 3.0 | 39 (31.2) | 56.9 | | | | |
| Cell grade | | | | | | |
| grade 1 | 56 (44.8) | 70.7 | 0.636 | 33 (44.8) | 81.3 | 0.692 |
| grade 2-3 | 69 (55.2) | 70.9 | | 41 (55.2) | 78.1 | |
| Lymphatic invasion | | | | | | |
| absent | 61 (48.8) | 75.7 | 0.136 | 42 (49.6) | 84.5 | 0.119 |
| present | 64 (51.2) | 66.6 | | 32 (50.4) | 73.5 | |
| Vascular invasion | | | | | | |
| absent | 42 (33.6) | 75.8 | 0.396 | 28 (32.8) | 89.0 | 0.198 |
| present | 83 (66.4) | 67.8 | | 46 (67.2) | 72.5 | |
| PD-L1 on tumor cells | | | | | | |
| low | 104 (83.2) | 68.7 | 0.114 | 62 (83.8) | 76.7 | 0.098 |
| high | 21 (16.8) | 82.4 | | 12 (16.2) | 100.0 | |
| PD-L1 on CAFs | | | | | | |
| negative | 94 (75.2) | 66.3 | 0.031 | 52 (70.3) | 75.4 | 0.078 |
| positive | 31 (24.8) | 84.5 | | 22 (29.7) | 87.9 | |

5-y RFP: 5-year relapse-free probability

revealed to be a positive prognostic factor of higher 5-y RFP (HR: 2.134, 95% CI: 1.101–4.135, $P = 0.025$), and the variable was not correlated with PD-L1 expression on CAFs in patients with pT1N0M0 NSCLC ($P = 0.079$, data not shown). A variable, PD-L1 expression intensity of tumor cells, was unable to be applied in the multivariate analysis. These data demonstrate that PD-L1 expressions status on CAFs is an independent prognostic factor of tumor relapse after radical surgery for non-metastatic pN0M0 as well as early stage, pT1N0M0, NSCLC.

## 4. Discussion

In this study, we examined the clinical significance of PD-L1 expression on CAFs in pN0M0 NSCLC. The data showed that PD-L1 expression on CAFs was reversibly regulated by IFN-γ from activated lymphocytes. In pN0M0 NSCLC, high PD-L1 expression on CAFs predicted a better clinical course after radical surgery and was significantly associated with RFS after surgery.

Since the emergence of anti-cancer therapeutics targeting the PD-1/PD-L1 immune check point mechanism, the clinical significance of PD-L1 has been examined in patients with NSCLC. In many previous studies, the focus was on PD-L1 on tumor cells [10–15]. However, PD-L1 expression is not limited to tumor cells and is also expressed on several cell types in tumor stroma [17,18]. Given that tumor-suppressive and tumor-promoting cell types interact with each other in tumor stroma (reviewed in Ref. [19]), stromal PD-L1 status may carry some clinical significance in NSCLC. In the present study, we reported that PD-L1 expression on CAFs was significantly associated with better prognosis after surgery in pN0M0 NSCLC. In other papers focusing on immune cells in tumor stroma, it was reported that patients with PD-L1–positive regulatory T cells in the tumor tissue had a better response to anti-PD-1/PD-L1 immunotherapy in NSCLC [17]. In other types of cancer, PD-L1 expression in tumor-infiltrating immune cells was reported to be an independent factor for improved prognosis in patients with colorectal cancer [25] and triple-negative breast cancer [26]. These data implicated that stromal PD-L1 is involved in determining the prognosis of cancer patients. Thus, when the PD-L1 status of patients with NSCLC is evaluated, PD-L1 expression on tumor cells as well as stromal cells should be analyzed.

Concerning the association of PD-L1 expression with the prognosis of patients with pN0M0 NSCLC, it is important to consider why PD-L1 expression on CAFs contributes to prolonged RFS after surgery. Following the interaction between PD-L1 and PD-1, PD-L1-expressed tumor cells are believed to suppress the activity of PD-1-expressed activated T lymphocytes (reviewed in Ref. [9]). Stromal PD-L1 is also reported to inhibit the immune responses of CD8-positive cells in colorectal cancer in vitro [27]. These findings suggest that PD-L1-expressing CAFs could suppress anti-tumor immune responses through the exhaustion of PD-1-positve activated lymphocytes, leading to poor prognosis of patients; however, the data in the present study

**Table 4**
Multivariate analysis on relapse-free survival after surgery.

| Variables | pN0M0 (n = 125) | | | pT1N0M0 (n = 74) | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *P* | HR | 95% CI | *P* |
| Age, years | | | | | | |
| < 70 vs. ≥ 70 | 0.8111 | 0.423–1.553 | 0.527 | 1.326 | 0.797–2.206 | 0.277 |
| Gender | | | | | | |
| male vs. female | 2.396 | 0.622–9.233 | 0.204 | 0.939 | 0.303–2.843 | 0.897 |
| Smoking | | | | | | |
| current/formerr vs.never | 1.370 | 0.371–5.058 | 0.637 | 1.833 | 0.594–5.967 | 0.282 |
| Adjuvant chemotherapy | | | | | | |
| performed vs. not performed | 0.906 | 0.386–2.125 | 0.820 | 1.420 | 0.741–2.720 | 0.290 |
| Tumor size, cm | | | | | | |
| > 3.0 vs. ≤ 3.0 | 1.962 | 0.911–4.225 | 0.085 | | | |
| Microvessel invasion | | | | | | |
| present vs. absent | 1.496 | 0.652–3.434 | 0.342 | 2.134 | 1.101–4.135 | 0.025 |
| PD-L1 on tumor cells | | | | | | |
| low vs. high | 2.391 | 0.707–8.090 | 0.161 | – | – | – |
| PD-L1 on CAFs | | | | | | |
| negative vs. positive | 3.225 | 1.144–9.086 | 0.027 | 5.275 | 1.118–24.899 | 0.036 |

HR: hazard ratio, 95% CI: 95% confidence interval.

demonstrated opposite results. On the basis of the results in the present study showing that PD-L1 expression on CAFs is induced by IFN-γ released from activated lymphocytes, PD-L1 expression on CAFs suggests the induction of anti-tumor immune responses in the tumor microenvironment; however, unfortunately, we did find no correlation between PD-L1-positive CAFs and infiltration of lymphocytes in tumor stroma in the sections because it might have be difficult to catch the scene of contact with IFN-γ-releasing lymphocytes which might be freely moving around in the tumor microenvironment. In cases of PD-L1-expressing CAFs during the early stages of NSCLC, the exhaustion of PD-1-positive activated lymphocytes by PD-L1-expressing CAFs is unlikely to be induced because the period of interaction between PD-L1 and PD-1 might be still insufficient. A limitation of the present study is that we have insufficient data to confirm this hypothesis, and we need to examine the clinical significance of PD-L1-expressing CAFs in advanced NSCLC in further studies.

The tumor stroma is a complicated microenvironment in which CAFs interplay with many types of immune cell to affect their function. Thus, profile analyses of CAFs are of value in predicting the clinical course and prognosis of patients with NSCLC. In the present study, we showed that the existence of PD-L1-expressing CAFs indicated infiltration of IFN-γ-releasing activated lymphocytes, and that, in the early stages of NSCLC, patients with PD-L1-expressing CAFs would benefit from the induction of anti-tumor immune responses, leading to better prognosis. In other studies of prognostic markers on CAFs in patients with NSCLC, patients with podoplanin-positive CAFs were reported to have a risk of tumor relapse after surgery for stage I pulmonary adenocarcinoma [28]. Podoplanin-positive CAFs have the potential to generate an immunosuppressive tumor microenvironment [29]; thus, in patients with podoplanin-positive CAFs, anti-tumor immune responses do not work on residual tumor cells after surgery, leading to tumor relapse. Matrix metalloproteinase-2 (MMP-2)-positive CAFs are also reported to have a role in tumor progression, and patients with MMP-2-positive CAFs showed poor overall survival as compared with patients with MMP-2-negative CAFs in NSCLC [30]. Conversely, Kilvaer et al. reported that fibroblast activation protein (FAP)-positive CAFs were an indicator of good prognosis after surgery for squamous cell carcinoma of the lung [31,32]. They also reported that FAP-positive CAFs positively influenced the effector function of cytotoxic tumor-infiltrating lymphocytes [32] in other types of cancer, and FAP-positive CAFs were demonstrated to suppress anti-tumor immune responses [29]. Edlund et al. reported that CD99 expression on CAFs led to good overall survival after surgery in NSCLC [33], and CD99 on CAFs was supposed to modulate anti-tumor immune responses. These data indicate that CAFs seem to consist of phenotypically different fibroblast types, and it is worth examining the expression status of surface molecules on CAFs and the dominant phenotype among them to predict the prognosis of patients with NSCLC.

Even in stage I NSCLC with no metastatic lesions, approximately 20% of patients experience tumor relapse after radical surgery [34]. In the present study, 5-y RFP was 71.8% for patients who had undergone complete resection for pN0M0 NSCLC. In patients who experience tumor relapse, some critical mechanisms of recurrence occur before surgery. Our data suggest that insufficient induction of anti-tumor immune responses may cause tumor recurrences. From the viewpoint of tumor immunology, as a predictive biomarker for tumor recurrence in pN0M0 NSCLC, Takahashi et al. reported that high neutrophil–lymphocytes ratios in peripheral blood were associated with poor patient prognosis [35]. Another predictive biomarker of tumor recurrence in pN0M0 NSCLC is reported to be vessel invasion of tumor cells [36,37]. Ko et al. reported that overexpression of cyclin A2 on tumor cells was associated with poor relapse-free survival because cyclin A2 positively regulated the cell cycle of tumor cells [38]. Concerning genetic risk factor, it is reported that cohypermethylation of *CDKN2A* and *FHIT* in tumor cells is associated with increased risk of tumor recurrence in pN0M0 NSCLC [39]. Many immunological, molecular, and genetic factors seem to be implicated in tumor recurrence of pN0M0 NSCLC. In this study, we focused on just one molecule, PD-L1, and examined its correlation with tumor recurrence in pN0M0 NSCLC. On the basis of the contribution of many prognostic factors on tumor recurrence in pN0M0 NSCLC, we need to focus on the combination of PD-L1 expression on CAFs with other prognostic factors in future studies, and evaluate it as a more reliable prognostic biomarker for tumor recurrence in pN0M0 NSCLC.

In conclusion, in the early stages of pN0M0 NSCLC, PD-L1 expression on CAFs suggests the induction of anti-tumor immune responses, and PD-L1 expression intensity can serve as a prognostic biomarker to predict tumor relapse after radical surgery in patients with pN0M0 NSCLC.

## Declaration of Competing Interest

The authors have declared no conflict of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.09.013.

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