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# Link between tumor-promoting fibrous microenvironment and an immunosuppressive microenvironment in stage I lung adenocarcinoma

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ABSTRACT

*Objectives:* Podoplanin-positive cancer-associated fibroblasts [PDPN (+) CAFs] play an important role in cancer progression in non-small-cell lung cancer. The aim of this study was to clarify the correlation between a fibrous microenvironment containing PDPN (+) CAFs and an immune microenvironment.

*Materials and methods:* A total of 174 patients with pathological stage I lung adenocarcinoma were analyzed. We evaluated PDPN (+) CAFs and immune-related cells, CD 204-positive tumor-associated macrophages [CD204 (+) TAMs], CD8-positive T cells, and FOXP3-positive T cells, in cancer stroma by using immunohistochemical staining. We compared the expression levels of immune-regulatory cytokines between the PDPN high and low expression groups by analyzing the gene expression profiles of lung adenocarcinoma (n = 442).

*Results:* Presence of PDPN (+) CAFs was a risk factor for recurrence ($P = 0.042$). The number of CD204 (+) TAMs was significantly higher ($P < 0.001$) and the CD8/FOXP3 T cell ratio was significantly lower in PDPN (+) CAFs cases than in PDPN (-) CAFs cases ($P = 0.027$). Within the same tumor, the number of CD 204 (+) TAMs was significantly higher ($P < 0.001$) and CD8/FOXP3 T cell ratio tended to be lower ($P = 0.062$) in PDPN (+) CAF areas. Microarray analysis revealed that the PDPN expression-high group had significantly higher gene expression levels of cytokines that inducing M2 macrophage polarization and suppressing immune-related lymphocytes.

*Conclusion:* The current results show that lung adenocarcinoma with PDPN (+) CAFs is typified by the immunosuppressive microenvironment, suggesting a close link between the tumor-promoting fibrous microenvironment and the immunosuppressive microenvironment.

## 1. Introduction

The prognosis of even early-stage non-small-cell lung cancer (NSCLC) is critical due to local recurrence and distant metastasis following surgery [1]. Some meta-analyses and randomized trials have shown that adjuvant chemotherapy improves survival in patients with NSCLC compared to surgical intervention alone; however, this remains a controversial issue [2,3]. Clarifying the process of cancer prognosis and identification of risk factors for recurrence would help establish effective therapeutic strategies for NSCLC treatment.

Cancer tissue consists not only of cancer cells, but of also several kinds of stromal cells. Various types of stromal cells, mainly fibroblast,

*Abbreviations:* NSCLC, non-small-cell lung cancer; CAFs, cancer-associated fibroblasts; PDPN, podoplanin; PDPN (+) CAFs, PDPN-positive CAFs; TAMs, tumor-associated macrophages; TILs, tumor-infiltrated lymphocytes; FAP, fibroblast activation protein-alpha; HE, hematoxylin and eosin; VVG, verhoeff-van-Gieson; M-CSF, macrophage colony-stimulating factor; IL, interleukin; IDO, indoleamine 2,3-dioxygenase; TGFβ1, transforming growth factor-β1; VEGFA, vascular endothelial growth factor A precursor; RFS, recurrence free survival

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endothelial, and immune-related cells create a specific microenvironment for tumor progression [4–6]. Several reports have demonstrated that these stromal cells can influence biological behavior of cancer cells through both direct and indirect mechanisms [6–8].

Fibroblasts in cancer tissue, known as cancer-associated fibroblasts (CAFs), are a major component of stromal cells. CAFs are the main source of collagen and other extracellular matrix proteins, and have critical early influence in tumorigenesis and tumor development: creating a specific fibrous microenvironment facilitating cancer progression [9–11]. Furthermore, CAFs directly interact with cancer cells, which results in a phenotypic change of cancer cells. In particular, podoplanin (PDPN), a glycoprotein also known as a marker for lymphatic vessels, is one of the functional molecules expressed by CAFs in several types of cancer including NSCLC [12,13]. PDPN-positive CAFs [PDPN (+) CAFs] contribute to cancer cell invasion and implantation [14,15]. Moreover, retrospective studies have reported that the presence of PDPN (+) CAFs was a risk factor for prognosis in NSCLC [16,17]. These results indicate that PDPN (+) CAFs are one of the subpopulation of tumor-promoting CAFs.

The immune microenvironment had a major impact on cancer prognosis in NSCLC [18–20]. Immune-related cells, such as tumor-associated macrophages (TAMs) and tumor-infiltrated lymphocytes (TILs), in cancer stroma regulate cancer progression in various ways. TAMs, mainly M2 TAMs, promote cancer progression by producing cytokines involved in angiogenesis, tumorigenesis, matrix remodeling, and immunosuppression [21–23]. TILs such as cytotoxic T cells, helper T cells, natural killer cells, and regulatory T cells regulate cancer progression by playing two conflicting roles: suppressing tumor growth and promoting tumor progression [24]. Recent studies have shown that regulatory T cells in cancer stroma could inhibit cytotoxic T cells and induce an immunosuppressive environment allowing cancer cells to escape host immune surveillance in several cancers [25–27].

Costa et al., revealed that a special subpopulation of CAFs; CAF-S1, which express five fibroblast markers; fibroblast activation protein-alpha (FAP), integrinβ1/CD29, αSMA, S100-A4/FSP1 (fibroblast-specific protein 1), and PDGFRβ (platelet-derived growth factor receptor-β) produce the immunosuppressive microenvironment via secretion of immunoregulatory cytokines in triple-negative breast cancer [28]. This result implies that CAF-S1 affects the immune microenvironment.

Investigating the correlation between the tumor-promoting fibrous microenvironment and immunosuppressive microenvironment is important for understanding cancer progression processes as well as to develop a microenvironment-targeted therapy. In the current study, we aimed to clarify the correlation between the fibrous microenvironment, containing PDPN (+) CAFs, and the immune microenvironment.

## 2. Materials and methods

### 2.1. Subjects

We retrospectively reviewed our database for patients who underwent complete surgical resection and lobectomy for their pathological stage I primary lung adenocarcinoma in Department of Thoracic Surgery, National Cancer Center Hospital East between January 2011 and December 2012. We excluded patients who were diagnosed with pathologically minimally invasive adenocarcinoma, variants of adenocarcinoma, usual interstitial pneumonia, multiple lung carcinomas, and had undergone preoperative chemotherapy and/or radiation therapy. The remaining 174 patients were included in this analysis. We obtained comprehensive informed consent from all the patients before this study (National Cancer Center Hospital IRB approval number; 2017-356).

### 2.2. Clinicopathological evaluation

All surgical specimens were fixed using 10% formalin, and embedded in paraffin. All tumors were cut at 5-mm intervals, and 4 μm-thick-sections were stained using the hematoxylin and eosin (HE) method. The Verhoeff-van-Gieson (VVG) method was also performed for visualizing elastic fibers. Lymphovascular and pleural invasion were identified using both HE and VVG, respectively. The diagnosis of histological type was based on the 2015 World Health Organization classification of lung tumors [29], and pathological stage was determined according to the 8$^{th}$ edition of TNM classification for lung cancer [30].

We reviewed the regularly updated clinicopathological database from the division of thoracic surgery, National Cancer Center Hospital East, Japan. We extracted patient information, including age, sex, smoking status, tumor size (total size and invasive size), lymphovascular and pleural invasion, pathological stage, subtype predominant, EGFR mutation status, recurrence, and survival status.

### 2.3. Patient follow-up

All patients were followed at 6-month intervals for 5 years after surgery. The follow-up evaluation included a physical examination, chest radiography, and blood examination. Computed tomography was performed at 1-year intervals. After recurrence was detected, all patients underwent anti-cancer therapies including chemotherapy, radiotherapy, immunotherapy, and molecular targeted therapy.

### 2.4. Immunohistochemistry

We performed immunohistochemical staining using 4 antibodies according to previously published methods [17]. We used anti-PDPN (mouse monoclonal, diluted at 1:200, D-40; Acris Antibodies Inc., San Diego, CA, USA) as a CAF marker, anti-CD204 (mouse monoclonal, diluted at 1:400, SRA-E5; Trans Genic Inc., Fukuoka, Japan) as the TAMs marker, and anti-CD8 (rabbit monoclonal, diluted at 1:800, P17-V; DB Biotech Inc., Kosice, Slovakia) and FOXP3 (rabbit monoclonal, diluted at 1:100, SP97; Acris Antibodies Inc., San Diego, CA, USA) to identify the lymphocyte subpopulation.

### 2.5. Calculation of immunohistochemical scores

All stained slides were scanned and captured using a digital slide scanner; Aperio VERSA SL200 (Leica Biosystems, Nußloch, Germany) and NanoZoomer-XR C12000-03 (Hamamatsu Photonics, Shizuoka, Japan), and reviewed by two different pathologists (TS and GI) who did not know the clinicopathological information of each slide. For anti-PDPN staining, spindle cells in cancer stroma were identified morphologically as CAFs, and classified as negative (-; stained at less than 10%), and positive (+; more than 10%). We also divided cases into 3 groups according to the expression grade of PDPN in CAFs: grade 0 (PDPN (+) CAF area/stromal area < 10%, n = 131), grade 1 (PDPN (+) CAF area/stromal area = 10–50%; n = 29), and grade 2 (PDPN (+) CAF area/stromal area > 50%; n = 14). The cut-off on PDPN-CAFs was set based on the previous study [16]. For anti-CD204, anti-CD8 and anti-FOXP3 staining, we selected 5 areas where the macrophages or lymphocytes most infiltrated the cancer stroma, enclosed them in high power fields (0.0625 mm$^2$ /field), counted and averaged the number for each case (**Supplementary Figure S1**).

### 2.6. Analysis of microarray data

We analyzed a messenger RNA expression dataset including 442 patients with primary lung adenocarcinoma (GSE 68465) by using the Affymetrix Human Genome U133 A 2.0 Array (Thermo Fisher Inc., Waltham, MD, USA) [31]. We identified the podoplanin gene (204879_at) and divided samples into either the group of high-expression level or the group of low-expression level, based on the median. We extracted the genes of cytokine polarizing M2 macrophages; macrophage colony-stimulating factor (M-CSF), interleukin (IL)-4, IL-10, IL-14 and cytokines related to immunosuppression; indoleamine 2,3-

dioxygenase (IDO), transforming growth factor-β1 (TGFβ1), vascular endothelial growth factor A precursor (VEGFA), and galectin family, and analyzed them in relation to PDPN expression [32–37].

### 2.7. Statistical analysis

Differences in categorical variables were analyzed by using Fisher's exact test, and continuous variables were analyzed by using Mann–Whitney's U test and Kruskal-Wallis test. Calculation of the recurrence-free survival (RFS) was performed using the Kaplan–Meier method, and compared using the log-rank test. Multivariate analysis was performed using the Cox-proportional hazard model. *P* -values were determined using two-sided analyses, and the statistical significance level was set at $< 0.05$. All statistical analyses were performed using JMP® ver.13.2.1 (SAS Institute Inc., Cary, NC, USA).

## 3. Results

### 3.1. Relationship between PDPN expression status and clinicopathological features

Fig. 1 shows representative PDPN staining in CAFs. All patients (n = 174) were classified into two groups according to the expression status of PDPN antibody, either negative (PDPN (-) CAFs; n = 131) or positive (PDPN (+) CAFs; n = 43). Univariate analysis of clinicopathological features for recurrence showed that pack-year smoking (HR = 1.01; 95% CI = 1.00–1.02; $P = 0.037$), invasive size (HR = 1.10; 95% CI = 1.05–1.16; $P < 0.001$), lymphatic permeation (HR = 3.594; 95% CI = 1.39–8.36; $P = 0.004$), vascular invasion (HR = 4.98; 95% CI = 2.23–11.56; $P < 0.001$), pleural invasion (HR = 2.87; 95% CI = 1.24–6.42; $P = 0.011$), and positivity of PDPN (+) CAFs (HR = 2.48; 95% CI = 1.07–5.54; $P = 0.029$) were risk factors (**Supplementary Table S1**). Invasive size (HR = 1.06; 95% CI = 1.00–1.12; $P = 0.039$) and vascular invasion (HR = 4.98; 95% CI = 1.07–7.05; $P = 0.035$) were independent prognostic factors for recurrence based on the multivariate analysis (**Supplementary Table S2**).

**Table 1**
Relationship between podoplanin expression status and clinicopathological features.

| Variables | | Podoplanin (-) (N = 131) | Podoplanin (+) (N = 43) | *p* value |
|---|---|---|---|---|
| Age (y) | mean ± SD | 67.8 ± 9.8 | 68.3 ± 7.9 | 0.70 |
| Sex: Male | number (%) | 69 (53.1) | 28 (63.6) | 0.28 |
| Pack Year Smoking | mean ± SD | 20.5 ± 26.7 | 32.0 ± 36.2 | 0.034 |
| Invasive size (cm) | mean ± SD | 1.6 ± 0.7 | 2.0 ± 0.8 | < 0.001 |
| Lymphatic invasion (+) | number (%) | 11 (8.5) | 10 (22.7) | 0.010 |
| Vascular invasion (+) | number (%) | 23 (17.7) | 19 (43.2) | 0.002 |
| Pleural invasion (+) | number (%) | 25 (19.2) | 14 (31.8) | 0.16 |
| EGFR mutation (+) | number (%) | 30 / 59 (50.8) | 9 / 22 (40.9) | 0.56 |

Pack-year smoking (20.5 ± 36.7 vs. 32.0 ± 36.2; $P = 0.034$), invasive size (1.6 ± 0.7 vs. 2.0 ± 0.8; $P < 0.001$), positivity of vascular invasion (17.7% vs. 43.2%; $P = 0.010$), and positivity of lymphatic permeation (8.5% vs. 22.7%; $P = 0.002$) were significantly higher in PDPN (+) CAFs cases (Table 1). With a median follow-up period of 5.5 years, PDPN (+) CAFs cases had significantly shorter RFS than PDPN (-) CAFs cases (66.7% vs. 84.1%, $P = 0.042$) (**Supplementary Figure S2**).

### 3.2. Relationship between PDPN expression status in CAFs and immune cells

Representative cases of immunohistochemical staining with CD204 antibody are shown in Fig. 2**A and B**. The number of CD204 (+) TAMs in PDPN (+) CAFs cases was significantly higher than in PDPN (-) CAFs cases (11.7 vs. 33.1; $P < 0.001$) (Fig. 2C).

To clarify the relationship between PDPN expression levels and immune cells for further confirmation, we divided cases into 3 groups according to the expression grade of PDPN in CAFs: grade 0 (PDPN (+)

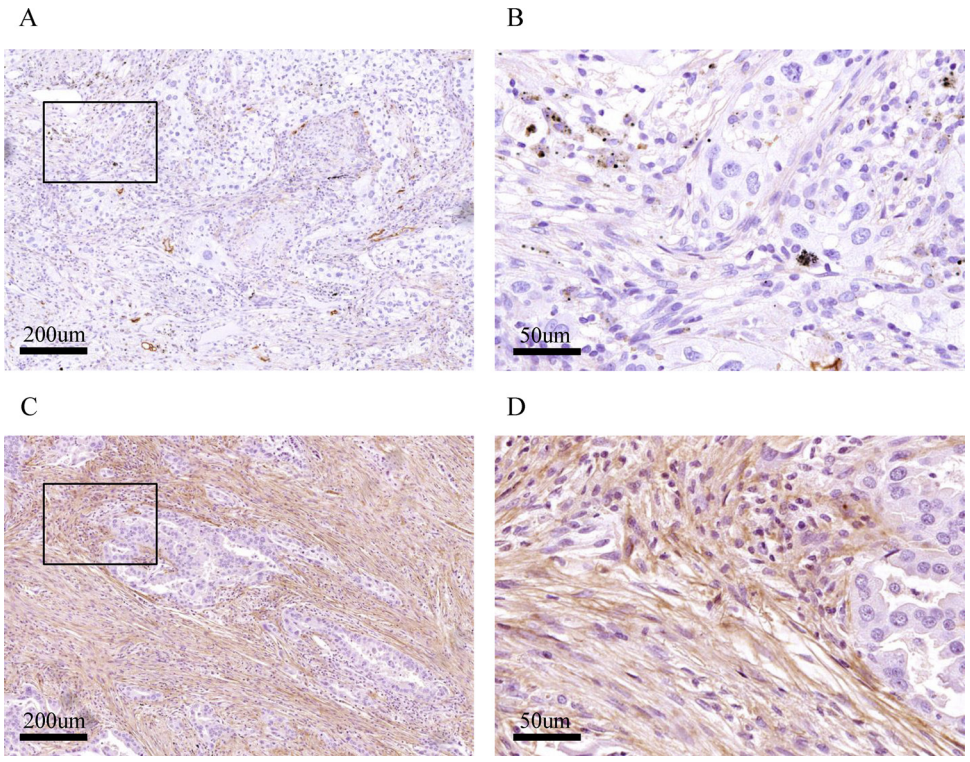


**Fig. 1.** Immunohistochemical staining of pathological stage I lung adenocarcinoma with PDPN antibody in CAFs.
A; Case with PDPN (-) CAFs at lower magnification.
B; Higher magnification of the square area of Figure A.
C; Case with PDPN (+) CAFs at lower magnification.
D; Higher magnification of the square area of Figure C.

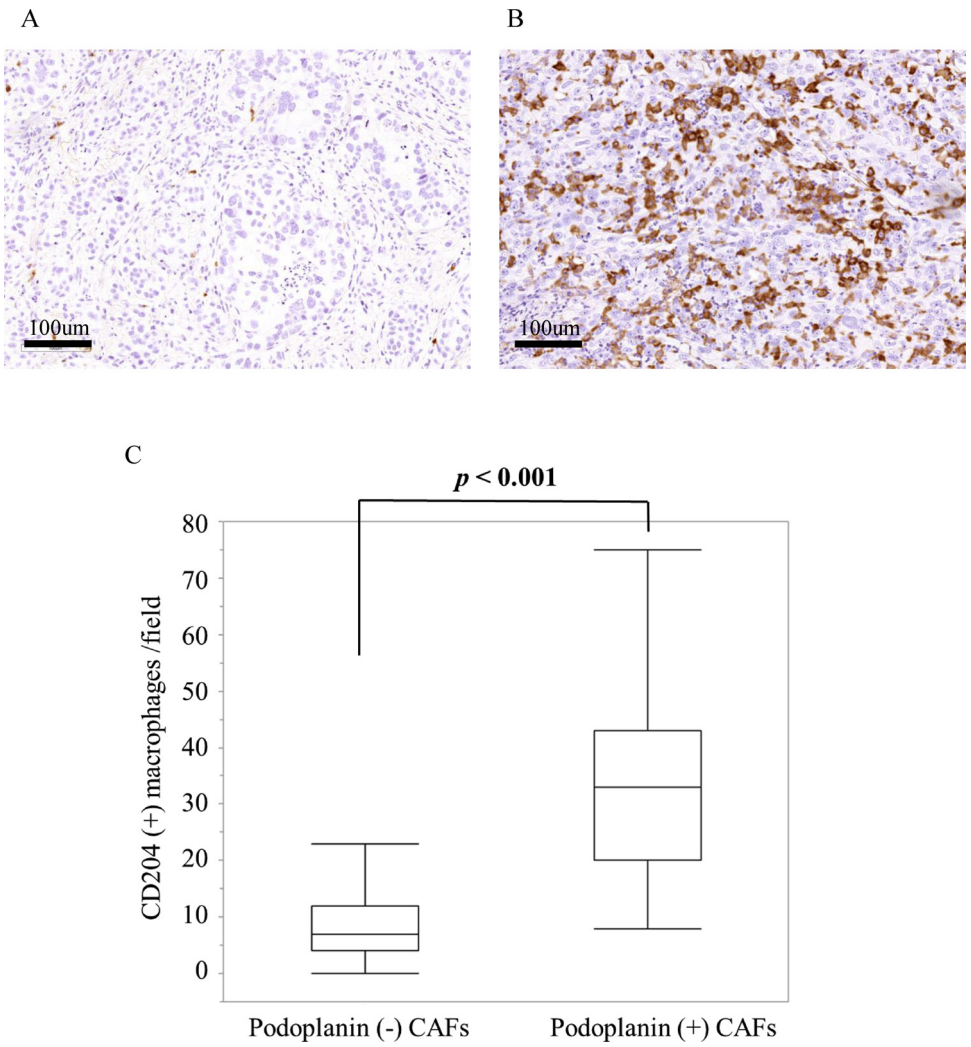


**Fig. 2.** Immunohistochemical staining with CD204 antibody.
A; Case with a low number of CD204 (+) TAMs.
B; Case with a high number of CD204 (+) TAMs.
C; Comparison of the number of CD204 (+) TAMs according to the expression status of PDPN in CAFs.

CAF area/stromal area < 10%, n = 131), grade 1 (PDPN (+) CAF area/stromal area = 10–50%; n = 29), and grade 2 (PDPN (+) CAF area/stromal area > 50%; n = 14). The number of CD204 (+) TAMs was significantly higher as the expression rate of PDPN increased (11.7 vs. 30.2 vs. 39.2; $P < 0.001$) (**Supplementary Figure S3**).

Next, we examined the number of CD8-positive T cells [CD8 (+) T cells] and FOXP3-positive T cells [FOXP3 (+) T cells] according to PDPN expression status (Fig. 3**A–D**). The number of FOXP3 (+) T cells in PDPN (+) CAFs cases was significantly higher than in PDPN (-) CAFs cases (17.4 vs. 22.3; $P = 0.010$), but the number of CD8 (+) T cells showed no significant difference according to PDPN expression in CAFs (48.4 vs. 52.0; $P = 0.30$) (**Supplementary Figure S4**). CD8/FOXP3 T cell ratio in PDPN (+) CAFs cases was significantly lower than in PDPN (-) CAFs cases (3.8 vs. 2.8; $P = 0.027$, Fig. 3E). The number of FOXP3 (+) T cells was significantly higher as the expression grade of PDPN in CAFs increased (17.4 vs. 20.5 vs. 26.1; $P = 0.010$), however; CD8 (+) T cells showed no significant difference according to PDPN expression grade (48.4 vs. 52.3 vs. 51.5; $P = 0.59$). The CD8/FOXP3 ratio tended to be lower as the expression grade of PDPN increased (3.8 vs. 2.9 vs. 2.6; $P = 0.081$) (**Supplementary Figure S3**).

### 3.3. Anatomical correlation between PDPN (+) CAFs and immune cells

To validate the anatomical correlation between PDPN (+) CAFs and immune cells, we examined CD204, CD8, and FOXP3 positive cells within PDPN-CAF (+) areas and PDPN-CAF (−) areas within the same tumor cases (**Supplementary Figure S5**). The number of CD 204 (+) TAMs was significantly higher in the PDPN (+) CAF areas than in PDPN (-) CAF areas ($P < 0.001$), and the CD8/FOXP3 T cell ratio tended to be lower in PDPN (+) CAF areas than in PDPN (-) CAFs areas ($P = 0.062$) (Fig. 4).

### 3.4. Relationships between gene expression levels of PDPN and immune-regulatory cytokines

To investigate gene expression of immune-regulatory cytokines according to expressions level of PDPN, we analyzed a messenger RNA expression dataset including 442 patients with primary lung adenocarcinoma (public database; GSE 68,465) [31]. We identified the gene of PDPN and divided samples into two groups, either PDPN expression-high group or the PDPN expression-low group, based on the median. We extracted cytokine genes which are involved in polarizing M2 macrophages; M-CSF, IL-4, IL-10, IL-14, and suppressing immune related lymphocytes; IDO, TGFβ1, VEGFA, galectin1, and galectin 9. In

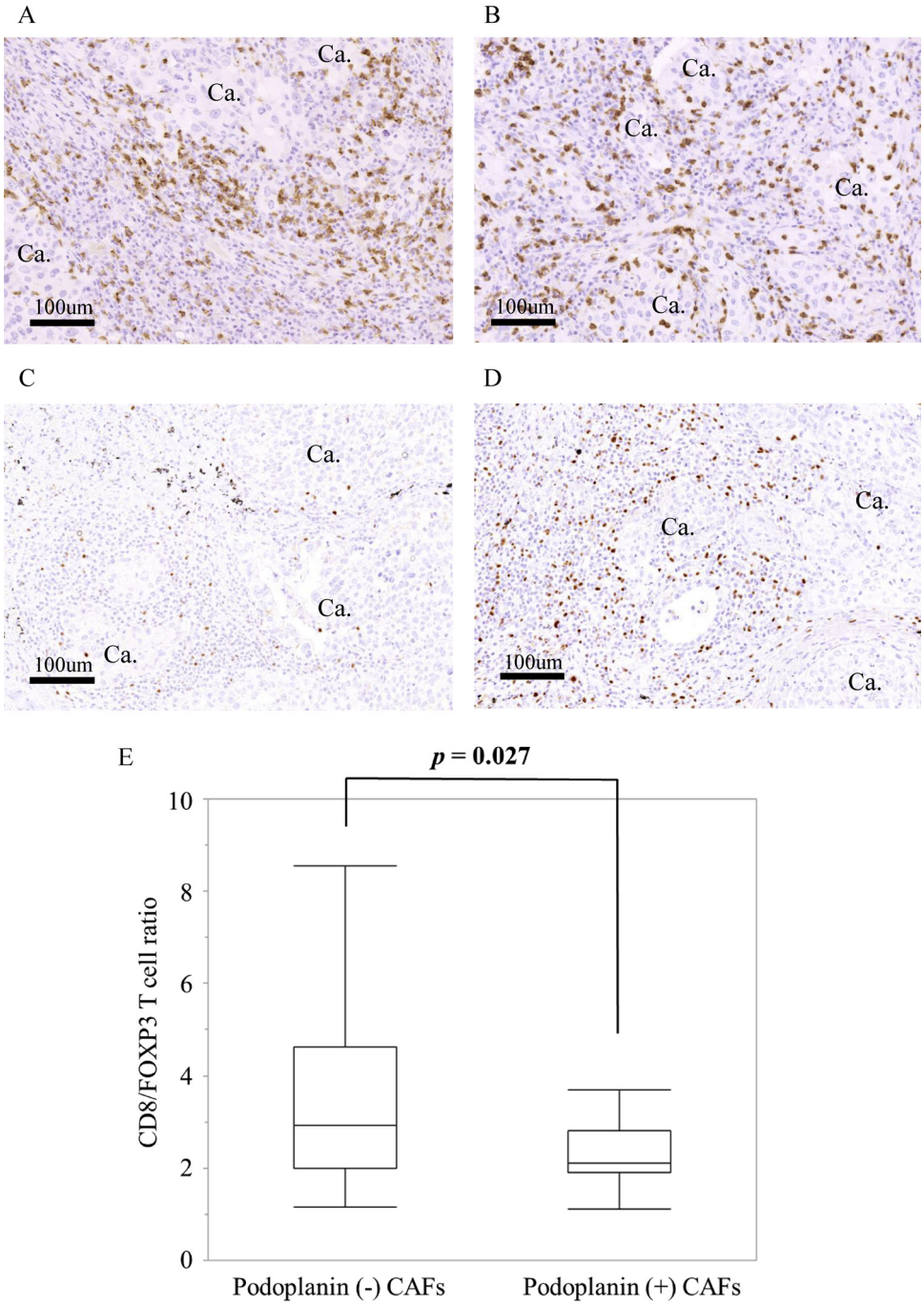


**Fig. 3.** Immunohistochemical staining with CD8 and FOXP3 antibody.
A; CD8 expression in PDPN (-) CAFs case.
B; CD8 expression in PDPN (+) CAFs case.
C; FOXP3 expression in PDPN (-) CAFs case.
D; FOXP3 expression in PDPN (+) CAFs case.
E, Comparison of CD8/FOXP3 T cell ratio according to the expression status of PDPN in CAFs.

the cytokine genes polarizing M2 macrophages, PDPN expression-high group had a significantly higher level of M-CSF than PDPN expression-low group ($P < 0.001$). In the cytokine genes suppressing immune related lymphocytes, the PDPN expression-high group had a significantly higher level of TGFβ1 ($P < 0.001$), IDO ($P < 0.001$), VEGFA ($P = 0.048$), and galectin 1 ($P < 0.001$) than the PDPN expression-low group, and had a higher level of galectin 9 ($P = 0.10$) (Fig. 5).

## 4. Discussion

In this study, we clarified that pathological Stage I lung adenocarcinoma with PDPN (+) CAFs display both a high number of CD204 (+) TAMs and a low ratio of CD8/FOXP3 T cells in the cancer stroma. These consequences were also confirmed by comparing the number of CD204 (+) TAMs and the CD8/FOXP3 T cell ratio between PDPN (+) CAF areas and PDPN (-) CAF areas within the same tumor. Moreover, PDPN-high lung adenocarcinoma had higher gene expression level of

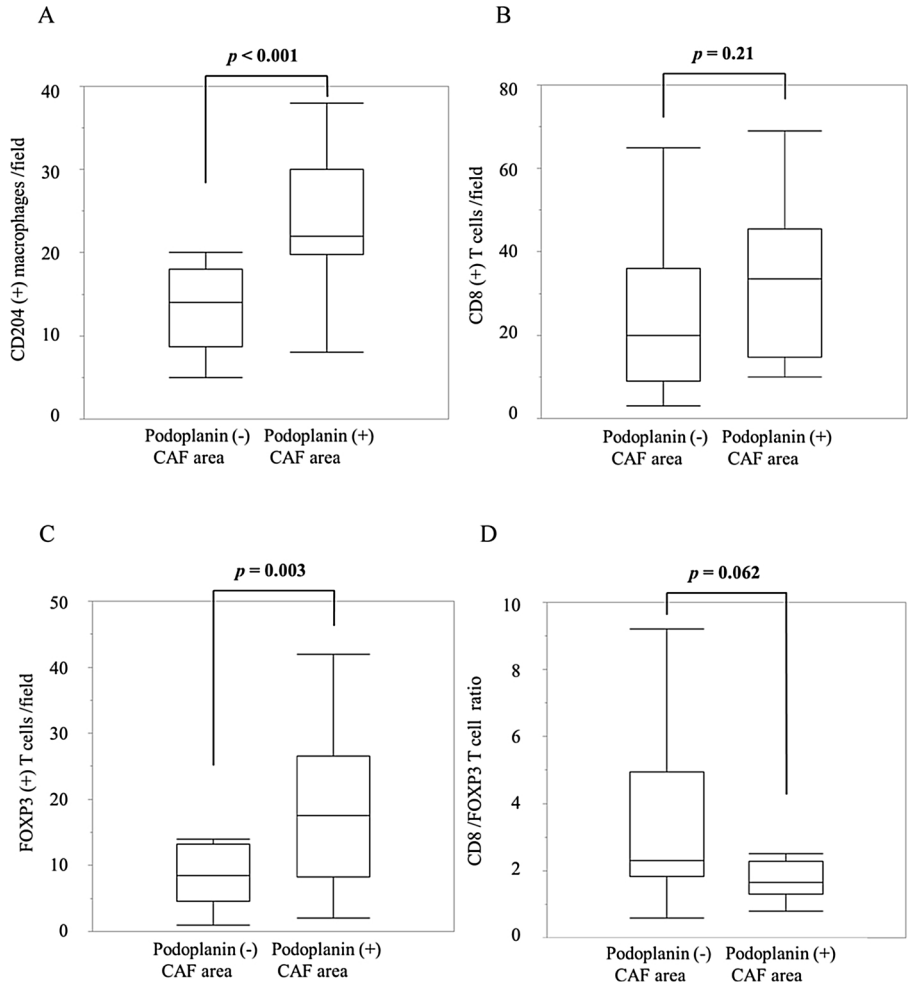


**Fig. 4.** Comparison of the immune-related cells according to the PDPN (+) CAFs area vs. PDPN (-) area in the same cases.
A; The number of CD204 (+) TAMs.
B; The number of CD8 (+) T cells.
C; The number of FOXP3 (+) T cells.
D; CD8/FOXP3 T cell ratio.

cytokines, polarizing M2 macrophage polarization, and immunosuppression according to the microarray data analysis. This is the first report elucidating that the tumor-promoting fibrous microenvironment containing PDPN (+) CAFs correlates with the immunosuppressive microenvironment in NSCLC.

TILs constitute an immune suppressing microenvironment by selecting the cancer cells that can evade immune surveillance through a multistep process: elimination, equilibrium, and escape [24,27]. Regulatory T cells have been known to play an important role in suppressing the immune microenvironment involving T cells. Forkhead box P3 (FOXP3) is a member of the forkhead/winged-helix family of transcriptional regulators, and some studies have shown that FOXP3 (+) regulatory T cells are related to prognosis in NSCLC [38,39]. Recent studies have shown that CAFs indirectly induce an immunosuppressing microenvironment via secretion of immunoregulatory cytokines [40,41]. Kinoshita et al., showed that culture supernatant of CAFs from FOXP3 (+) T cells-high lung adenocarcinoma expressed higher mRNA levels of TGFβ and VEGF; cytokines that involve in inducing regulatory T cells [42]. These results suggested that a subpopulation of CAFs might produce immunosuppressing cytokines and create a tumor-promoting microenvironment in lung adenocarcinoma. Besides, Costa et al., revealed that one of subpopulations of CAFs, CAF-S1, creates the immunosuppressing microenvironment via secretion of immunoregulatory cytokines in triple-negative breast cancer [28]. In a murine cancer model, FAP-positive CAFs create the immunosuppressing microenvironment by attracting myeloid-derived suppressor cells via STAT3–CCL2 signaling [43,44]. We have already demonstrated that PDPN (+) CAFs directly promote cancer cell invasion using an *in vitro* model [45] and implantations in animal models [46,47], our present study raises the possibility that PDPN (+) CAFs might promote cancer progression via indirect mechanisms, to alter the host immune surveillance system, as well as direct mechanisms.

We previously reported that solid predominant lung adenocarcinoma displayed a higher number of PDPN (+) CAFs, CD204 (+) TAMs and FOXP3 (+) T cells. Saruwatari et al. reported that solid predominant lung adenocarcinoma displayed higher levels of PDPN (+) CAFs and CD204 (+) TAMs significantly than non-solid predominant lung adenocarcinoma, and Kinoshita et al. reported that the number of FOXP3 (+) T cells in solid predominant tumors was significantly higher than in non-solid predominant tumors in lung adenocarcinoma [42,48]. In this study, pathological Stage I lung adenocarcinoma with solid predominant displayed a higher number of PDPN (+) CAFs and CD204 (+) TAMs significantly, and tended to display a higher number of FOXP3 T cells than with non-solid predominant (**Supplementary Table S3**). These results also confirm the previous reports that solid predominant adenocarcinomas display higher levels of PDPN (+) CAFs and immune cells related to immunosuppression.

There are some limitations in this study. This study was

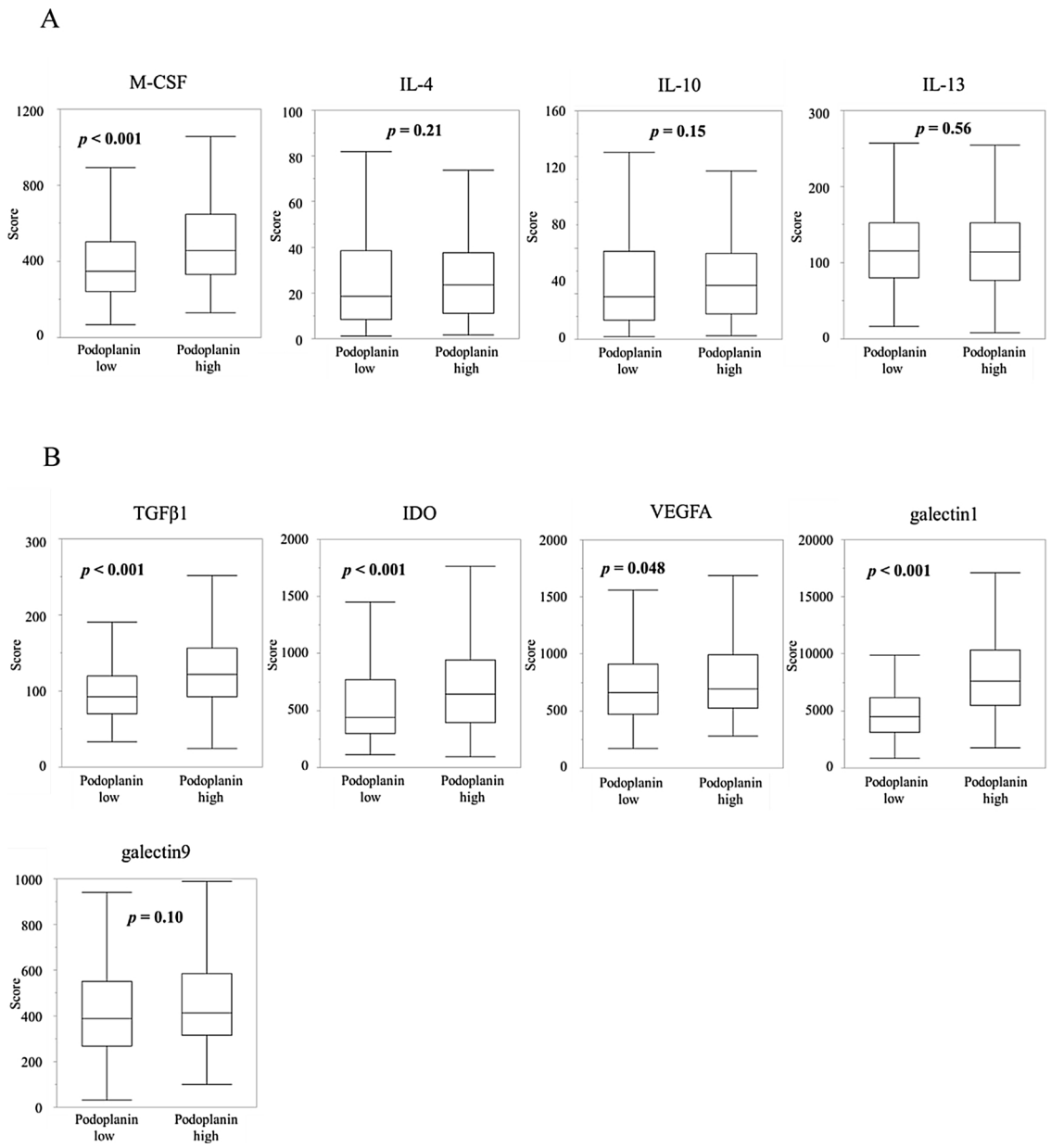


**Fig. 5.** Comparison of the gene expression score in the microarray data.
A; Gene expression status of cytokines inducing M2 macrophage polarization.
B; Gene expression status of immunosuppressive cytokines.

retrospective and was a single-center study. Prospective and multicenter studies with more cases will be required in the future. Furthermore, it is also important to investigate whether the subpopulation of PDPN (+) CAFs can secrete high levels of immunosuppressive cytokines and affect the immune microenvironment. Alternatively, cancer cells might secrete master-regulatory factors, which result in the recruitment of PDPN (+) CAFs, CD204 (+) TAMs, and FOXP3 T cells.

In conclusion, we showed the close link between the tumor-promoting fibrous microenvironment and the immunosuppressive microenvironment of NSCLC, for the first time. Immunotherapy using an immune checkpoint inhibitor has been incorporated in a treatment strategy for NSCLC with excellent results in clinical trials [49]. However, as the features of TILs differ in clinicopathological factors especially for adenocarcinoma, it may influence the efficiency of immunotherapy [20]. The current study suggests the possibility that PDPN (+) CAFs could be a novel biomarker for predicting the effect of immunotherapy in NSCLC.

## Ethics approval and consent to participate

We obtained comprehensive informed consent from all the patients before this study (National Cancer Center Hospital IRB approval number; 2017-356).

## Author contributions

Conception and design: TS, and GI; development of methodology: TS, HN and GI; acquisition of data and analysis and interpretation of data: TS, KA, SNe, SNo, KT, TM, MT and GI; writing, review and/or revision of the manuscript: TS, KA, SNe, HN, SNo, KT, TM, MS, MK, SF, TK, AO, AI, MT and GI; administrative, technical, or material support: KA, SNe, SNo, MT and IG; study supervision: KA, and GI.

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## Conflict of 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.2018.10.021.

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