Virchows Archiv (2019) 475:181–189
https://doi.org/10.1007/s00428-019-02587-9

ORIGINAL ARTICLE

Prognostic significance of combining immunohistochemical markers for cancer-associated fibroblasts in lung adenocarcinoma tissue

Chihiro Inoue[1] · Daisuke Tamatsuki[2] · Yasuhiro Miki[3] · Ryoko Saito[1] · Yoshinori Okada[4] · Hironobu Sasano[1]

Received: 5 March 2019 / Revised: 16 April 2019 / Accepted: 7 May 2019 / Published online: 27 May 2019

Abstract

Cancer-associated fibroblasts (CAFs), activated fibroblasts in a cancer microenvironment, exert various effects upon carcinoma cells including lung adenocarcinoma cells. Various markers identifying CAFs have been proposed, but the correlations among these markers proposed and their clinicopathological significance have remained largely unknown. Therefore, in this study, we immunohistochemically evaluated the expression of alpha-smooth muscle actin (α-SMA), podoplanin, and periostin among these proposed markers in 92 cases of lung adenocarcinoma. These three markers were weakly correlated, but the relative abundance of α-SMA was significantly associated with high Ki-67 labelling index (LI), lymph node metastasis, and low 5-year overall survival (OS) rate of the patients. That of podoplanin was significantly associated with high pT and Ki-67 LI, distant metastasis, and low 5-year OS rate and that of periostin with high pT and Ki-67 LI. We then tentatively subclassified these cases into four groups according to high or low status of each of paired markers: α-SMA/podoplanin, α-SMA/periostin, and periostin/podoplanin. The α-SMA high/podoplanin high group was associated with the lowest survival rate (53.3%) among the four groups with significance. However, there were no significant differences in overall survival when the patients were classified according to the combinations of α-SMA/periostin or periostin/podoplanin. Results of our study firstly revealed the heterogeneity of CAFs in human lung adenocarcinoma tissue, and the analysis employing multiple markers of CAFs is generally required to study the clinical significance of CAFs in clinical materials.

Keywords Alpha-smooth muscle actin · Periostin · Podoplanin · Cancer-associated fibroblasts · Lung adenocarcinoma · Immunohistochemistry

Introduction

Cancer-associated fibroblasts (CAFs) are activated fibroblasts in a cancer microenvironment. CAFs interact with carcinoma cells and other components of the microenvironment, including immune cells and blood vessels, thereby influencing the biological behavior of carcinoma cells, such as their proliferation, migration, and invasion. In lung adenocarcinoma, CAFs have been reported to influence the prognosis and resistance to chemotherapy [1]. In addition, CAFs have been proposed to be derived from various cells such as resident fibroblasts, bone marrow-derived progenitor cells, or even epithelial carcinoma cells undergoing epithelial-to-mesenchymal transition [1–4]. Therefore, considering the various origins above, CAFs that have undergone genetic and/or epigenetic regulation are considered to be highly heterogeneous and vary in their function [1–4] but this has not necessarily been studied in detail in human malignancy.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00428-019-02587-9) contains supplementary material, which is available to authorized users.

✉ Chihiro Inoue
chihiro_inoue@med.tohoku.ac.jp

[1] Department of Anatomic Pathology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan

[2] Tohoku University School of Medicine, Sendai, Miyagi, Japan

[3] Department of Disaster Obstetrics and Gynecology, International Research Institute of Disaster Science, Tohoku University, Sendai, Miyagi, Japan

[4] Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Miyagi, Japan

Morphologically, CAFs are known to be similar to activated fibroblasts detected in wound healing, but it is pivotal to identify the marker proteins that could characterize CAFs. Among the proteins proposed, alpha-smooth muscle actin (α-SMA) has been widely used to detect CAFs at both in vivo and in vitro levels [1, 5–7]. In addition to α-SMA, various other proteins, such as podoplanin, periostin, fibroblast activation protein (FAP), platelet-derived growth factor receptor-α/β (PDGFR-α/β), paladin, tenascin-C, neural/glial antigen 2 (NG2), and CD90, have been proposed as CAF markers [3, 4]. Among these CAF markers, in this study, we examined the expression of α-SMA, podoplanin, and periostin in lung adenocarcinoma tissue.

α-SMA has been considered as a potential marker of activated fibroblasts, which could proliferate, migrate, and secrete cytokines and extracellular matrix [1–4]. Results of in vitro studies did demonstrate that CAFs in non–small cell lung cancer (NSCLC) expressed abundant amounts of α-SMA than fibroblasts that resided in normal tissue [5, 6]. α-SMA was also reported abundant in tumor stroma of the cases harboring aggressive biological behavior in various human malignancies including NSCLC [7].

Podoplanin is expressed in the endothelium of lymphatic vessels and podocytes [8], and its expression in cancer stroma was also regarded as a prognostic marker as mentioned below. Kitano et al. reported that the status of podoplanin in stroma of lung adenocarcinoma was associated with lymphangiogenesis and poor clinical outcome [9]. Neri et al. also demonstrated that the presence of podoplanin-positive CAFs in metastatic lymph nodes was associated with shorter overall survival in the patients with stage III lung adenocarcinoma [10]. In addition, Saruwatari et al. reported that solid-predominant adenocarcinoma of the lung, which is associated with aggressive biological behavior, harbors much more podoplanin-positive CAFs [11].

Periostin belongs to a group of matricellular or nonstructural ECM protein and is secreted by fibroblasts especially at the sites of inflammation. Periostin was also reported to be abundant in cancer tissue and plays pivotal roles in promoting cancer invasion and metastasis [12]. Soltermann et al. did reveal that periostin status in tumor stroma was significantly associated with relapse of NSCLC patients [13].

As described above, the biological or clinical significance of CAFs detected by the single marker has been relatively well studied but the correlation among these markers has not necessarily been studied in human malignancies including lung adenocarcinoma in which cancer stroma is particularly considered important in its biological behavior [14]. Therefore, in this study, we first examined the association between the clinicopathological features and the expression of individual markers in lung adenocarcinoma tissues. We then tentatively classified the patients into four different groups according to low or high expression status of paired markers. We subsequently assessed the prognostic value of each pair of these markers to evaluate which pair(s) could predict clinical outcome of the patients with lung adenocarcinoma in order to further explore the potential value of the markers detecting CAFs in clinical materials of lung adenocarcinoma.

Materials and methods

Patients

This study included 92 patients who had undergone surgical resection for lung adenocarcinoma at Tohoku University Hospital from 2003 to 2005. None of the patients received chemotherapy nor radiation therapy before the surgery. The clinicopathological characteristics of the patients were summarized in Supplementary Table S1 (Online Resource 1). The protocol for this study was approved by the Ethics Committee at the Tohoku University School of Medicine (2018-193). This study has been performed in accordance with the Declaration of Helsinki.

Pathological evaluation

Pathological diagnosis was made according to the 2015 World Health Organization classification of lung tumors [14]. All the specimens examined were classified into variants according to the predominant subtype after the percentage of each subtype was evaluated. We also classified specimens according to the presence or absence of solid component, micropapillary component, mucinous component, and necrosis.

Immunohistochemistry

Specimens of lung adenocarcinoma tissues were fixed in 10% neutral formalin and embedded in paraffin. The antibodies used in this study were α-SMA (dilution, 1/3000; clone, 1A4; DAKO, Carpinteria, CA, USA), periostin (dilution, 1/3000; catalog number, ab14041; Abcam, Cambridge, UK), podoplanin (dilution, 1/200; clone, D2-40; Signet Laboratories Inc., Dedham, MA, USA), Ki-67 (dilution, 1/100; clone, MIB-1; DAKO). Antigen retrieval for podoplanin immunostain was performed by microwaving the tissues in citric acid for 15 min and that for Ki-67 by autoclaving the tissues in citric acid for 5 min at 121 °C. Immunostaining of α-SMA and periostin was conducted using a Histofine Kit (Nichirei Bioscience, Tokyo, Japan), according to the manufacturer's instructions. Envision (Dako) was used for immunostaining of podoplanin. Immune complexes were detected with 3, 3-diaminobenzidine (DAB), and tissues were counterstained with hematoxylin.

All the slides were evaluated independently by two of the authors (C.I. and D.T.) using a light microscope. The percentage of the tumor stromal area positive for α-SMA, podoplanin, and periostin was evaluated respectively according to the

methodologies of previous reports [15–18]. The Ki-67 labelling index (LI) was determined by counting 1000 tumor cells in the hot spots.

Statistical analysis

All statistical analyses were performed using JMP Pro 14.0.0 (SAS Institute, Tokyo, Japan). Correlation between CAF markers was evaluated by Spearman's rank correlation coefficient. Statistical differences between the groups were evaluated by Wilcoxon signed-rank test, Pearson's chi-square test, Fisher's exact test, or Spearman's rank correlation coefficient. Five-year overall survival curves were generated according to the Kaplan-Meier method, and the statistical significance was calculated using the log-rank test. Statistical significance was defined as $p < 0.05$.

Results

Immunoreactivity of α-SMA, podoplanin, and periostin was detected in the cytoplasm of fibroblast-like stromal cells in lung adenocarcinoma tissues (Fig. 1). α-SMA and podoplanin immunoreactivity was detected in intratumoral vascular smooth muscle cells and lymphatic epithelial cells, respectively. No immunoreactivities of α-SMA and podoplanin were detected in carcinoma cells, while that of periostin was detected very weakly in carcinoma cells.

The relatively weakly positive correlations were detected between the percentage of CAFs identified by the markers employed and total tumor stromal area as follows (α-SMA vs podoplanin: $\rho = 0.3677$, $p = 0.0003$; periostin vs α-SMA: $\rho = 0.2789$, $p = 0.0071$; podoplanin vs periostin: $\rho = 0.2998$, $p = 0.0037$) (Supplementary Fig. S1; Online Resource 1).

The median values of α-SMA-, podoplanin-, and periostin-positive areas to total tumor stromal area were 60, 0, and 40%, respectively (Supplementary Fig. S1; Online Resource 1). The patients were then tentatively classified into two groups according to the median percentage value of each marker as follows: α-SMA high- (≥ 60%) and α-SMA low-expression group (< 60%); podoplanin high- (≥ 1%) and podoplanin low-expression group (< 1%); and periostin high- (≥ 40%) and periostin low-expression group (< 40%).

The intratumoral status of α-SMA was significantly positively associated with the presence of solid component ($p = 0.003$) and necrosis ($p = 0.002$) in the tumor tissues and high pN ($p = 0.045$) and Ki-67 LI ($p = 0.006$) of the cases examined, and tended to be associated with high pStage ($p = 0.070$) (Table 1). That of podoplanin was significantly associated with the presence of necrosis in the tumors ($p = 0.005$), smoking history ($p = 0.005$), high Brinkman index ($p = 0.003$), pStage ($p = 0.022$), pT ($p = 0.032$), cM ($p = 0.020$), and Ki-67 LI ($p < 0.0001$). In addition, the intratumoral podoplanin-positive areas were significantly higher in men than in women ($p = 0.021$) (Table 1). The intratumoral periostin status tended to be associated with large size of tumor ($p = 0.063$), high pT ($p = 0.099$), and Ki-67 ($p = 0.055$), but these correlations did not reach statistical significance. The intratumoral periostin-positive area was also significantly higher in men than in women ($p = 0.036$). There were no statistically significant associations between the intratumoral status of periostin and histological features including variants and the presence or absence of intratumoral necrosis (Table 1). Intratumoral α-SMA and podoplanin status were significantly associated with a lower 5-year overall survival (OS) rate (α-SMA high, 61.2%; α-SMA low, 88.4%, $p = 0.0044$; podoplanin high, 60.5%; podoplanin low, 85.7%, $p = 0.0096$) (Fig. 2a, b). There were no statistically significant associations between the intratumoral status of periostin and the 5-year OS rate (periostin high, 72.5%; podoplanin low, 75.6%, $p = 0.7678$) (Fig. 2c).

We then subclassified the patients into four groups according to high or low expression of each of paired markers, α-SMA/podoplanin, α-SMA/periostin, and periostin/podoplanin. When the patients were classified into four groups according to α-SMA and podoplanin status, survival analysis did reveal statistically significant differences among these groups ($\chi^2 = 9.1555$, $p = 0.0273$) (Fig. 3a). Those in the α-SMA high/podoplanin high group of patients had the lowest survival rate among these four groups (53.3%). The survival curve of the α-SMA high/podoplanin high group also revealed statistically significant difference compared to the survival curves of the α-SMA high/podoplanin low group ($\chi^2 = 3.8353$, $p = 0.0402$) and the α-SMA low/podoplanin low group ($\chi^2 = 6.9347$, $p = 0.0085$) (Table 2). No statistically significant differences were detected between survival curves among those groups above when the patients were divided into four groups according to the status of α-SMA/periostin or periostin/podoplanin (α-SMA/periostin: $\chi^2 = 3.8666$, $p = 0.2762$; periostin/podoplanin: $\chi^2 = 7.0783$, $p = 0.0694$) (Fig. 3b, c).

Discussion

CAFs are well known to harbor various features and also to be derived from diverse origins of the cells [1–4]. CD68-positive macrophages are well known to be classified into M1 and M2 macrophages according to the expression profiles of functional protein markers, and these intratumoral macrophages are called tumor-associated macrophages (TAMs) [19]. Characterizing CAFs according to their functions, however, has still remained unestablished compared to TAMs above. However, employment of multiple markers could make it possible to further classify CAFs according to their functions as in the cases of TAMs above. This will also help us identify novel prognostic factors and therapeutic targets related to carcinoma

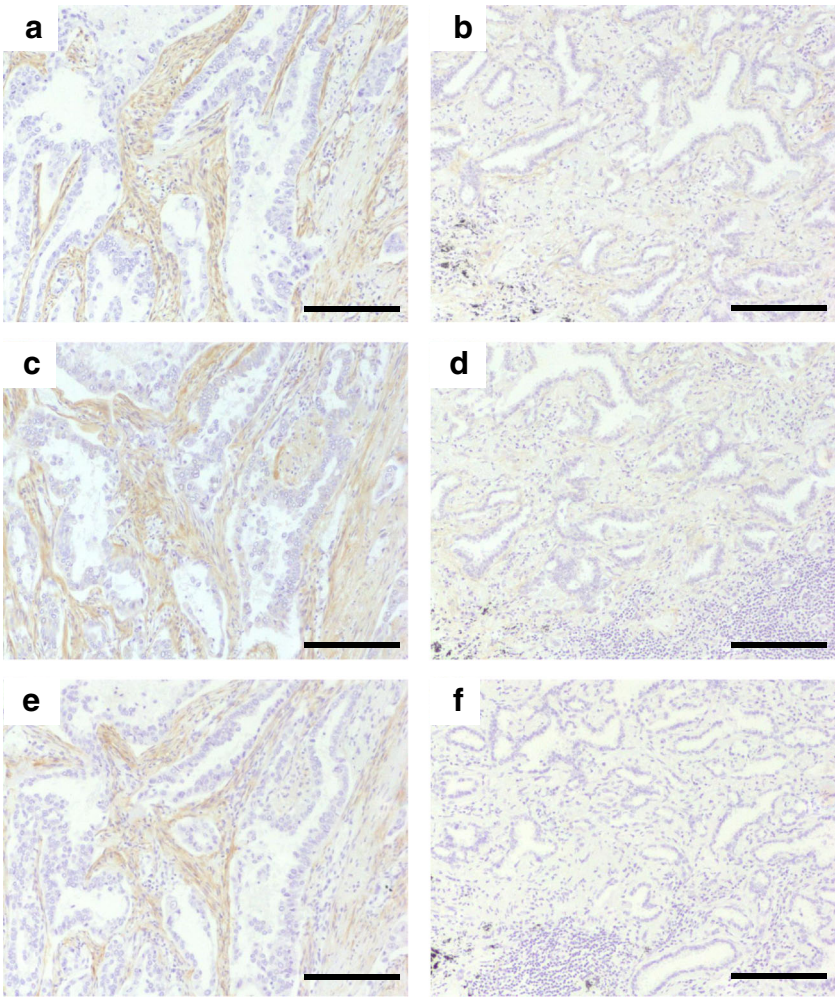

Fig. 1 Immunolocalization of α-SMA (**a** high, **b** low expression), periostin (**c** high, **d** low), and podoplanin (**e** high, **f** low) in samples of tissues with lung adenocarcinoma. Immunolocalization of CAF markers, α-SMA, periostin, and podoplanin was detected in the cytoplasm of fibroblast-like stromal cells. Bar = 200 μm

stromal interaction. In our present study, we did detect a heterogeneous intratumoral distribution of CAFs according to each marker, and the degree of this particular heterogeneity of the CAFs varied among different cases of lung adenocarcinoma. Therefore, considering this rather enormous intratumoral heterogeneity, multiple markers are definitively considered to be required to identify CAFs in the whole tissue sections and the examinations using tissue-microarray or biopsy specimens could be associated with difficulties in interpretation of the findings obtained. Therefore, in this study, we employed the whole slide in all the cases examined in order to more accurately explore the status of CAFs.

The status of relatively abundant α-SMA was significantly associated with high Ki-67 LI, lymph node metastasis (pN), and low 5-year overall survival (OS) rate. These results are all consistent with those of previously reported studies [7]. In addition, the status of intratumoral α-SMA-positive CAFs ranged from 1 to 90%, indicating that α-SMA is a highly sensitive surrogate marker of CAFs, and the amount of CAFs also varies among the cases of lung adenocarcinoma.

Compared to α-SMA, intratumoral podoplanin status was lower than 1% in nearly half of the cases examined in our present study. The status of high podoplanin was significantly associated with tumor proliferation or growth (pT, Ki-67 LI), distant metastasis (cM), and low 5-year OS rate. Koriyama et al. also reported that the presence of podoplanin-positive CAFs was significantly associated with a shorter period of progression-free survival in lung adenocarcinoma patients treated with platinum-based chemotherapy [20]. Yoshida et al. further reported that podoplanin-positive CAFs induced therapeutic resistance to EGFR-TKI [21]. Therefore, in our present study, resistance to chemotherapy, induced by podoplanin-positive CAFs, was considered to result in a lower 5-year OS rate of the patients with relatively high intratumoral podoplanin status. These findings all indicated that podoplanin could be a rather specific surrogate marker of those CAFs. The Brinkman index was also significantly higher in those with relatively high intratumoral podoplanin abundance. Inflammatory cytokines are known to upregulate podoplanin expression [22], suggesting that smoking-related inflammation induced the expression of podoplanin in the

Table 1 Clinicopathological parameters of patients with high/low expression of each CAF marker

		α-SMA		*p* value	Podoplanin		*p* value	Periostin		*p* value
		High	Low		High	Low		High	Low	
Age (years)	Median	65	70	0.087	66	68	0.897	68	66	0.261
	Max	78	82		79	82		82	79	
	Min	30	45		37	30		37	30	
Sex	Men	32	20	0.092	30	22	0.021*	34	18	0.036*
	Women	17	23		13	27		17	23	
Smoking	Smoker	33	23	0.203	33	23	0.005*	35	21	0.132
	Never	16	20		10	26		16	20	
Brinkman index	Median	520	240	0.122	700	0		520	60	0.144
	Max	1840	1920		1920	1560	0.003*	1920	1840	
	Min	0	0		0	0		0	0	
Size of tumor (mm)	Median	23	25	0.174	25	23	0.126	25	24	0.063
	Max	75	70		70	75		75	55	
	Min	10	10		10	10		10	10	
pStage	I	28	31	0.070	23	36	0.022*	31	28	0.718
	II	5	5		5	5		8	2	
	III	11	7		10	8		10	8	
	IV	5	0		5	0		2	3	
pT	1	28	26	0.704	20	34	0.032*	26	28	0.099
	2	15	13		17	11		18	10	
	3	2	1		2	1		3	0	
	4	4	3		4	3		4	3	
pN	0	35	38	0.045*	31	42	0.094	41	32	0.749
	1	3	1		2	2		3	1	
	2	10	4		9	5		6	8	
	3	1	0		1	0		1	0	
cM	0	0	0	0.058	0	0	0.020*	49	38	0.653
	1	5	0		5	0		2	3	
Ki-67 LI (%)	Median	17.5	9.1	0.006*	23.9	7.6	<0.0001*	15.9	9.3	0.055
	Max	87.2	86.7		86.7	87.2		86.7	87.2	
	Min	1.1	0.9		2.6	0.9		0.9	1.1	
Variant	Lepidic	7	15	0.008*	7	15	0.236	11	11	0.848
	Acinar	7	2		6	3		5	4	
	Papillary	19	18		15	22		19	18	
	Solid	10	0		7	3		7	3	
	Mucinous	5	7		7	5		8	4	
	Pleomorphic	1	1		1	1		1	1	
Solid component	Presence	14	2	0.003*	9	7	0.424	10	6	0.590
	Absence	35	41		34	42		41	35	
Micropapillary component	Presence	3	3	1.000	4	2	0.413	4	2	0.689
	Absence	46	40		39	47		47	39	
Mucinous component	Presence	2	6	0.537	7	5	0.537	8	4	0.538
	Absence	47	37		36	44		43	37	
Necrosis	Presence	17	3	0.002*	15	5	0.005*	15	5	0.074
	Absence	32	40		28	44		36	36	

*Statistically significant

lung adenocarcinoma tissue microenvironment. It is therefore possible to classify inflammation-related CAFs according to the status of intratumoral podoplanin status, but further investigations are required for clarification.

High intratumoral periostin status was also significantly associated with tumor growth (high pT and Ki-67 LI), but no statistical association was detected between intratumoral periostin status and distant/lymph node metastasis (high pN and cM) in our present study. Soltermann et al. reported similar results, i.e., that periostin status in tumor stroma of NSCLC was significantly associated with a higher clinical stage, pT, larger tumor size, and tumor recurrence [13]. A previous in vitro study demonstrated that overexpression of periostin in lung adenocarcinoma cells promoted their proliferation and migration [23]. In other types of human malignancies, increased periostin secretion by CAFs was also reported to promote the cell proliferation of carcinoma cells via activation of the Erk pathway [24].

In this study, the intratumoral status of α-SMA was significantly associated with the presence of solid component and necrosis in the tumor tissues, and that of podoplanin was associated with the presence of necrosis in the tumor tissues. Saruwatari et al. reported that solid-predominant adenocarcinoma of the lung harbored much more podoplanin-positive

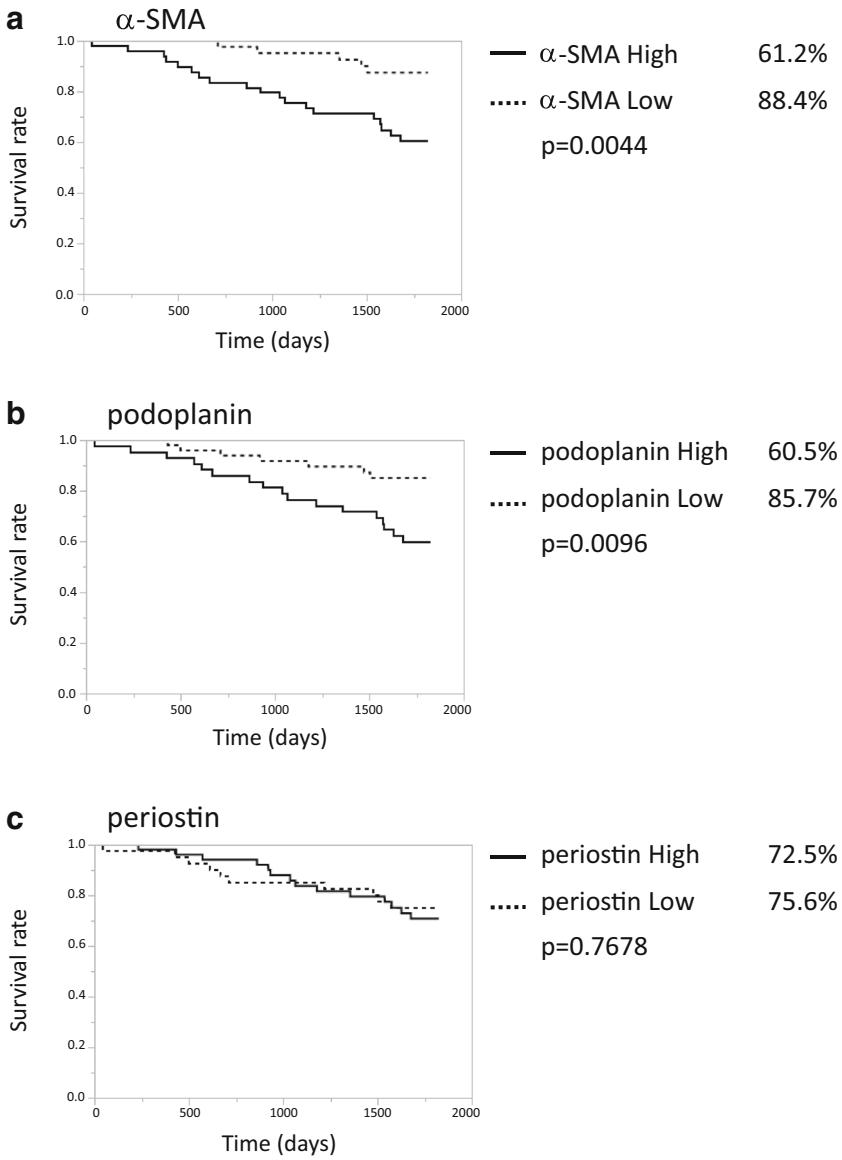

Fig. 2 The association between overall survival and the expression status of α-SMA (**a**), periostin (**b**), and podoplanin (**c**) immunoreactivity of the patients with lung adenocarcinoma cases

CAFs, and they suggested that podoplanin-positive CAFs could contribute to enhanced carcinoma cell invasion and development of metastasis [11]. α-SMA-positive CAFs could also play a more crucial role for the development of solid-predominant adenocarcinoma than podoplanin-positive CAFs do. Previous in vitro studies showed that hypoxia promoted an activation of fibroblasts [25, 26]. Hypoxia generally induces necrosis and may also activate fibroblasts in the tumor to increase CAFs. CAFs are also well known to promote the proliferation of carcinoma cells [1–4] and could result in lung adenocarcinoma with solid component. The presence of necrosis was associated with much α-SMA-positive CAFs and podoplanin-positive CAFs in the tissue. However, hypoxia was also reported to inactivate CAFs [27]. Further in vitro and/or in vivo studies will be required to clarify the correlation among histological variant, necrosis, and CAF markers.

Kilvaer et al. reported that the expression of FAP-1, but not that of α-SMA, was significantly correlated with the status of HIF2, LDH5, FOXP3, CSF1R, and miR21 in NSCLC l tumor stroma [16]. Nishishita et al. also reported α-SMA subtraction (obtained using α-SMA-positive and desmin-negative regions) revealed marked correlation with regions positive for collagen I and a weak correlation in the regions positive for PDGFRβ and D2-40 (also known as podoplanin-positive region) in colon cancer [28]. In this study, we firstly did demonstrate relatively weak positive correlations between the status of α-SMA and podoplanin, α-SMA and periostin, and periostin and podoplanin. Podoplanin was immunolocalized in a rather small area of tumor stroma in lung adenocarcinoma tissue compared to the much wider immunolocalization of α-SMA and periostin. The weakest correlation was actually detected between the status of α-SMA and periostin. Periostin-positive

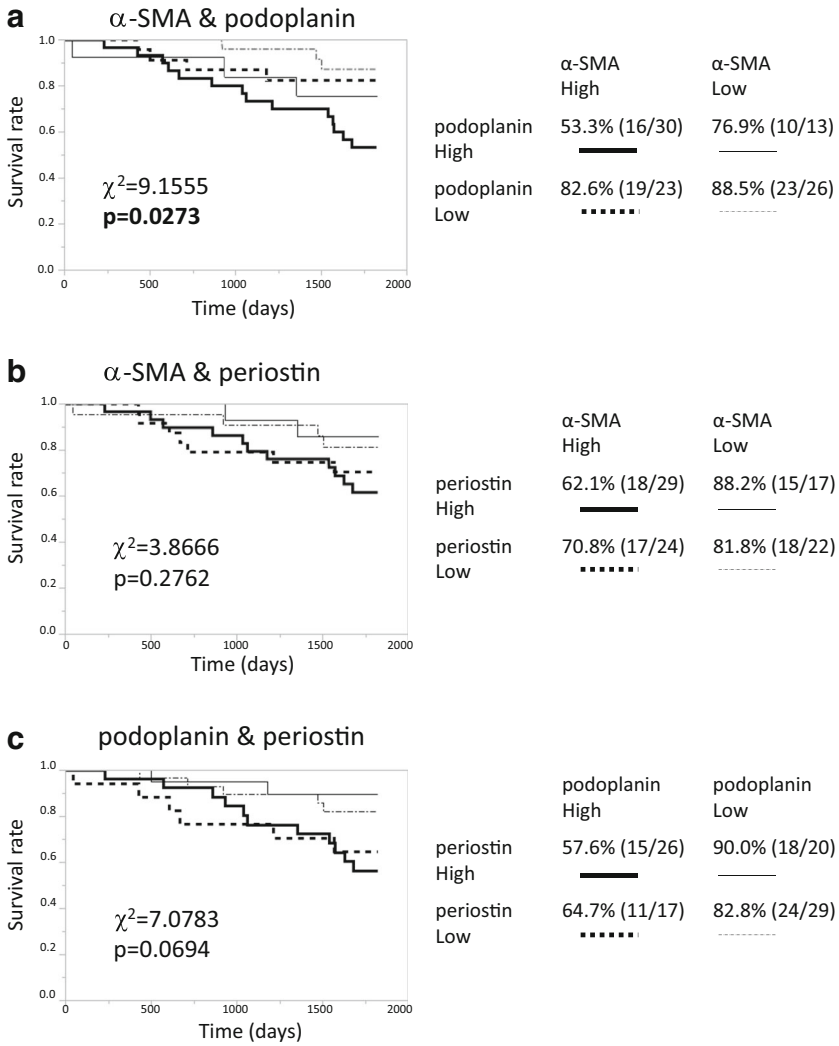

Fig. 3 The association between overall survival and the expression status of each pair of CAF markers in the patients with adenocarcinoma (**a** α-SMA and podoplanin, **b** α-SMA and periostin, **c** periostin and podoplanin)

areas were larger than α-SMA-positive areas in 23 patients, indicating that not all α-SMA-positive CAFs were positive for periostin. Soltermann et al. examined periostin expression in cancer cells and reported its association with sex (men > women), higher stage, higher pT, and larger tumor size [13], whereas, in our study, negative or weakly positive expression of periostin in adenocarcinoma cells. Stromal cells, including CAFs, could be the main source of periostin in lung adenocarcinoma tissue. Further investigations such as double immunohistochemistry could provide detailed information as to CAFs.

We used the expression of α-SMA and podoplanin, α-SMA and periostin, and periostin and podoplanin in order to analyze the survival of patients with lung adenocarcinoma. We detected no significant differences in overall survival between four groups when the patients were grouped according to the combinations of α-SMA/periostin or periostin/podoplanin. The group with high expression of α-SMA and podoplanin was significantly associated with a lower overall survival rate compared with that with high α-SMA and low podoplanin, and that with low α-SMA and podoplanin. These

Table 2 Survival analyses between the patients divided into four groups according to the expression of α-SMA and podoplanin

	α-SMA high podoplanin high	α-SMA high podoplanin low	α-SMA low podoplanin high
α-SMA high, podoplanin low	$\chi^2 = 3.8353$, $p = 0.0402$*		
α-SMA low, podoplanin high	$\chi^2 = 1.4366$, $p = 0.2307$	$\chi^2 = 0.1707$, $p = 0.6795$	
α-SMA low, podoplanin low	$\chi^2 = 6.9347$, $p = 0.0085$*	$\chi^2 = 0.3647$, $p = 0.5459$	$\chi^2 = 0.9848$, $p = 0.3210$

*Statistically significant

results did indicate poor prognosis for patients with lung adenocarcinoma containing podoplanin-positive CAFs and massive α-SMA-positive CAF populations in the tumor stroma. The 5-year overall survival rate of the group with high α-SMA and podoplanin was 53.3% (Fig. 3a), lower than that of the group with high α-SMA (61.2%) (Fig. 2a) and high podoplanin (60.5%) (Fig. 2b). CAFs arise from various types of cells and demonstrated heterogeneity in cancer tissues. The origins of CAFs include resident tissue fibroblasts and bone marrow-derived mesenchymal stem cells [29]. The expression of α-SMA and podoplanin is lower in normal fibroblasts than in CAFs [30], indicating that α-SMA- and podoplanin-positive CAFs might arise from marrow-derived mesenchymal stem cells. In addition, as described previously, the status of podoplanin could be related to inflammation in the cancer microenvironment. Periostin is also recognized as a marker of inflammation in the airway and pulmonary fibrosis in inflammatory lung disease [31]. Evaluating CAF markers could provide a novel classification system of the patients with lung adenocarcinoma. However, further studies evaluating various CAF markers should help to clarify the significance of heterogeneous CAF populations.

Conclusion

In this study, we evaluated the status of three different CAF markers in lung adenocarcinoma tissue in order to identify CAFs that could promote tumor malignancy and indicate poor prognosis. The presence of podoplanin-positive CAFs and massive α-SMA-positive CAFs in tumor stroma of lung adenocarcinoma indicated the poor prognosis of the patients. Immunohistochemical analysis using multiple CAF markers in the same patient cohort should help identify a combination of markers of CAFs corresponding to specific CAF functions.

Acknowledgments C.I. is supported by a scholarship from the Takeda Science Foundation (Osaka, Japan).

Author contributions C.I. and Y.M. designed the study and wrote the initial draft of the manuscript. C.I. and D.T. performed the experiments and analyzed the data. R.S. evaluated immunohistochemistry and supervised the interpretation of pathology. Y.O. collected tissue samples and supervised the interpretation of clinical data. H.S. supervised all experiments and edited the manuscript. All authors approved the submission of the final manuscript.

Compliance with ethical standards The protocol for this study was approved by the Ethics Committee at the Tohoku University School of Medicine (2018-193). This study has been performed in accordance with the Declaration of Helsinki.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Mahale J, Smagurauskaite G, Brown K, Thomas A, Howells LM (2016) The role of stromal fibroblasts in lung carcinogenesis: a target for chemoprevention? Int J Cancer 138:30–44. https://doi.org/10.1002/ijc.29447
2. Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. Nat Rev Cancer 6:392–401
3. Kalluri R (2016) The biology and function of fibroblasts in cancer. Nat Rev Cancer 16:582–598
4. Togo S, Polanska UM, Horimoto Y, Orimo A (2013) Carcinoma-associated fibroblasts are a promising therapeutic target. Cancers (Basel) 5:149–169
5. Kim S-H, Choe C, Shin Y-S, Jeon M-J, Choi S-J, Lee J, Bae GY, Cha HJ, Kim J (2013) Human lung cancer-associated fibroblasts enhance motility of non-small cell lung cancer cells in co-culture. Anticancer Res 33:2001–2009
6. Horie M, Saito A, Mikami Y, Ohshima M, Morishita Y, Nakajima J, Kohyama T, Nagase T (2012) Characterization of human lung cancer-associated fibroblasts in three-dimensional in vitro co-culture model. Biochem Biophys Res Commun 423:158–163
7. Chen Y, Zou L, Zhang Y, Chen Y, Xing P, Yang W, Li F, Ji X, Liu F, Lu X (2014) Transforming growth factor-β1 and α-smooth muscle actin in stromal fibroblasts are associated with a poor prognosis in patients with clinical stage I-IIIA nonsmall cell lung cancer after curative resection. Tumor Biol 35:6707–6713
8. Ugorski M, Dziegiel P, Suchanski J (2016) Podoplanin - a small glycoprotein with many faces. Am J Cancer Res 6:370–386
9. Kitano H, Kageyama SI, Hewitt SM, Hayashi R, Doki Y, Ozaki Y, Fujino S, Takikita M, Kubo H, Fukuoka J (2010) Podoplanin expression in cancerous stroma induces lymphangiogenesis and predicts lymphatic spread and patient survival. Arch Pathol Lab Med 134:1520–1527
10. Neri S, Ishii G, Hashimoto H, Kuwata T, Nagai K, Date H, Ochiai A (2015) Podoplanin-expressing cancer-associated fibroblasts lead and enhance the local invasion of cancer cells in lung adenocarcinoma. Int J Cancer 137:784–796
11. Saruwatari K, Ikemura S, Sekihara K, Kuwata T, Fujii S, Umemura S, Kirita K, Matsumoto S, Yoh K, Niho S, Ohmatsu H, Ochiai A, Kohrogi H, Tsuboi M, Goto K, Ishii G (2016) Aggressive tumor microenvironment of solid predominant lung adenocarcinoma subtype harboring with epidermal growth factor receptor mutations. Lung Cancer 91:7–14
12. Liu AY, Zheng H, Ouyang G (2014) Periostin, a multifunctional matricellular protein in inflammatory and tumor microenvironments. Matrix Biol 37:150–156
13. Soltermann A, Tischler V, Arbogast S, Braun J, Probst-Hensch N, Weder W, Moch H, Kristiansen G (2008) Prognostic significance of epithelial-mesenchymal and mesenchymal- epithelial transition protein expression in non-small cell lung cancer. Clin Cancer Res 14:7430–7437
14. Travis WD, Brambilla E, Burke AP, Marx A, Nicholson AG (2015) WHO classification of tumours of the lung, pleura, thymus and heart, 4th edn. International Agency for Research on Cancer, Lyon
15. Liu L, Liu L, Yao HH, Zhu ZQ, Ning ZL, Huang Q (2016) Stromal myofibroblasts are associated with poor prognosis in solid cancers: a meta-analysis of published studies. PLoS One 11:1–16
16. Kilvaer TK, Khanehkenari MR, Hellevik T et al (2015) Cancer associated fibroblasts in stage I-IIIA NSCLC: prognostic impact and their correlations with tumor molecular markers. PLoS One 10:1–15
17. Tsujino T, Seshimo I, Yamamoto H, Ngan CY, Ezumi K, Takemasa I, Ikeda M, Sekimoto M, Matsuura N, Monden M (2007) Stromal myofibroblasts predict disease recurrence for colorectal cancer. Clin Cancer Res 13:2082–2090

18. Yamashita M, Ogawa T, Zhang X, Hanamura N, Kashikura Y, Takamura M, Yoneda M, Shiraishi T (2012) Role of stromal myofibroblasts in invasive breast cancer: stromal expression of alpha-smooth muscle actin correlates with worse clinical outcome. Breast Cancer 19:170–176
19. Lievense LA, Bezemer K, Aerts JGJV, Hegmans JPJJ (2013) Tumor-associated macrophages in thoracic malignancies. Lung Cancer 80:256–262
20. Koriyama H, Ishii G, Yoh K, Neri S, Morise M, Umemura S, Matsumoto S, Niho S, Ohmatsu H, Tsuboi M, Goto K, Ochiai A (2015) Presence of podoplanin-positive cancer-associated fibroblasts in surgically resected primary lung adenocarcinoma predicts a shorter progression-free survival period in patients with recurrences who received platinum-based chemotherapy. J Cancer Res Clin Oncol 141:1163–1170
21. Yoshida T, Ishii G, Goto K, Neri S, Hashimoto H, Yoh K, Niho S, Umemura S, Matsumoto S, Ohmatsu H, Iida S, Niimi A, Nagai K, Ohe Y, Ochiai A (2015) Podoplanin-positive cancer-associated fibroblasts in the tumor microenvironment induce primary resistance to EGFR-TKIs in lung adenocarcinoma with EGFR mutation. Clin Cancer Res 21:642–651
22. Kunita A, Baeriswyl V, Meda C, Cabuy E, Takeshita K, Giraudo E, Wicki A, Fukayama M, Christofori G (2018) Inflammatory cytokines induce podoplanin expression at the tumor invasive front. Am J Pathol 188:1276–1288. https://doi.org/10.1016/j.ajpath.2018.01.016
23. Hong L, Sun H, Lv X, Yang D, Zhang J, Shi Y (2010) Expression of periostin in the serum of NSCLC and its function on proliferation and migration of human lung adenocarcinoma cell line (A549) in vitro. Mol Biol Rep 37:2285–2293
24. Kikuchi Y, Kunita A, Iwata C, Komura D, Nishiyama T, Shimazu K, Takeshita K, Shibahara J, Kii I, Morishita Y, Yashiro M, Hirakawa K, Miyazono K, Kudo A, Fukayama M, Kashima TG (2014) The niche component periostin is produced by cancer-associated fibroblasts, supporting growth of gastric cancer through ERK activation. Am J Pathol 184:859–870
25. Zhao B, Guan H, Liu JQ, Zheng Z, Zhou Q, Zhang J, Su LL, Hu DH (2017) Hypoxia drives the transition of human dermal fibroblasts to a myofibroblast-like phenotype via the TGF-β1/Smad3 pathway. Int J Mol Med 39:153–159
26. Senavirathna LK, Huang C, Yang X, Munteanu MC, Sathiaseelan R, Xu D et al (2018) Hypoxia induces pulmonary fibroblast proliferation through NFAT signaling. Sci Rep 8:1–16
27. Madsen CD, Pedersen JT, Venning FA, Singh LB, Moeendarbary E, Charras G et al (2015) Hypoxia and loss of PHD2 inactivate stromal fibroblasts to decrease tumour stiffness and metastasis. EMBO Rep 6:1394–1408
28. Nishishita R, Morohashi S, Seino H, Wu Y, Yoshizawa T, Haga T, Saito K, Hakamada K, Fukuda S, Kijima H (2018) Expression of cancer-associated fibroblast markers in advanced colorectal cancer. Oncol Lett 15:6195–6202
29. Shiga K, Hara M, Nagasaki T, Sato T, Takahashi H, Takeyama H (2015) Cancer-associated fibroblasts: their characteristics and their roles in tumor growth. Cancers (Basel) 7:2443–2458
30. Gascard P, Tlsty TD (2016) Carcinoma-associated fibroblasts: orchestrating the composition of malignancy. Genes Dev 30:1002–1019. https://doi.org/10.1101/gad.279737.116
31. O'Dwyer DN, Moore BB (2015) The role of periostin in lung fibrosis and airway remodeling. Cell Mol Life Sci 74:4305–4314