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PRECLINICAL STUDY

Expression of cancer-associated fibroblast-related proteins differs between invasive lobular carcinoma and invasive ductal carcinoma

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Abstract Cancer-associated fibroblasts (CAFs) are classified into various functional subtypes such as fibroblast activation protein-α (FAP-α), fibroblast specific protein-1 (FSP-1), platelet-derived growth factor receptor-α (PDGFR-α), and PDGFR-β. In this study, we compared the expression of CAF-related proteins in invasive lobular carcinoma (ILC) with those in invasive carcinoma of no special type (NST) and assessed the implications of the differences observed. Using tissue microarrays of 104 ILC and 524 invasive carcinoma (NST) cases, immunohistochemistry for CAF-related proteins [podoplanin, prolyl 4-hydroxylase, FAP-α, FSP-1/S100A4, PDGFR-α, PDGFR-β, and chondroitin sulfate proteoglycan (NG2)] was conducted. In invasive carcinoma (NST), tumor cells expressed a high level of PDGFR-α, whereas ILC tumor cells expressed high levels of podoplanin, prolyl 4-hydroxylase, FAP-α, and FSP-1/S100A4. In stromal cells of invasive carcinoma (NST), high expression levels of prolyl 4-hydroxylase, PDGFR-α, and NG2 were observed, whereas ILC stromal cells expressed high levels of FAP-α, FSP-1/S100A4, and PDGFR-β. In ILC, tumoral FSP-1/S100A4 positivity was associated with higher Ki-67 labeling index ($p = 0.010$) and non-luminal A type cancer ($p = 0.014$). Stromal PDGFR-α positivity was associated with lymph node metastasis ($p = 0.011$). On survival analysis of entire cases, tumoral FSP-1/S100A4 positivity ($p = 0.002$), stromal podoplanin positivity ($p = 0.041$), and stromal FSP-1/S100A4 negativity ($p = 0.041$) were associated with shorter disease-free survival; only tumoral FSP-1/S100A4 positivity ($p = 0.044$) was associated with shorter overall survival. In ILC, the expression of FAP-α and FSP-1/S100A4 was higher in both tumor and stromal cells than that observed in invasive carcinoma (NST). These results indicate that CAFs are a potential target in ILC treatment.

Keywords Breast cancer · Invasive ductal carcinoma · Invasive lobular carcinoma · Cancer-associated fibroblast · Tumor stroma

Introduction

Breast cancer is one of the most common cancers in females. Among various types of breast cancer, invasive carcinomas can be classified according to histologic subtypes, such as invasive carcinoma of no special type (NST) and invasive lobular carcinoma (ILC) [1]. ILC accounts for approximately 5–15 % of invasive carcinomas [2, 3], and its incidence has increased to a greater extent than that of invasive carcinoma (NST) due to hormone replacement therapy and increased alcohol intake [4, 5]. ILC differs from invasive carcinoma (NST) in several aspects; clinically, ILC shows frequent multiplicity and bilaterality [6, 7], and histologically, non-cohesive cancer cells are observed in ILC due to the loss of E-cadherin [8]. In addition, sites of metastasis with ILC are different from those of invasive carcinoma (NST). For example, bone, gastrointestinal tract, uterus, meninges, ovary, and diffuse serosal involvement are observed frequently with ILC [7, 9, 10].

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As research on cancer has progressed, recognition of the importance of the tumor microenvironment has gradually increased. Among several elements comprising the tumor microenvironment, the most important and frequently investigated factor is cancer-associated fibroblasts (CAFs) [11]. CAFs are located adjacent to cancer cells and are associated with tumor initiation, tumor-stimulatory inflammation, metabolism, metastasis, drug responses, and immune surveillance [12]. Despite the important impact of CAFs on cancer, the exact origin of these cells has not been elucidated. In addition, an accurate definition of CAFs remains controversial [11, 12].

Several molecules have been suggested as CAF markers. These include α-smooth muscle actin (αSMA) [13], tenascin-C [14], chondroitin sulfate proteoglycan (NG2) [15], platelet-derived growth factor receptor-α/β (PDGFR-α/β) [16], fibroblast activation protein (FAP) [17], podoplanin [18], prolyl 4-hydroxylase [19], and fibroblast specific protein-1 (FSP-1) [15]. Each CAF marker plays a characteristic role in CAF cross-talk with cancer cells (Table 1). Based on these markers, CAFs can be categorized into various functional subsets. According to one study, CAFs can be divided into 4 types: FAP-α, FSP-1, PDGFR-α, and PDGFR-β. Each type shows different characteristics [20], supporting the hypothesis that CAF phenotypes may be diverse.

In breast cancer, several studies have been conducted about the cross-talk between CAFs and cancer cells. CAFs are associated with tumor progression, invasion or metastasis, therapeutic resistance, and prognosis in breast cancer. Due to varying amounts of tumor stroma, it is expected that the impact of CAFs on breast cancer is greater than that on other cancers. CAFs are involved in various clinicopathologic parameters of breast cancer [21]. In a recent study, the tissue microenvironment, such as CAFs and intratumor vessels, differed between invasive carcinoma (NST) and ILC [22]. This finding raises the possibility of differences in CAF phenotypes between invasive carcinoma (NST) and ILC. However, a comparative analysis about CAF-related proteins between invasive carcinoma (NST) and ILC has not been performed. In the current study, we investigated the difference in expression of CAF-related proteins between ILC and invasive carcinoma (NST) and attempted to determine its clinical implications.

Materials and methods

Patient selection and clinicopathologic evaluation

Formalin-fixed paraffin-embedded (FFPE) tissue samples from ILC patients who underwent surgical resection from January 2000 to December 2012 in Severance Hospital, Seoul, South Korea, were used in this study. Cases diagnosed in 2006 as invasive carcinoma (NST) were used as the control group. Patients who underwent neoadjuvant chemotherapy were excluded from this study. All cases were reviewed retrospectively by a breast pathologist (Koo, JS), and histologic evaluations were performed on hematoxylin- and eosin-stained slides. The histological grade was assessed based on the Nottingham grading system [23]. Tumor staging was based on the 7th American Joint Committee on Cancer criteria. Disease-free survival (DFS) time was calculated from the date of the first curative surgery to the date of the first loco-regional or systemic relapse, or death without any type of relapse. Overall survival time was estimated from the date of the first curative operation to the date of the last follow-up, or death from any cause. Clinicopathologic parameters evaluated in each breast cancer patient included age at initial diagnosis, lymph node metastasis, tumor recurrence, distant metastasis, and survival. This study was approved by the Institutional Review Board of the Severance Hospital.

Tissue microarray

After reviewing the hematoxylin- and eosin-stained slides, the most appropriate FFPE tumor tissue samples were collected retrospectively. The most representative tumor area was marked on the FFPE tissue blocks and then extracted using a punch machine. The extracted 3 mm tissue core was transferred to a 6 × 5 recipient block. Two tissue cores were extracted from each case for tissue microarray construction.

Table 1 Function of CAF-related proteins

Molecules	Functions in tumor-stroma cross-talk	References
Podoplanin	Activated CAF, Tumor cell invasion	[18, 57]
Prolyl 4-hydroxylase	ECM remodeling	[58]
FAP-α	Activated CAF, modulation of ECM, immunomodulatory function	[59–61]
FSP-1/S100A4	Metastatic colonization, macrophage infiltration	[44, 47]
PDGFR-α	Tumor cell growth, angiogenesis, macrophage recruitment	[62–64]
PDGFR-β	Metastatic spread, high interstitial fluid pressure	[65–67]
NG2	Tumor cell invasion/metastasis	[68]

Immunohistochemistry

The antibodies used for immunohistochemistry (IHC) on FFPE tissue sections are shown in Table 2. After sectioning the paraffin blocks to 3-μm thickness, the sections were deparaffinized and rehydrated using xylene and ethanol solutions. IHC was conducted with the Ventana Discovery XT automated stainer (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's protocol. Cell Conditioning 1 buffer (EDTA, pH 8.0, Ventana Medical Systems) was used for antigen retrieval. IHC was performed with inclusion of appropriate positive and negative controls.

Interpretation of IHC results

A cut-off value of 1 % or more positively stained nuclei was used to define estrogen receptor (ER) and progesterone receptor (PR) positivity [24]. Human epidermal growth factor receptor-2 (HER-2) staining was analyzed according to the American Society of Clinical Oncology/College of American Pathologists guidelines, which used the following categories: 0, no immunostaining or incomplete faint/barely perceptible membranous staining in less than 10 % of tumor cells; 1+, incomplete faint/barely perceptible membranous staining in more than 10 % of tumor cells; 2+, incomplete circumferential weak/moderate membranous staining in more than 10 % of tumor cells or complete circumferential intense membranous staining in less than 10 % of tumor cells; and 3+, complete circumferential intense membranous staining in more than 10 % of tumor cells [25]. HER-2 immunostaining was considered positive when strong (3+) membranous staining was observed. Cases scored 0 to 1+ were regarded as negative. Cases showing 2+ HER-2 expression were evaluated for HER-2 amplification by fluorescent in situ hybridization. IHC markers for CAF-related proteins were assessed by light microscopy. The stained slides were evaluated semiquantitatively according to a method reported previously [26]. Tumor and stromal cell staining were assessed as 0, negative or weak immunostaining in <1 % of the tumor/stroma; 1, focal expression in 1–10 % of tumor/stroma; 2, positive in 11–50 % of tumor/stroma; and 3, positive in 51–100 % of tumor/stroma. The evaluation of stained slides was performed on the entire tumor area, and scores of 2 or higher were regarded as positive.

Tumor phenotype classification

Breast cancer phenotypes were classified based on the IHC results for ER, PR, HER-2, and Ki-67 labeling index (LI), and the fluorescent in situ hybridization results for HER-2. Breast cancer phenotypes were defined as follows [27]: (1) Luminal A type—ER and/or PR positive, HER-2 negative, and Ki-67 LI <14 %; (2) Luminal B type (HER-2 negative)—ER and/or PR positive, HER-2 negative, and Ki-67 LI ≥14 %; and Luminal B type (HER-2 positive)—ER and/or PR positive and HER-2 overexpressed and/or amplified; (3) HER-2 type—ER and PR negative, and HER-2 overexpressed and/or amplified; and (4) Triple negative breast cancer (TNBC) type—ER, PR, and HER-2 negative.

Statistical analysis

Data were analyzed using SPSS for Windows version 21.0 (SPSS Inc., Chicago, IL, USA). Student's t test and Fisher's exact test were used for continuous and categorical variables, respectively. For data analyses involving multiple comparisons, p values were corrected using the Bonferroni multiple comparison procedure. Statistical significance was assumed when $p < 0.05$. Kaplan–Meier

Table 2 Sources, clones, and dilutions of antibodies

Antibody	Company	Isotype control antibody	Positive control	Clone	Dilution
CAF phenotype related proteins					
Podoplanin	Abcam, Cambridge, UK	IgG1	Placenta	18H5	1:100
Prolyl 4-hydroxylase	Abcam, Cambridge, UK	N/A	Colon cancer	Polyclonal	1:200
FAP-α	Abcam, Cambridge, UK	N/A	Breast cancer	Polyclonal	1:100
FSP-1/S100A4	Abcam, Cambridge, UK	N/A	Tonsil	Polyclonal	1:100
PDGFR-α	Abcam, Cambridge, UK	N/A	Brain	Polyclonal	1:100
PDGFR-β	Abcam, Cambridge, UK	IgG	Prostate cancer	Y92	1:100
NG2	Abcam, Cambridge, UK	IgG1	Melanoma	NG2	1:50
Molecular subtype related proteins					
ER	Thermo Scientific, San Siego, CA, USA	IgG	Breast tissue	SP1	1:100
PR	DAKO, Glostrup, Denmark	IgG1	Breast tissue	PgR	1:50
HER-2	DAKO, Glostrup, Denmark	N/A	Breast cancer	Polyclonal	1:1500
Ki-67	Abcam, Cambridge, UK	IgG	Tonsil	SP6	1:100

Table 3 Clinicopathologic characteristics of invasive lobular carcinoma

Parameters	Total $n = 104$ (%)	Classic type $n = 93$ (%)	Pleomorphic type $n = 11$ (%)	p value
Age (years)				**0.010**
<50	58 (55.8)	56 (60.2)	2 (18.2)	
≥50	46 (44.2)	37 (39.8)	9 (81.8)	
Nuclear grade				**<0.001**
1/2	93 (89.4)	93 (100.0)	0 (0.0)	
3	11 (10.6)	0 (0.0)	11 (100.0)	
Histologic grade				**<0.001**
I/II	100 (96.2)	93 (100.0)	7 (63.6)	
III	4 (3.8)	0 (0.0)	4 (36.4)	
T stage				**0.027**
T1	62 (59.6)	59 (63.4)	3 (27.3)	
T2/T3	42 (40.4)	34 (36.6)	8 (72.7)	
Lymph node metastasis				0.734
Absent	72 (69.2)	65 (69.9)	7 (63.6)	
Present	32 (30.8)	28 (30.1)	4 (36.4)	
ER				0.498
Negative	6 (5.8)	5 (5.4)	1 (9.1)	
Positive	98 (94.2)	88 (94.6)	10 (90.9)	
PR				**0.016**
Negative	17 (16.3)	12 (12.9)	5 (45.5)	
Positive	87 (83.7)	81 (87.1)	6 (54.5)	
HER-2				**0.002**
Negative	97 (93.3)	90 (96.8)	7 (63.6)	
Positive	7 (6.7)	3 (3.2)	4 (36.4)	
Ki-67 LI				**<0.001**
≤14 %	85 (81.7)	81 (87.1)	4 (36.4)	
>14 %	19 (18.3)	12 (12.9)	7 (63.6)	
Molecular type				**<0.001**
Luminal A	79 (76.0)	76 (81.7)	3 (27.3)	
Luminal B	20 (19.2)	13 (14.0)	7 (63.6)	
HER-2	1 (1.0)	0 (0.0)	1 (9.1)	
TNBC	4 (3.8)	4 (4.3)	0 (0.0)	

Bold values represents p-values that are < 0.05

survival curves and log-rank statistics were employed to evaluate time to tumor metastasis and time to survival. Multivariate regression analysis was performed using a Cox proportional hazards model.

Results

Basal characteristics of ILC

The clinicopathologic characteristics of 104 ILC cases are summarized in Table 3. Of these cases, 93 (89.4 %) were the classic type and 11 (10.6 %) were the pleomorphic type. Compared with the classic type, the pleomorphic type was associated with older age ($p = 0.010$), higher nuclear grade ($p < 0.001$), higher histologic grade ($p < 0.001$), higher T stage ($p = 0.027$), PR negativity ($p = 0.016$), HER-2 positivity ($p = 0.002$), higher Ki-67 LI ($p < 0.001$), and the non-luminal A subtype ($p < 0.001$).

Expression of CAF-related proteins in ILC according to histologic type

When comparing the expression of CAF-related proteins in ILC according to histologic subtypes, PDGFR-β and NG2 were not expressed in tumor cells. In contrast, PDGFR-α and NG2 were not expressed in stromal cells. There were no significant differences in the expression of CAF-related proteins between the classic and pleomorphic types of ILC (Supplementary Table 1).

Table 4 Expression of cancer-associated fibroblast-related proteins in invasive lobular and invasive carcinoma of no special type (NST)

Parameters	Total $n = 628$ (%)	Invasive carcinoma (NST) $n = 524$ (%)	ILC $n = 104$ (%)	p value
Cancer cell compartment				
Podoplanin				**<0.001**
Negative	536 (85.4)	479 (91.4)	57 (54.8)	
Positive	92 (14.6)	45 (8.6)	47 (45.2)	
Prolyl 4-hydroxylase				**0.001**
Negative	299 (47.6)	265 (50.6)	34 (32.7)	
Positive	329 (52.4)	259 (49.4)	70 (67.3)	
FAP-α				**<0.001**
Negative	525 (83.6)	515 (98.3)	10 (9.6)	
Positive	103 (16.4)	9 (1.7)	94 (90.4)	
FSP-1/S100A4				**<0.001**
Negative	424 (67.5)	379 (72.3)	45 (43.3)	
Positive	204 (32.5)	145 (27.7)	59 (56.7)	
PDGFR-α				**<0.001**
Negative	454 (72.3)	355 (67.7)	99 (95.2)	
Positive	174 (27.7)	169 (32.3)	5 (4.8)	
Stromal compartment				
Podoplanin				0.061
Negative	515 (82.0)	423 (80.7)	92 (88.5)	
Positive	113 (18.0)	101 (19.3)	12 (11.5)	
Prolyl 4-hydroxylase				**0.001**
Negative	447 (71.2)	359 (68.5)	88 (84.6)	
Positive	181 (28.8)	165 (31.5)	16 (15.4)	
FAP-α				**<0.001**
Negative	597 (95.1)	513 (97.6)	84 (80.8)	
Positive	31 (4.9)	11 (2.1)	20 (19.2)	
FSP-1/S100A4				**0.001**
Negative	203 (32.3)	184 (35.1)	19 (18.3)	
Positive	425 (67.7)	340 (64.9)	85 (81.7)	
PDGFR-α				**<0.001**
Negative	553 (88.1)	449 (85.7)	104 (100.0)	
Positive	75 (11.9)	75 (14.3)	0 (0.0)	
PDGFR-β				**<0.001**
Negative	576 (91.7)	503 (96.0)	73 (70.2)	
Positive	52 (8.3)	21 (4.0)	31 (29.8)	
NG2				**0.043**
Negative	608 (96.8)	504 (96.2)	104 (100.0)	
Positive	20 (3.2)	20 (3.8)	0 (0.0)	

Bold values represents p-values that are < 0.05

Underlined Bold values represents p-values that is statistically significant ($p<0.05$)

Comparison of the expression of CAF-related proteins between invasive carcinoma (NST) and ILC

Differences were observed using IHC in the expression of CAF-related proteins between invasive carcinoma (NST) and ILC. In the cancer cell compartment, PDGFR-α was expressed more highly in invasive carcinoma (NST) than that in ILC ($p < 0.001$), whereas podoplanin, prolyl 4-hydroxylase, FAP-α, and FSP-1/S100A4 were expressed more highly in ILC than those in invasive carcinoma (NST) ($p \leq 0.001$). In the stromal cell compartment, the expression of prolyl 4-hydroxylase ($p = 0.001$) and PDGFR-α ($p < 0.001$) was higher in invasive carcinoma (NST) than in ILC, while FAP-α, FSP-1/S100A4, and PDGFR-β

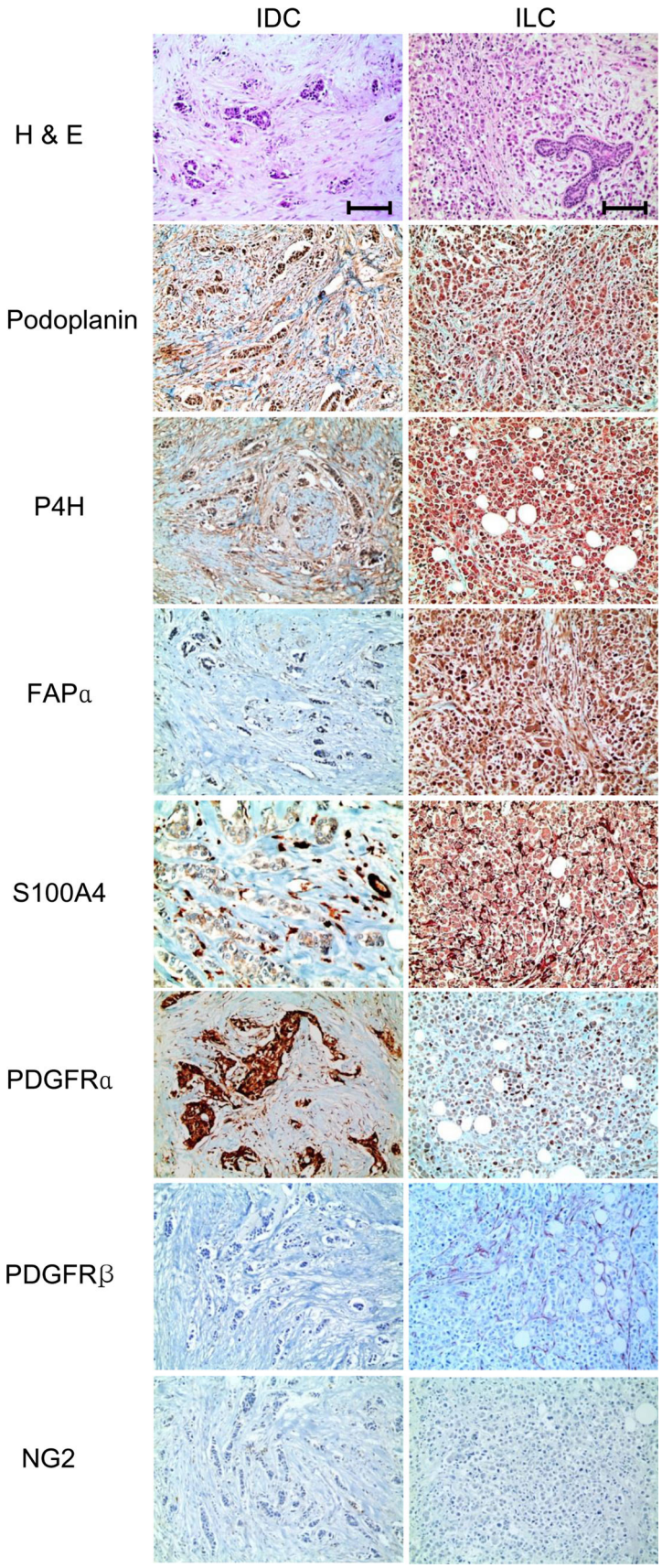

Fig. 1 Comparison of the expression of CAF-related proteins between invasive carcinoma (NST) and ILC. In the cancer cell compartment, invasive carcinoma (NST) showed a higher expression of PDGFR-α whereas ILC showed a higher expression of podoplanin, prolyl 4-hydroxylase, FAP-α, and FSP-1/S100A4. In the stromal cell compartment, a higher expression of prolyl 4-hydroxylase, PDGFR-α, and NG2 was observed in invasive carcinoma (NST) compared to ILC. However, ILC showed a higher expression of FAP-α, FSP-1/S100A4, and PDGFR-β. Scale bar represents 300 μm

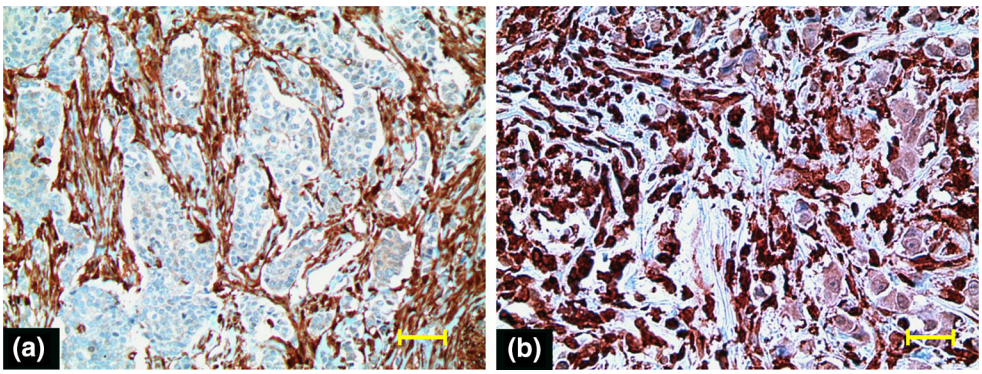

Fig. 2 Comparison of the expression of FSP-1/S100A4 between invasive carcinoma (NST) and ILC. For invasive carcinoma (NST), FSP-1/S100A4-positive cells showed a fascicular pattern with group formation. In contrast, ovoid or round cells were FSP-1/S100A4 positive in ILC. These cells exhibited a scattered but not grouped pattern. Scale bar represents 100 μm

($p \leq 0.001$) were expressed more highly in ILC than in invasive carcinoma (NST) (Table 4 and Fig. 1). The expression of FSP-1/S100A4 between invasive carcinoma (NST) and ILC differed not only in amount but also in the pattern. In invasive carcinoma (NST), a fascicular pattern was observed, whereas in ILC, a scattered pattern was observed (Fig. 2).

The majority of ILC cases were classified as the luminal type. Thus, we selected luminal type invasive carcinoma (NST) cases and compared the expression of CAF-related proteins with ILC. The results showed that differences in the expression of these proteins between ILC and luminal type invasive carcinoma (NST) were similar to those between ILC and invasive carcinoma (NST) (Table 5).

All ILC cases were classified into two groups according to the number of expressed CAF markers in stromal cells: cases with no more than one expressed CAF marker and those with two or more expressed CAF markers. Comparing the clinicopathologic features between the two groups, significant differences were observed in lymph node metastasis ($p = 0.033$) and estrogen receptor (ER) status ($p = 0.038$); in particular, cases expressing no more than one CAF-related protein showed higher proportions of lymph node metastasis and ER negativity (Table 6). In addition, cases expressing two or more CAF-related proteins showed a trend toward younger age ($p = 0.066$), higher nuclear grade ($p = 0.097$), HER-2 positivity ($p = 0.083$), luminal B type ($p = 0.079$), and pleomorphic type ($p = 0.097$).

Correlations between CAF-related proteins and clinicopathologic factors in ILC

Our analysis of the relationship between various clinicopathologic factors and the expression of CAF-related proteins in ILC indicated that positivity for tumoral FSP-1/S100A4 was associated with a higher Ki-67 LI ($p = 0.010$) and the non-luminal A type ($p = 0.014$). Stromal PDGFR-α positivity was associated with lymph node metastasis ($p = 0.011$) (Fig. 3).

Impact of the expression status for CAF-related proteins on the prognosis of ILC and invasive breast cancer

To investigate the impact of the expression of CAF-related proteins on the prognosis of ILC, a univariate analysis was performed. The expression of CAF-related proteins was not associated with the DFS or OS times (Supplementary Table 2).

The impact of the expression of CAF-related proteins on the prognosis was analyzed by performing a univariate analysis of all of 628 invasive breast cancer cases. Tumoral FSP-1/S100A4 positivity ($p = 0.002$), stromal podoplanin positivity ($p = 0.041$), and stromal FSP-1/S100A4 negativity ($p = 0.041$) were associated with shorter DFS, and only tumoral FSP-1/S100A4 positivity ($p = 0.044$) was associated with shorter OS (Table 7; Fig. 4). In addition, another univariate analysis was performed to investigate the impact of the expression of CAF-related proteins on the prognosis based on ER status. In ER-positive breast cancer ($n = 441$), stromal podoplanin positivity ($p = 0.045$) was associated with shorter DFS. In ER-negative breast cancer ($n = 168$), stromal FSP-1/S100A4 negativity ($p = 0.023$) and tumoral FSP-1/S100A4 positivity ($p = 0.023$) were associated with shorter DFS (Fig. 4).

On multivariate Cox analysis of all of 628 invasive breast cancer cases, higher T stage (hazard ratio 4.057, 95 % CI 1.683–9.779, $p = 0.002$), tumoral FSP-1/S100A4 positivity (hazard ratio 3.462, 95 % CI 1.414–8.479, $p = 0.007$), and stromal FSP-1/S100A4 negativity (hazard ratio 2.465, 95 % CI 1.113–5.461, $p = 0.026$) were independent factors associated with shorter DFS. For OS, higher T stage (hazard ratio 2.239, 95 % CI 1.034–4.849, $p = 0.041$) and lymph node metastasis (hazard ratio 2.011, 95 % CI 1.014–3.989, $p = 0.046$) were independent factors associated with shorter OS (Table 8).

Table 5 Expression of cancer-associated fibroblast-related proteins in invasive lobular carcinoma and the luminal type of invasive carcinoma of no special type (NST)

Parameters	Total $n = 332$ (%)	Invasive carcinoma (NST), luminal type $n = 228$ (%)	ILC $n = 104$ (%)	p value
Cancer cell compartment				
Podoplanin				**<0.001**
Negative	268 (80.7)	211 (92.5)	57 (54.8)	
Positive	64 (19.3)	17 (7.5)	47 (45.2)	
Prolyl 4-hydroxylase				0.088
Negative	131 (39.5)	97 (42.5)	34 (32.7)	
Positive	201 (60.5)	131 (57.5)	70 (67.3)	
FAP-α				**<0.001**
Negative	237 (71.4)	227 (99.6)	10 (9.6)	
Positive	95 (28.6)	1 (0.4)	94 (90.4)	
PDGFR-α				**<0.001**
Negative	277 (83.4)	178 (78.1)	99 (95.2)	
Positive	55 (16.6)	50 (21.9)	5 (4.8)	
FSP-1/S100A4				**<0.001**
Negative	229 (69.0)	184 (80.7)	45 (43.3)	
Positive	103 (31.0)	44 (19.3)	59 (56.7)	
Stromal compartment				
Podoplanin				0.355
Negative	285 (85.8)	193 (84.6)	92 (88.5)	
Positive	47 (14.2)	35 (15.4)	12 (11.5)	
Prolyl 4-hydroxylase				0.019
Negative	254 (76.5)	166 (72.8)	88 (84.6)	
Positive	78 (23.5)	62 (27.2)	16 (15.4)	
FAP-α				**<0.001**
Negative	312 (94.0)	228 (100.0)	84 (80.8)	
Positive	20 (6.0)	0 (0.0)	20 (19.2)	
FSP-1/S100A4				**<0.001**
Negative	118 (35.5)	99 (43.4)	19 (18.3)	
Positive	214 (64.5)	129 (56.6)	85 (81.7)	
PDGFR-α				**<0.001**
Negative	309 (93.1)	205 (89.9)	104 (100.0)	
Positive	23 (6.9)	23 (10.1)	0 (0.0)	
PDGFR-β				**<0.001**
Negative	298 (89.8)	225 (98.7)	73 (70.2)	
Positive	34 (10.2)	3 (1.3)	31 (29.8)	
NG2				0.174
Negative	328 (98.8)	224 (98.2)	104 (100.0)	
Positive	4 (1.2)	4 (1.8)	0 (0.0)	

Bold values represents p-values that are < 0.05

Discussion

In this study, we compared the expression of these proteins in ILC and invasive carcinoma (NST). Using IHC, the expression of CAF-related proteins differed between ILC and invasive carcinoma (NST). In the cancer cell compartment, invasive carcinoma (NST) showed higher expression of PDGFR-α whereas a higher expression of podoplanin, prolyl 4-hydroxylase, FAP-α, and FSP-1/S100A4 was observed in ILC. The expression of PDGFR-α was increased in TNBC [28, 29], a subtype of invasive carcinoma (NST). The results of these previous studies

Table 6 Clinicopathologic characteristics of invasive lobular carcinoma according to the number of expressed CAF markers in stromal cells

Parameters	Total $n = 104$ (%)	Number of expressed CAF markers ≤ 1 $n = 62$ (%)	Number of expressed CAF markers ≥ 2 $n = 42$ (%)	p value
Age (years)				0.066
<50	58 (55.8)	30 (48.4)	28 (66.7)	
≥50	46 (44.2)	32 (51.6)	14 (33.3)	
Nuclear grade				0.097
1/2	93 (89.4)	58 (93.5)	35 (83.3)	
3	11 (10.6)	4 (6.5)	7 (16.7)	
Histologic grade				0.689
I/II	100 (96.2)	60 (96.8)	40 (95.2)	
III	4 (3.8)	2 (3.2)	2 (4.8)	
T stage				0.107
T1	62 (59.6)	33 (53.2)	29 (69.0)	
T2/T3	42 (40.4)	29 (46.8)	13 (31.0)	
Lymph node metastasis				**0.033**
Absent	72 (69.2)	38 (61.3)	34 (81.0)	
Present	32 (30.8)	24 (38.7)	8 (19.0)	
ER				**0.038**
Negative	6 (5.8)	6 (9.7)	0 (0.0)	
Positive	98 (94.2)	56 (90.3)	42 (100.0)	
PR				0.313
Negative	17 (16.3)	12 (19.4)	5 (11.9)	
Positive	87 (83.7)	50 (80.6)	37 (88.1)	
HER-2				0.083
Negative	97 (93.3)	60 (96.8)	37 (88.1)	
Positive	7 (6.7)	2 (3.2)	5 (11.9)	
Ki-67 LI				0.229
≤14 %	85 (81.7)	53 (85.5)	32 (76.2)	
>14 %	19 (18.3)	9 (14.5)	10 (23.8)	
Molecular type				0.079
Luminal A	79 (76.0)	49 (79.0)	30 (71.4)	
Luminal B	20 (19.2)	8 (12.9)	12 (28.6)	
HER-2	1 (1.0)	1 (1.6)	0 (0.0)	
TNBC	4 (3.8)	4 (6.5)	0 (0.0)	
Histologic type				0.097
Classic	93 (89.4)	58 (93.5)	35 (83.3)	
Pleomorphic	11 (10.6)	4 (6.5)	7 (16.7)	

Bold values represents p-values that are < 0.05

accord well with our finding that the expression of PDGFR-α in tumor cells is higher in invasive carcinoma (NST) than that in ILC.

The expression of podoplanin in tumor cells is associated with cancer cell migration and invasion [30, 31], and the expression of prolyl 4-hydroxylase is associated with disease progression and metastasis in breast cancer [32]. The expression of podoplanin and prolyl 4-hydroxylase in tumor cells also correlates with cell motility. Histologically, ILC shows discohesive and infiltrative features [1], and it is expected that ILC will show higher cell motility than invasive carcinoma (NST). ILC does show a higher rate of lymph node metastasis than invasive carcinoma (NST) [33]. In addition, a previous study reported that the expression of a pseudopodial constituent such as α-parvin was only observed in ILC [34]. These previous findings are consistent with the results of the current study.

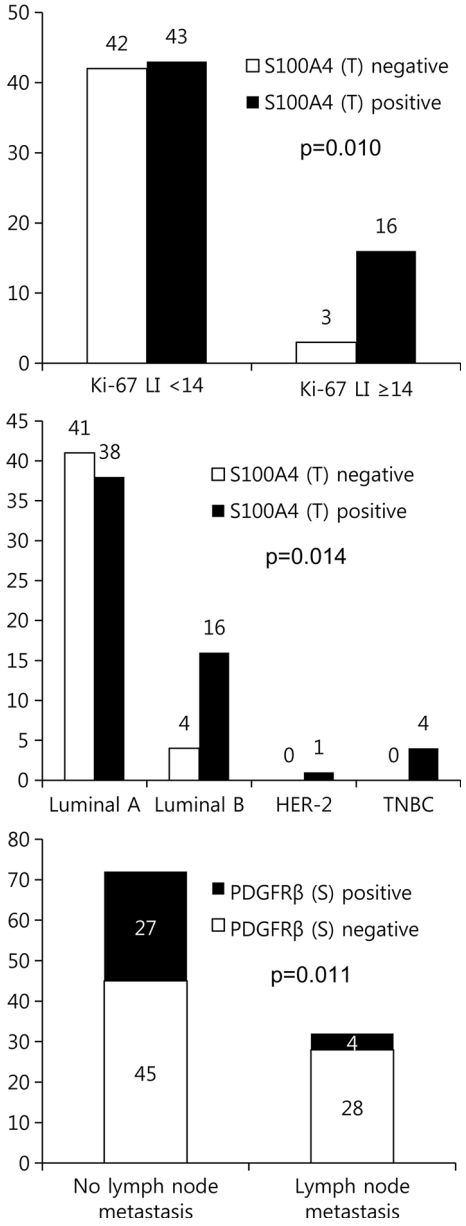

Fig. 3 Correlation between CAF-related proteins and clinicopathologic factors in ILC. The positivity for tumoral FSP-1/S100A4 is associated with a higher Ki-67 LI and the non-luminal A type. Stromal PDGFR-α positivity is associated with lymph node metastasis

The current results showed that tumor stroma exhibited different expression levels of CAF-related proteins between invasive carcinoma (NST) and ILC. A higher expression of prolyl 4-hydroxylase, PDGFR-α, and NG2 was observed in invasive carcinoma (NST) stromal cells, while ILC stromal cells showed higher expression of FAP-α, FSP-1/S100A4, and PDGFR-β. These findings suggested the possibility of differences in CAF characteristics of tumor stroma between invasive carcinoma (NST) and ILC. According to a previous study examining differences in the tumor microenvironment between ILC and invasive carcinoma (NST), ILC showed more conspicuous proliferation of CAFs and endothelial cells than invasive carcinoma (NST), whereas invasive carcinoma (NST) showed more prominent maturation of newly formed microvessels than ILC [22, 35]. Alpha-smooth muscle actin was used as a marker of CAF in the previous study [22], while we used seven different CAF markers. Thus, a direct comparison between the two studies is difficult. However, NG2 is a marker for mature pericytes that is only expressed in invasive carcinoma (NST), and PDGFR-β, which is highly expressed in ILC, is a marker for immature pericytes [36]. Thus, our findings correspond well with the results of previous studies. In a study performed using a mouse xenograft model for breast cancer, αSMA-positive CAFs showed 96 % accordance with CAFs expressing PDGFR-β [15]. This finding supports a previous report on the more prominent proliferation of αSMA-positive CAFs in ILC than in invasive carcinoma (NST) [22] and is consistent with our study showing higher PDGFR-β expression in stroma in ILC than in invasive carcinoma (NST). Desmoplastic stroma, which is a frequently observed histologic finding in invasive carcinoma (NST) [37], is caused by PDGFR-α type CAFs [38]. We also found higher expression of PDGFR-α in the stroma of invasive carcinoma (NST), which is consistent with these prior findings.

The expression of FAP-α in breast cancer cells is associated with cell motility and invasion [39, 40]. In addition, FSP-1/S100A4 expression in breast cancer cells correlates with cell motility and invasion [41, 42]. Our results show that the expression of FAP-α and FSP-1/S100A4 in ILC is higher than in invasive carcinoma (NST) in both the cancer cell and stromal compartments. Thus, these findings are consistent with the clinical, histologic, and biologic features of ILC. FAP-α type CAFs are associated with activation of CAFs, modulation of the extracellular matrix, and immunomodulatory functions [20]. In previous studies, increases in the number of αSMA-positive CAFs were more prominent in ILC than those in invasive carcinoma (NST) [22]. This suggests that the number of activated CAFs may increase in ILC. In addition, the immune-related subtype may be one of the two biologically distinct subtypes in ILC [43]. Thus, it is possible that CAFs with an immunomodulatory function may be more prominent in ILC. However, further studies are required to confirm this possibility.

In a previous colocalization study using a mouse xenograft model for breast cancer, αSMA-positive CAFs, and FSP-1/S100A4 CAFs, showed minimal overlap [15]. Although not investigated fully, we expect that the FSP-1/S100A4 type CAFs may have a unique function. For example, the FSP-1/S100A4 type CAF is associated with metastatic colonization [44] and carcinogen protection [45]. Further studies are necessary to elucidate the role of FSP-1/S100A4 in ILC.

Table 7 Univariate analysis via log-rank test of the impact of cancer-associated fibroblast-related protein expression in invasive breast cancer on disease-free and overall survival times

Parameters	Disease-free survival		Overall survival	
	95 % CI	*p* value	95 % CI	*p* value
Cancer cell compartment				
Podoplanin		0.346		0.723
Negative	107 (102–111)		108 (106–111)	
Positive	178 (153–202)		169 (145–194)	
Prolyl 4-hydroxylase		0.777		0.655
Negative	108 (104–111)		110 (107–114)	
Positive	163 (125–201)		161 (124–198)	
FAP-α		0.206		0.391
Negative	108 (106–111)		109 (106–111)	
Positive	174 (151–196)		177 (159–194)	
FSP-1/S100A4		**0.002**		**0.044**
Negative	177 (156–198)		175 (155–194)	
Positive	102 (95–109)		114 (109–119)	
PDGFR-α		0.151		0.235
Negative	170 (148–192)		176 (161–191)	
Positive	101 (97–105)		100 (96–104)	
Stromal compartment				
Podoplanin		**0.041**		0.181
Negative	170 (148–191)		178 (163–192)	
Positive	103 (98–107)		98 (88–108)	
Prolyl 4-hydroxylase		0.105		0.588
Negative	172 (152–192)		177 (163–192)	
Positive	90 (87–94)		91 (87–95)	
FAP-α		n/a		0.450
Negative	n/a		176 (161–191)	
Positive	n/a		106 (97–114)	
FSP-1/S100A4		**0.041**		0.164
Negative	176 (165–187)		**181 (173–189)**	
Positive	105 (102–108)		**115 (108–121)**	
PDGFR-α		0.735		0.380
Negative	170 (150–190)		175 (161–189)	
Positive	81 (78–84)		83 (81–85)	
PDGFR-β		0.528		0.448
Negative	177 (161–192)		176 (161–191)	
Positive	102 (98–106)		105 (99–111)	
NG2		n/a		n/a
Negative	n/a		n/a	
Positive	n/a		n/a	

Bold values represents *p*-values that are < 0.05

Underlined Bold values represents *p*-values that is statistically significant ($p<0.05$)

In addition to the higher expression of FSP-1/S100A4 in ILC than in invasive carcinoma (NST), we found that the expression patterns of FSP-1/S100A4 differed between both types of breast cancer. In invasive carcinoma (NST), FSP-1/S100A4 was expressed in a fascicular pattern that involved grouped spindle-shaped CAFs. In contrast, FSP-1/S100A4 expression in ILC was scattered in ovoid or round cells. In addition to expression in CAFs and malignant cells in breast cancer tissue, FSP-1/S100A4 is expressed in macrophages [46]. FSP-1/S100A4-type CAFs induce macrophage recruitment in the tumor microenvironment via the secretion of monocyte chemotactic protein-1 [47].

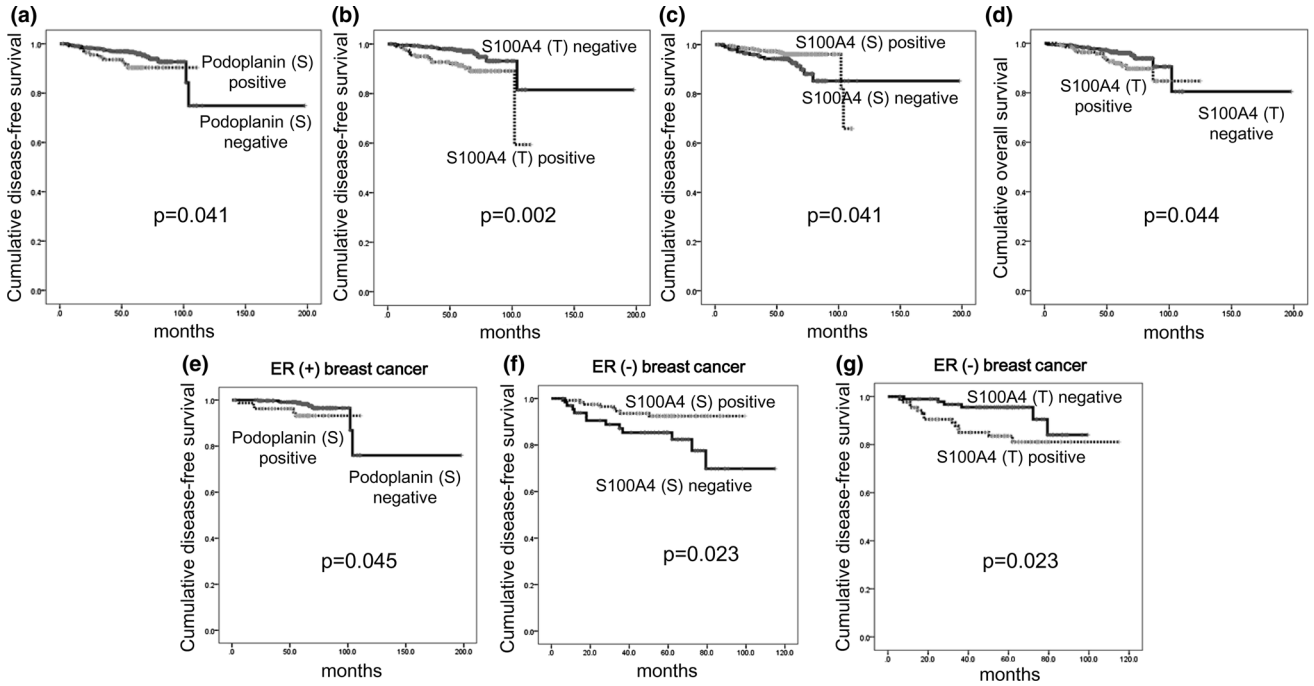

Fig. 4 Disease-free survival (DFS) and overall survival (OS) according to the expression of CAF-related proteins in invasive breast cancer, ER-positive breast cancer, and ER-negative breast cancer. (**a**) Stromal podoplanin positivity, (**b**) tumoral FSP-1/S100A4 positivity, and (**c**) stromal FSP-1/S100A4 negativity are associated with shorter DFS, and (**d**) tumoral FSP-1/S100A4 positivity is associated with shorter OS in invasive breast cancer. In ER-positive breast cancer, (**e**) stromal podoplanin positivity is associated with shorter DFS. In ER-negative breast cancer, (**f**) stromal FSP-1/S100A4 negativity and (**g**) tumoral FSP-1/S100A4 positivity are associated with shorter DFS

Table 8 Multivariate Cox analysis of invasive breast cancer survival

Included parameters	Disease-free survival			Overall survival		
	Hazard ratio	95 % CI	*p* value	Hazard ratio	95 % CI	*p* value
T stage			**0.002**			**0.041**
T1 versus T2–3	4.057	1.683–9.779		2.239	1.034–4.849	
Lymph node metastasis			0.238			**0.046**
Absent versus Present	1.522	0.758–3.055		2.011	1.014–3.989	
Histologic grade			0.780			0.553
I/II versus III	1.126	0.491–2.581		1.278	0.568–2.880	
ER status			0.223			0.599
Negative versus positive	2.007	0.654–6.156		1.328	0.461–3.989	
PR status			0.228			0.145
Negative versus positive	1.983	0.652–6.032		2.111	0.772–5.768	
HER-2 status			0.839			0.575
Negative versus positive	0.913	0.379–2.198		0.770	0.308–1.921	
Ki-67 LI			0.871			0.849
$\leq$14 % versus >14 %	0.932	0.397–2.186		1.082	0.478–2.448	
FSP-1/S100A4 in cancer cells			**0.043**			0.276
Negative versus positive	2.060	1.024–4.143		1.458	0.740–2.870	
Podoplanin in stromal cells			**0.007**			0.091
Negative versus positive	3.462	1.414–8.479		2.103	0.888–4.980	

Table 8 continued

Included parameters	Disease-free survival			Overall survival		
	Hazard ratio	95 % CI	*p* value	Hazard ratio	95 % CI	*p* value
FSP-1/S100A4 in stromal cells			**0.026**			0.576
Negative versus positive	2.465	1.113–5.461		0.576	0.272–1.217	

Bold values represents *p*-values that are < 0.05

These findings indicate that macrophages in ILC stroma, like CAFs, may be positive for FSP-1/S100A4. Further studies are required to assess this possibility.

No significant differences in the expression of CAF-related proteins between classic type and pleomorphic type of ILC in this study. In general, pleomorphic type is more aggressive and shows poor prognosis than classic type ILC [48, 49]. There are several possible causes that can explain the insignificant differences in the expression of CAF-related proteins between the two subtypes. First, such differences could have been caused by limitations in the statistical analysis due to the difference in the number of cases between the two subtypes. Second, there may have been no significant differences in the expression of CAF-related proteins between the two subtypes. One previous study reported no significant differences between the prognosis of the two subtypes after matching patients' age and the year of diagnosis [50]. In addition, the pleomorphic type is known to show genetic alteration that is similar to that of the classic type [51]. Thus, these findings raise the possibility of insignificant differences in tumor stroma between the two subtypes of ILC, and further studies are required.

Clinically, our study indicates that CAFs are a potential target in cancer therapy. There are several reasons that these cells are a promising drug target [52]. First, compared with cancer cells, CAFs are genetically stable. Second, CAFs show different epigenetic changes from normal stromal cells. Finally, CAFs accompany and support cancer cells through the entire neoplastic spectrum. Thus, we can manage and treat neoplasms in any stage of the disease.

In conclusion, the expression of CAF-related proteins differed between invasive carcinoma (NST) and ILC in both the cancer and stromal cell compartments. ILC showed a particularly elevated expression of FAP-α and FSP-1/S100A4 in both tumor and stromal cells compared to invasive carcinoma (NST). Therapy targeted to the CAF markers used in our study has shown an inhibitory effect on tumor growth [53–56]. This supports our contention that the application of targeted therapy for CAFs can be applied to ILC, which expresses high levels of CAF-related proteins.

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Compliance with ethical standards

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

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