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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Cellular Senescence, Represented by Expression of Caveolin-1, in Cancer-Associated Fibroblasts Promotes Tumor Invasion in Pancreatic Cancer

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ABSTRACT

Background. The role of senescence of cancer-associated fibroblasts (CAFs) in the development of cancer is controversial. In this study, we investigated whether cellular senescence of CAFs, represented by CAV1 expression, affects tumor progression in pancreatic cancers (PC).

Methods. Because CAV1 plays a major role in cellular senescence, we used CAV1 expression to monitor cellular senescence. A total of 157 consecutive patients with PC who underwent curative resection were enrolled in the study. Patients were divided into two groups according to CAV1 expression in CAFs by immunohistochemistry. We investigated the relationship between the CAV1 expression in CAFs and the patients' clinicopathological characteristics, including survival. We also established ten CAFs cell lines using PC clinical samples and chose one of them to knock down *CAV1* expression. Finally, we cultured a PC cell line (MIAPaCa-2) in CAF-conditioned medium (CM).

Results. Regarding patients' clinicopathological characteristics, the serum levels of carbohydrate antigen 19-9 and the rate of advanced tumor stage (pT2, 3, and 4) were significantly higher in the high-CAV1 group. The high-CAV1 group had significantly worse outcomes in both overall and disease-free survival ($p < 0.01$). Additionally, in co-culture assays using CAFs-CM and MIAPaCa-2 cells, we found that knockdown of *CAV1* in CAFs negatively affected the invasion of PC cells.

Conclusions. In PC, CAV1 expression in CAFs is associated with patients' poor prognosis and the downregulation of CAV1 in CAFs reduces the invasiveness of PC cells. Therefore, CAV1 of CAFs might be a new target for the treatment of PC.

Pancreatic cancer (PC) is the seventh most common cause of cancer-related death worldwide and causes more than 331,000 deaths each year.[1] Approximately 80% of PC cases are unresectable progressive cancers.[2] Additionally, even when surgery is possible, multidisciplinary treatment is typically required because of the high recurrence rate of PC.[2,3] To date, few targeted therapies have been shown to be effective for treating PC. Therefore, studies of the detailed molecular mechanisms involved in PC are urgently needed to identify novel PC targets and improve patient prognosis.

Recently, the relationship between cancer cell progression and the tumor microenvironment (TME) has received increased attention.[4–6] Specifically, the cancer stroma, which is abundant in PC, was reported to contribute to tumor progression, invasion, metastasis, and chemoresistance.[7–9] Studies of TME may lead to the development of new therapies; For example, previous reports showed that the expression of secreted protein acid rich in cysteine, a calcium-binding glycoprotein that interacts with the

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extracellular matrix, is associated with poor prognosis in patients with PC treated with gemcitabine plus albumin-bound paclitaxel (nab-paclitaxel).[10,11]

In this study, we focused on the role of cellular senescence of cancer-associated fibroblasts (CAFs) in the progression of PC. Although several studies have shown that CAFs induce cancer progression and that CAF activation predicts poor prognosis,[12–14] the importance of cellular senescence of CAFs remains unclear. The secretion of inflammatory cytokines, chemokines, growth factors, and extracellular matrix is increased in senescent cells.[15,16] These phenomena are collectively known as the senescence-associated secretory phenotype (SASP), which facilitates oncogenesis, cancer invasion, and metastasis by causing inflammation.[16,17] Therefore, we hypothesized that the senescence of CAFs promotes the invasion and metastasis of PC by causing inflammation in cancer cells.

In recent years, caveolin-1 (CAV1), which is expressed in CAFs, was reported to have a major role in cellular senescence.[18,19] CAV1 is a membrane protein and major constituent of caveolae, which are invaginations of the cell surface that play important roles in physiological functions such as cell surface signaling, endocytosis, and intracellular cholesterol transport.[20–24] CAV1 was recently reported to control cellular senescence through the murine double minute/p53-mediated pathway.[25] Therefore, we also hypothesized that the expression of CAV1 indicates cellular senescence in CAFs and causes tumor progression in PC through SASP-related factors.

To evaluate our hypotheses, we detected CAV1 expression in CAFs by immunohistochemistry using resected clinical PC samples, and conducted in vitro analyses using newly established CAF cell lines.

PATIENTS AND METHODS

Patients and Tissue Samples

A total of 157 patients with PC who underwent curative pancreatectomy at Kumamoto University Hospital (Kumamoto, Japan) from April 2004 to December 2016 were included in this study. We isolated primary CAFs from ten patients, and used the cell lines generated (CAF1–4) for co-culture and enzyme-linked immunosorbent assays (ELISAs). The T/N status of the patients was determined according to the seventh edition of the American Joint Committee on Cancer/International Union Against Cancer staging manual.[26] All patients provide informed consent before treatment according to the provisions of the Declaration of Helsinki. This study was approved by the Medical Ethics Committee of the Kumamoto University (IRB approval no. 711 and 1291).

Cell Line, Chemicals, and siRNA Transfection

These items are described in detail in the Supplementary Information.

IHC, Reverse Transcription, Quantitative Polymerase Chain Reaction (qPCR), and Western Blotting (WB)

Immunohistochemistry (IHC), reverse transcription, qPCR, and WB were performed as previously described.[27] Additional information is provided in the Supplementary Information.

Co-culture Assays, ELISAs, and Gene Expression Microarray

Co-culture assays with PC cells and CAF-conditioned medium (CM) were performed to evaluate invasion, migration, and proliferation. ELISAs and gene expression microarray were performed using CAF-CM. Please refer to the Supplementary Information.

Statistical Analysis

Survival curves were constructed using the Kaplan–Meier method, and the log-rank test was used to evaluate the statistical significance of differences. Continuous variables were expressed as median and range and compared using the Mann–Whitney U test. Categorical variables were compared by using the Chi square test or Fisher's exact test, as appropriate. Cox regression analysis was utilized for univariate and multivariate analyses of overall survival (OS) and disease-free survival (DFS). Repeated-measurement analysis of variance was used to compare cell proliferation. Statistical analyses were performed using JMP software (version 10, SAS Institute, Cary, NC, USA), and p values < 0.05 were considered significant.

RESULTS

CAV1 Expression in PC-CAFs Correlates with Tumor Progression and Poor Prognosis in Patients with PC

We first investigated CAV1 levels in samples from patients with PC. CAV1 expression was detected in the cytoplasm of stromal CAFs (Fig. 1a, b), and high CAV1 expression was observed in 49 of 157 patients (31.2%). Accordingly, we classified the patients into high and low expression groups. Next, we evaluated the relationship between CAV1 expression in CAFs and clinicopathological factors. As shown in Table 1, high CAV1 expression

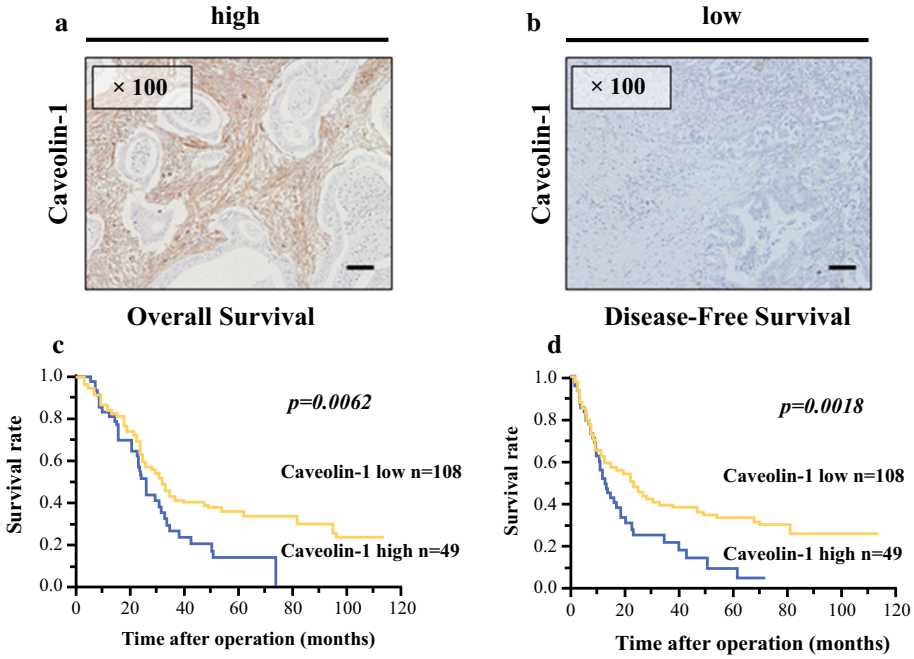

FIG. 1 High caveolin-1 (CAV1) expression in cancer-associated fibroblasts (CAFs) is implicated in poor prognosis in patients with pancreatic cancer (PC). **a**, **b** Representative immunohistochemistry staining of CAV1 expression in 157 PC tissues. Scale bar = 100 μm. Relationship between CAV1 expression in CAFs and overall survival (**c**) or disease-free survival (**d**) using Kaplan–Meier method

TABLE 1 Clinicopathological features and caveolin-1 expression in PC-CAFs

	CAV1 expression in CAFs		*p* value
	High (*n* = 49)	Low (*n* = 108)	
Patient factors			
Age (years)	69 (43–90)	69 (37–83)	0.90
Sex (male/female)	25/24	58/50	0.86
Preoperative chemistry (±)	13/36	24/84	0.55
Tumor-related factors			
Tumor size (mm)	30 (18–70)	29.5 (3–65)	0.096
Neural invasion (ne0/ne1, 2, 3)	2/45	14/92	0.15
Vascular or lymphatic invasion (v·ly0/v·ly1, 2,3)[a]	4/44	17/91	0.31
Differentiation (well, mod/poor)	46/3	89/20	0.16
pT (T0, 1/T2, 3, 4)	1/48	19/88	0.014
pN (N0/N1)	21/28	50/58	0.73
CEA (ng/mL)	2.6 (0.5–13.6)	2.3 (0.2–2089)	0.48
CA19-9 (U/mL)	108.8 (10.4–4764)	52.3 (0.1–4760)	0.024

Values presented as median

PC pancreatic cancer, *CAFs* cancer-associated fibroblasts, *CAV1* caveolin-1, *pT* pathological tumor stage, *pN* pathological lymph node stage, *CEA* carcinoembryonic antigen, *CA19-9* carbohydrate antigen 19-9

[a]Not available in one patient

was significantly associated with high CA19-9 levels ($p = 0.024$), and advanced tumor stage (T2–4; $p = 0.014$). Kaplan–Meier analysis revealed that high CAV1 expression significantly correlated with poor prognosis, in terms of both OS ($p = 0.0062$, Fig. 1c) and DFS ($p = 0.0018$, Fig. 1d). These results suggest that high CAV1 expression in CAFs strongly associated with tumor progression and poor prognosis in patients with PC.

High CAV1 Expression in CAFs is an Independent Poor Prognostic Factor for OS and DFS

The results of univariate and multivariate analyses of prognostic factors for OS and DFS are summarized in Supplementary Tables 1 and 2. Multivariate analysis revealed that the independent prognostic factors for OS were carcinoembryonic antigen (CEA) > 5 ng/mL (hazard

ratio, HR: 1.90, 95% confidence interval, CI 1.07–3.18, $p = 0.030$), advanced tumor stage (T2–4; HR: 3.64, 95% CI 1.76–8.88, $p = 0.0004$), and high CAV1 expression in CAFs (HR: 1.54, 95% CI 1.01–2.33, $p = 0.046$) (Supplementary Table 1). The independent prognostic factors for DFS were CEA > 5 ng/mL (HR: 2.41, 95% CI 1.44–3.87, $p = 0.0012$), poor differentiation (HR: 2.04, 95% CI 1.17–3.37, $p = 0.013$), advanced tumor stage (T2–4; HR: 3.39, 95% CI 1.77–7.36, $p < 0.0001$), and high CAV1 expression in CAFs (HR: 1.61, 95% CI 1.09–2.37, $p = 0.018$) (Supplementary Table 2).

Characteristics of Primary CAF Cell Lines Established from Resected Tissues

We established a total of ten CAF cell lines from resected PC samples (PC-CAFs). We assessed CAV1 expression in PC-CAFs by WB and qPCR (Fig. 2a, b) to determine which cell line was suitable for use in an indirect co-culture assay. Senescence-associated proteins such as p21 and p53 and inflammation-related protein such as phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells (p-NF-κB) were positively correlated with CAV1 expression (Fig. 2a). In CAF1 cells, the expression of CAV1 and senescence-associated proteins was not affected by cell passaging. (Supplementary Fig. 1a). In the two pairs of CAFs and normal fibroblasts (NFs), CAV1 and senescence-associated proteins were expressed at higher levels in CAFs than in NFs (Supplementary Fig. 1d). The expression of CAV1 (24-kDa) was higher in CAF1-4 (Fig. 2a, b). Therefore, we used CAF1-4 in co-culture assays with the MIAPaCa-2 cell line and to isolate CAF-CM.

Knockdown of CAV1 Expression in CAFs Reduces the Invasiveness and Motility of PC cells

Of the three siCAV1s tested, we selected siCAV1#1 and #3, which had the greatest effect on lowering the expression of CAV1, at both the protein (24-kDa) and messenger RNA (mRNA) level (Supplementary Fig. 1b, c). To evaluate whether CAV1 expression in CAFs induces the

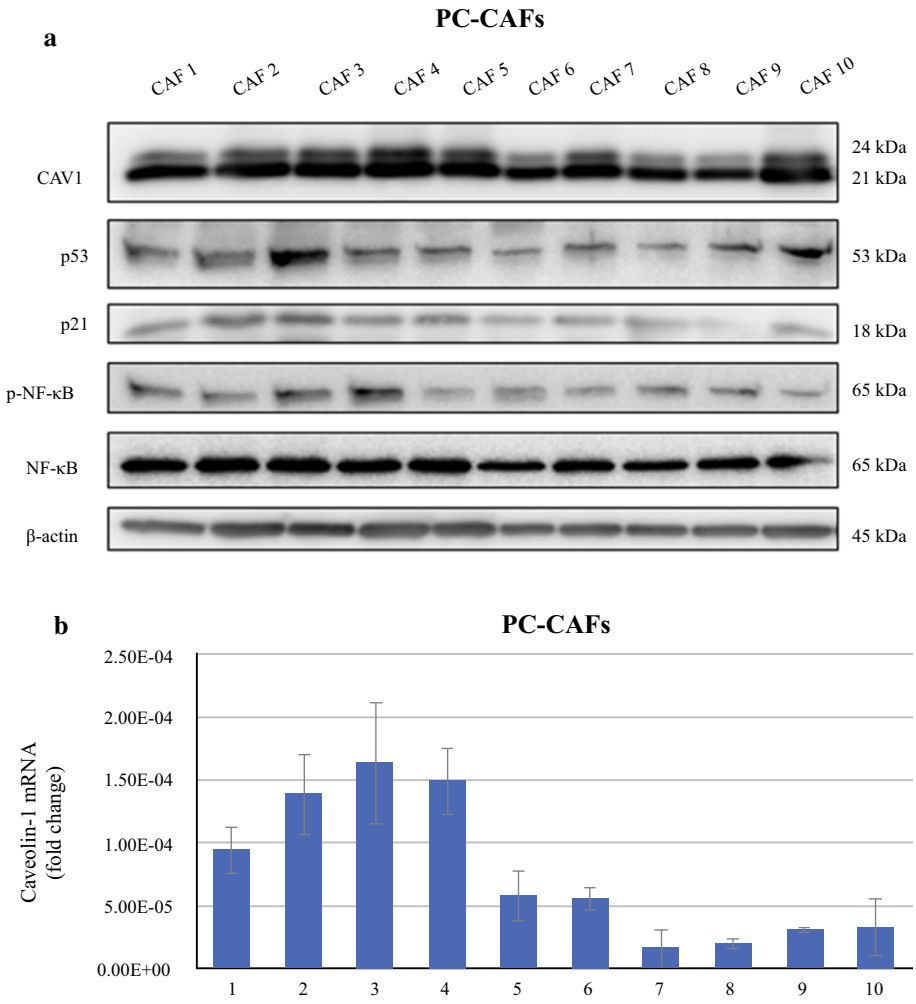

FIG. 2 Characteristics of ten cancer-associated fibroblast (CAF) cell lines established from resected pancreatic cancer tissues. **a** Expression of caveolin-1 (CAV1) in CAF cell lines by Western blot analysis. **b** Quantitative PCR analysis of *CAV1* expression in CAF cell lines. Data normalized to *β-actin* levels and shown as mean ± standard error (SE) of three independent experiments

invasive phenotype of PC, we performed proliferation, invasion, and migration assays in MIAPaCa-2 cells exposed to CAF-CM isolated from CAF1-4 cells in which *CAV1* expression was knocked down; we confirmed the knockdown of *CAV1* by qPCR and WB (Supplementary Fig. 1b, c). Significantly fewer cells invaded the Matrigel when the CM in the lower chamber was isolated from CAFs with knocked-down *CAV1* expression than when the CM was isolated from CAFs transfected with control siRNA (siCtrl) (Fig. 3a). Similar results were obtained in the wound-healing assay. Knockdown of *CAV1* expression in CAFs reduced the motility of PC cells (Fig. 3b). In contrast, in proliferation assays, we found no significant differences depending on the CAV1 levels (Fig. 3c; comparison among siCAV1#1, siCAV1#3, and siCtrl). These results indicate that knockdown of *CAV1* in CAFs negatively affected the invasiveness and motility of MIAPaCa-2 cells but did not affect cell proliferation.

Expression of CAV1 in PC-CAFs Controls the Secretion of Interleukin (IL)-6 and IL-8 Through p-NF-κB

To evaluate if the levels of secretory factors were changed by downregulation of *CAV1* in PC-CAFs, we assessed the secretion of IL-6 and IL-8 in CAF-CM by ELISA. We found that both IL-6 and IL-8 levels decreased in CM isolated from CAFs in which *CAV1* had been knocked down (Fig. 4a, b). Next, we assessed the gene expression of CAV1 downstream by using siCAV1 for CAFs. We found that senescence-associated proteins such as p21 and p-NF-κB were decreased in siCAV1-CAF (Fig. 4c). These results indicate that CAFs with high CAV1 levels induce the secretion of more IL-6 and IL-8 through p-NF-κB.

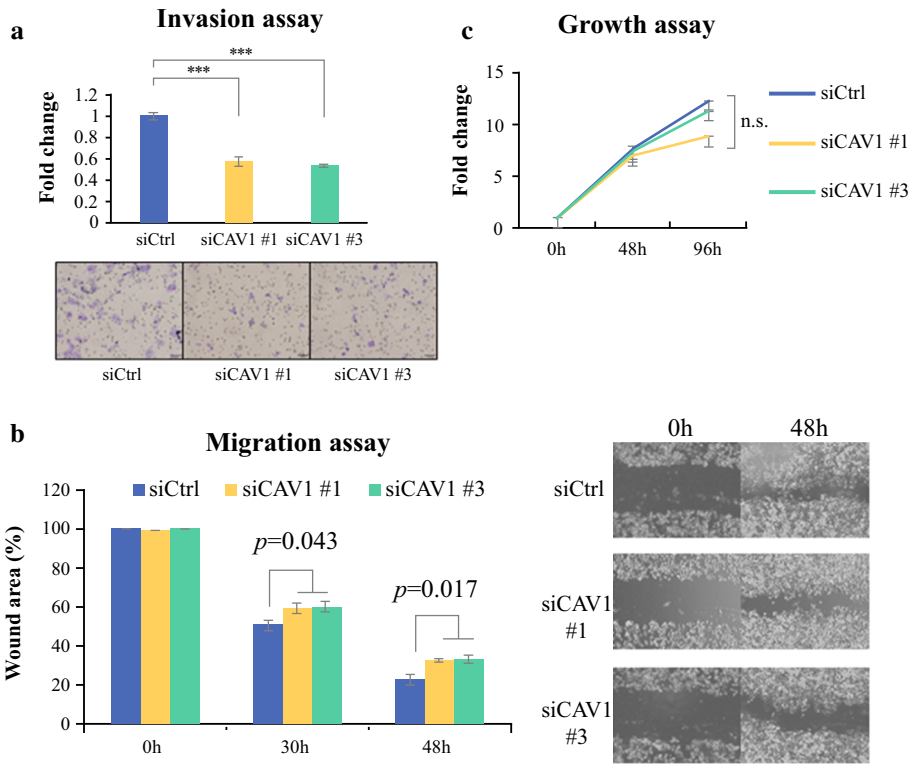

FIG. 3 Knockdown of *CAV1* expression in cancer-associated fibroblasts (CAFs) reduces the invasiveness and motility of pancreatic cancer cells. **a** Invasion ability in MIAPaCa-2 cells co-cultured with conditioned medium (CM) of CAF4 cells transfected with siRNA targeting CAV1 or with control siRNA was evaluated by invasion assay. Data presented as treated/control cell ratio. Scale bar = 20 μm. **b** Motility ability in MIAPaCa-2 cells co-cultured with CM of CAF1 cells transfected with siRNA targeting CAV1 or with control siRNA was evaluated by wound-healing assay. Data presented as treated/control cell ratio. **c** MIAPaCa-2 cells that were co-cultured with CM of CAF4 cells transfected with siRNAs targeting CAV1 or with control siRNA were incubated for up to 96 h and the cells were counted; data presented as treated/control (time = 0) cell ratio. ***$p < 0.001$; *n.s.* not significant

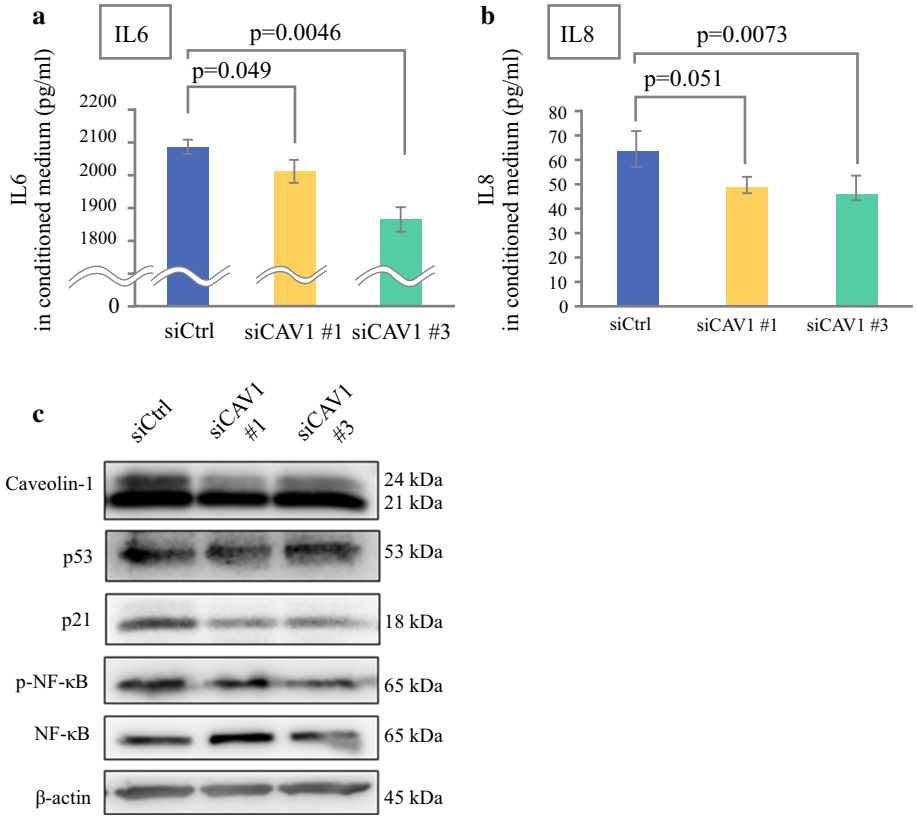

FIG. 4 Knockdown of *CAV1* expression in cancer-associated fibroblasts (CAFs) derived from PC samples is associated with lower secretion of IL-6 and IL-8 by suppressing p-NF-κB. Concentration of IL-6 (**a**) and IL-8 (**b**) in conditioned medium from CAF1-4 cells transfected with siCAV1 or with siCtrl was evaluated by ELISA. **c** Knockdown of *CVA1* suppressed the expression of p53, p21, and p-NF-κB

Knockdown of CAV1 Is Correlated with the Suppression of Cell-Cycle-Related Genes in PC-CAF Cells

To determine the comprehensive gene expression profiles of CAF cells transfected with siCtrl and siCAV1, we conducted gene expression microarray analysis using total RNA from both CAF-cell lines and compared the gene expression by Gene Set Enrichment Analysis using the HALLMARK gene sets. As a result, the siCAV1 group was correlated with increases in cell-cycle-related genes such as G2/M checkpoint or Myc targets (Supplementary Fig. 2a, b). Moreover, the siCAV1 group showed a correlation with downregulation of senescence-associated genes such as p53 (Supplementary Fig. 2c).

DISCUSSION

Recent research revealed that TME plays an important role in tumor invasion, proliferation, and metastasis.[4–6] However, the relationship between senescence in the TME and cancer progression still remains unclear. This study was conducted to investigate the relationship between the senescence of CAFs, using CAV1 as a senescence marker, and tumor progression in PC. Our data obtained using 157 PC resected samples showed that high CAV1 expression was associated with poor prognosis. Additionally, multivariate analyses revealed that high CAV1 expression in PC-CAFs is an independent prognostic factor for OS and DFS. Several CAF proteins have been reported as prognostic markers in patients with PC,[28,29] such as fibroblast activation protein-α.[28] In contrast, negative expression of CD146 in CAFs is an independent factor of poor prognosis, as CD146 suppresses the expression of growth and proinflammatory factors in CAFs.[29] Therefore, crosstalk between cancer and TME is important for tumor development. In recent years, immunotherapy targeting TME factors, such as PD-1/PD-L1, was shown to be advantageous,[30,31] and treatments targeting the cancer stroma, including CAFs, have been attempted;[10,32,33] however, favorable results have not been obtained, and further studies are necessary.

Co-culture assays showed that CM from CAFs in which *CAV1* had been knocked down reduced the invasiveness of MIAPaCa-2, possibly by downregulating IL-6 and IL-8 secretion. These results suggest that the expression of CAV1 in PC-CAFs contributes to tumor invasion in PC by secreting inflammatory proteins. CAFs secrete large amounts of growth factors, cytokines, and chemokines, including IL-6 and IL-8, which promote tumor proliferation and migration.[34,35] Specifically, IL-6 may promote invasion in PC cells via the signal transducer and activator of transcription 3 (STAT3) pathway.[34] Although several studies showed that IL-6/STAT3 promotes cross-talk

between CAF and cancer cells in TME, single inhibition of STAT3 in CAFs does not suppress cancer progression.[36,37] One study showed that remodeling of the TME was promoted in PC by targeted inhibition of STAT3 combined with gemcitabine using AZD1480, an inhibitor of STAT3.[38] Thus, combination therapy with STAT3 inhibitor and anticancer chemotherapy may become a new strategy for treating PC. The relationship between CAV1 expression in CAFs and tumor development has been reported in several cancers; however, the data are controversial.[39–44] Most studies indicated that the loss of CAV1 expression in CAFs is associated with poor prognosis.[39–42] Mechanistically, the absence of CAV1 in CAFs appears to induce a myofibroblast phenotype via transforming growth factor-β signaling, oxidative stress, autophagy, and glycolysis in stromal cells.[45–47] However, some reports showed opposite results;[43,44] For example, high expression of CAV1 in CAFs was found to inhibit Rho GTPase, resulting in increased expression of α-smooth muscle actin, a marker of myofibroblasts. As a result, the study associated high expression of CAV1 in CAFs with cancer invasion and metastasis, resulting in poor prognosis in patients with breast carcinoma, kidney carcinoma, colon carcinoma, and melanoma metastasis.[43] In the study, CAV1 expression in CAFs promoted remodeling of collagen fiber alignments in breast cancers; this appears to support our current results. In a different study of lung cancer, CAV1 expression in CAFs was associated with more frequent solid predominant adenocarcinoma, which is associated with lymphatic vascular invasion and poor prognosis.[44] Similarly, our study showed that high expression of CAV1 in PC-CAF induces invasion in PC cells and is associated with early recurrence and poor prognosis. These data are in contrast to those from a previous report.[39] It is possible that CAV1 has more than one function depending on the tumor type, tumor stage, and relationship with other tumor-associated stromal components, which would explain this discrepancy. However, the detailed mechanisms require further analysis.[43]

There are some limitations to this study. First, although we performed in vitro assays using CAF-CM and a PC cell line, we did not reproduce the complete TME because, in our system, there were no tumor-associated stromal components except the CAFs. Second, we could not evaluate the role of cellular interactions between PC cells and CAF because we performed co-culture assays using CAF-CM and MIAPaCa-2 cells. Third, CAV1 expression in CAFs was not completely suppressed by siRNA at the protein level, particularly the 21-kDa isoform of CAV1. Previous reports showed that CAV1 has two isoforms, CAV1α (24-kDa) and CAV1β (21-kDa), which have distinct phenotypes in the lung; the alveolar type I cell expresses the β isoform predominantly, while the endothelium expresses the α isoform.[48] However, the relationship between the CAV1 isoform and its functions in cancer cells or CAFs is unknown, and further studies are needed to evaluate this issue in CAFs. Finally, although we evaluated the secretion of IL-6 and IL-8 by CAFs, other secreted factors may be important.

In summary, we determined the relationship between senescence of PC-CAFs and PC cell progression using clinical specimens and in vitro assays. The high expression of CAV1 in CAFs may be associated with early recurrence and poor prognosis, and contribute to the invasiveness of PC cells by secreting IL-6 and IL-8.

CONFLICT OF INTEREST The authors declare that they have no conflicts of interest.

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