Tohoku J. Exp. Med., 2025, **266**, 279-293

Cancer-Associated Fibroblasts in Gallbladder Cancer Modify the Migration and Invasion of Gallbladder Cancer Cells

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The importance of the microenvironment in cancer progression is widely recognized, and interactions between cancer cells and stromal cells play an important role in the progression of the disease. A major component of stromal cells are fibroblasts, known as cancer-associated fibroblasts (CAFs). CAFs are thought to enhance the malignant properties of cancer cells through various secreted proteins. It is also known that CAFs function as a leading cell in cancer invasion, and their migratory ability is involved in local cancer invasion. The aim of this study was to elucidate the function of CAF in gallbladder cancer, which is one of the gastrointestinal malignancies with the worst prognosis.

CAFs were primarily cultured from surgical specimens of gallbladder cancer patients. We investigated the migration ability of established CAFs and the effects of conditioned medium obtained from CAFs on the growth and invasion ability of gallbladder cancer cell lines. Functional analysis showed that the migration ability of certain CAFs was enhanced compared to control, and that conditioned medium enhanced the migration and invasion of gallbladder cancer cell lines. Gene expression analysis of CAFs revealed that *tenascin-C* (*TNC*) and *podoplanin* (*PDPN*) were highly expressed in CAFs with the enhancing functions. Immunohistochemical staining of *TNC* and *PDPN* on surgical specimens was performed to investigate the relationship with the prognosis. Disease-free survival and overall survival were found to be reduced in patients with high expression of those genes. The results of this study indicate that CAFs expressing *TNC* and *PDPN* promote cancer progression in gallbladder cancer.

Keywords: cancer-associated fibroblast; gallbladder cancer; invasion; podoplanin; tenascin-C
Tohoku J. Exp. Med., 2025 July, **266** (3), 279-293.
doi: 10.1620/tjem.2024.J126

Introduction

Gallbladder cancer is one of the gastrointestinal malignancies with the worst prognosis. Its 5-year relative survival rate is 20.2%, and its poor prognosis is striking compared with gastric cancer (62.1%), colon cancer (68.9%) and esophageal cancer (31.6%) (Matsuda et al. 2011). Currently, radical surgery is considered the most effective treatment and it has been reported that the 5-year survival rate for resected cases is 40% and only 1% for those who cannot undergo surgery (Kayahara et al. 2008). In addition, since the recurrence rate is high even in surgically resected cases, there is an urgent need to improve the prognosis by developing new diagnostic and therapeutic methods.

The epidemiological features of gallbladder cancer include a high prevalence in women, two to three times higher than in men, and a wide variation in prevalence between regions (Sharma et al. 2017). The regions with the highest prevalence are Chile (27/100,000), northern India (21.5/100,000), Poland (14/100,000), southern Pakistan (11.3/100,000), and Japan (7 cases/100,000). Areas of low prevalence are those with a high proportion of people of European ancestry, such as the United States, Australia, Canada and the United Kingdom. In Japan, the incidence of gallbladder cancer is higher than that in Europe and the United States, and Japan should be in a position to take the lead in research and treatment development.

In recent years, it has become clear that not only cancer cells but also the microenvironment surrounding the cancer is an important factor associated with cancer malignancy (De Wever and Mareel 2003). Cancer tissue is composed of cancer cells, stromal cells and extracellular matrix,

Received February 26, 2024; revised and accepted October 28, 2024; J-STAGE Advance online publication November 7, 2024
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and fibroblasts are the major component cells of the cancer stroma and are called cancer-associated fibroblasts (CAFs). Some CAFs are thought to promote the proliferation, invasion and metastasis of cancer cells, and it is thought that these CAFs are stimulated by cancer and acquire their malignant properties (Shiga et al. 2015). It has been reported that CAFs affect the prognosis of many types of cancer (Calon et al. 2015; Sha et al. 2018; Yin et al. 2019).

It has been shown that the mechanism by which CAFs contribute to cancer progression is regulated by the secretion of various growth factors, cytokines and chemokines (Yamamura et al. 2015). Orimo et al. (2005) subcutaneously injected CAFs isolated from breast cancer tissue together with breast cancer cell lines into immunodeficient mice and found that the levels of SDF1, a chemokine that induces angiogenesis, were significantly higher than in breast cancers with normal fibroblasts (NF). Stuelten et al. (2005) reported that TNFα and TGFβ secreted by breast cancer cells induce the expression of MMP-9 in CAFs, which selectively degrades type IV collagen and laminin that make up the basement membrane, contributing to tumor growth and invasion.

In addition, cancer invasion is not only regulated by secretory proteins, but a mechanism called CAF-led invasion has also been elucidated, in which CAFs are the first cells to invade surrounding tissues before cancer cells (Gaggioli et al. 2007). The preceding infiltration of CAFs followed by cancer cells is reported in squamous cell carcinoma, lung adenocarcinoma and gastric cancer (Gaggioli et al. 2007; Neri et al. 2015; Satoyoshi et al. 2015). This mode of invasion does not depend on the invasive properties of the cancer cells themselves, and it has been suggested that the invasive properties of CAFs contribute to cancer cell invasion and are rate-limiting factors (Gaggioli et al. 2007; Neri et al. 2015).

In particular, gallbladder cancer is highly desmoplastic, and the stromal volume is known to correlate with the prognosis (Li et al. 2017). Therefore, CAF is thought to have a major impact on cancer progression.

There are five reports on CAF in gallbladder cancer (Wang et al. 2019; Chen et al. 2020; Pan et al. 2020; Shi et al. 2021; Wang et al. 2021). In gallbladder cancer, CAF has the effect of promoting its progression (Pan et al. 2020; Wang et al. 2021), and elucidation of the mechanism in CAF is thought to provide useful knowledge for the treatment of gallbladder cancer.

The aim of this study was to clarify the characteristics of CAF in gallbladder cancer and its effect on cancer.

Materials and Methods

This study was approved by the Tohoku University Ethics Committee, and informed consent was obtained from the patients before the study (approval number: 2016-1-745, 2016-1-061).

Cell culture

Primary culture of the fibroblasts was performed by the outgrowth method, described below, on resected specimens of gallbladder with gallbladder cancer or other diseases operated from 2018 to 2020 at Tohoku University Hospital. Fibroblasts obtained from a gallbladder cancer specimen were used as CAF, and fibroblasts obtained from a normal gallbladder were used as NF. The primary culture method was performed according to the literature (Aizawa et al. 2019).

Primary culture of the fibroblasts was performed by the outgrowth method. First, cancerous tissues or normal gallbladder walls were collected from resected specimens and washed with PBS. Then, they were cut into pieces of approximately 2 mm and transferred to a culture dish for primary culture. The medium was DMEM (product number 12320032, Thermo Fisher Scientific, MA, USA) with 10% fetal bovine serum (FBS) (product number 175012, NICHIREI BIOSCIENCE INC., Tokyo, Japan), 1% penicillin-streptomycin-amphotericin B (product number 161-23181, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and cultured at 37°C in 5% CO_2. Although no treatment was performed to remove cancer cells, the spindle-shaped morphology was confirmed by light microscopy to confirm that the cultured cells were fibroblasts. RT-PCR was performed to confirm the expression of actin (αSMA) and the absence of E-cadherin. The RT-PCR method is described below. Each fibroblast was used for functional analysis between passages 2-5.

The human gallbladder cancer cell line OCUG1 (Yamada et al. 1997) was obtained from the JRCB Cell Bank (Osaka, Japan) of the National Institutes of Biomedical Innovation, Health and Nutrition. The human gallbladder cancer cell line TGBC2TKB was obtained from Riken BRC (Ibaraki, Japan). These cell lines were cultured in the same medium and culture conditions as CAF and NF.

Preparation of conditioned medium (CM)

Conditioned medium (CM) was prepared from each cell of NF and CAF obtained from the primary culture, according to the method referenced (Aizawa et al. 2019). To prepare CM, NFs or CAFs were cultured to 100% cell density in a 100 mm dish, washed twice with 5 ml of PBS (product number 10000023, Thermo Fisher Scientific, MA, USA), and then cultured for 3 days in DMEM medium supplemented with 1% penicillin-streptomycin-amphotericin B without FBS. After 3 days, the supernatant of the medium was collected and cell components were removed using a syringe filter (product number SLGVR33RS, Merck KGaA, Darmstadt, Germany) to obtain CM. CM was stored frozen at –80°C until used for experiments.

Cell migration assay

The migration ability of CAFs and NFs obtained from the primary culture was measured by migration assay using 8.0 μm transparent PET membrane (product number

353097, CORNING, NY, USA) according to the method noted (Sato et al. 2020). DMEM containing 10% FBS was added to the bottom layer of the chamber, and a cell solution containing 1.5×10^4 CAFs or NFs suspended in 500 μL FBS-free DMEM was added to the top layer. After culturing for 16 hours, the chamber was removed and the cells remaining on the upper layer of the membrane were removed. Diff-Quick (product number 16,920, SYSMEX CORPORATION, Hyogo, Japan) was used to fix and stain the cells adhering to the lower layer of the membrane. Photographs were taken at high magnification. The number of cells in the captured images was counted using HistoQuest Tissue Analysis Software ver.3.5 (TissueGnostics, Vienna, Austria) (hereafter referred to as HistoQuest).

Cell proliferation assay

The cell proliferation ability was assessed by the MTS test (Aizawa et al. 2019). The gallbladder cancer cell lines, OCUG1 and TGBC2TKB, were each seeded in a 96-well plate at 5×10^3 cells/well and cultured for 24 hours. After removing the culture medium by aspiration, 100 μL/well of CM supplemented with 2% FBS were added and cultured for 96 hours. Since FBS has a strong cell growth-promoting effect, it is assumed that the effect of CAF will be relatively small at 10% concentration, which is usually used in cell cultures, and the FBS concentration was set at 2% according to Aizawa et al. (2019). The cell number was evaluated using CellTiter 96® AQueous One Solution Reagent (Promega, WI, USA). The relative absorbance to the average absorbance of NF was evaluated as the proliferative capacity.

Wound healing test

A wound healing test was performed by scratch assay according as noted (Aizawa et al. 2019). 9.5×10^5 each of OCUG1 and TGBC2TKB were seeded in a 6-well plate and cultured for 24 hours. After confirming that the cell density reached 90% or more, the medium was changed to DMEM without FBS and cultured for 24 hours. The cells were then removed linearly by scraping with the tip of a 200 μL micropipette. After washing with PBS, the cells were observed at three locations with a 5x objective field under a light microscope, and the area of the gap from which the cells were removed was measured using Axio Vert.A1 (Zeiss, Oberkochen, Germany). The medium was replaced with CM supplemented with 1% FBS and, after 20 hours of culturing for OCUG1 and 8 hours for TGBC2TKB, the area of the gap was measured in the same way at the same location. Cell migration ability was calculated as follows: Cell migration rate = 1– (gap area after 24 hours incubation/gap area before incubation in CM).

Invasion assay

The effect of CM on the invasion ability of OCUG1 and TGBC2TKB was evaluated by an invasion assay using a Matrigel invasion chamber (CORNING, NY, USA). The method was performed according to the manufacturer's protocol. A CM containing 10% FBS was added to the bottom layer of the chamber, and 500 μL of a DMEM solution containing 4.0×10^4 cells of OCUG1 and 3.0×10^4 cells of TGBC2TKB suspended in FBS were added to the top layer. After 40 hours for OCUG1 and 20 hours for TGBC2TKB, the chamber was removed and the cells remaining on the upper layer of the membrane were also removed. The cells adhering to the lower layer of the membrane were fixed and stained with Diff-Quick and photographed with a light microscope (BZ-X800, KEYENCE, Osaka, Japan) at 4x magnification. The number of cells in the captured images was counted using HistoQuest analysis software.

Comprehensive microarray gene expression analysis

Comprehensive gene expression analysis of the fibroblasts was performed by microarray. After each cultured sample was washed with PBS, the cells were isolated using 0.25% Trypsin EDTA (Thermo Fisher Scientific, MA, USA) and centrifuged at 200 rcf for 4 minutes at 4°C to remove the supernatant, and then were subjected to RNA extraction using NucleoSpin® RNA (Takara Bio, Shiga, Japan) according to the enclosed product instructions. The extracted RNA was confirmed to be of sufficient concentration using NanoDrop2000c (Thermo Fisher SCIENTIFIC, MA, USA) and a quality check was performed by electrophoresis to confirm that the RNA had not degenerated.

Microarray was performed on the extracted RNA using the GeneChip WT PLUS Reagent Kit (Thermo Fisher SCIENTIFIC, MA, USA) according to the enclosed product instructions. Human GeneChip Clariom™ S Array (Thermo Fisher Scientific, MA, USA), GeneChip Hybridisation Oven 645 (Thermo Fisher Scientific, MA, USA), GeneChip Fluidics Station 450 (Thermo Fisher Scientific, MA, USA) and GeneChip Scanner 3000 7G (Thermo Fisher Scientific, MA, USA) were used for the analysis. The results were analyzed using analysis software, Transcriptome Analysis Console 4.0 (Thermo Fisher Scientific, MA, USA).

Gene expression analysis by RT-PCR

Each sample was isolated and centrifuged as described above and the RNA was extracted using NucleoSpin® RNA. Reverse transcription was performed using PrimeScrip™ RT Master Mix (Takara Bio, Shiga, Japan) according to the enclosed product instructions for cDNA synthesis. The concentration was measured using NanoDrop 2000c and adjusted to 40 ng/μL. RT-PCR was performed using the Step One Plus Real Time PCR System (Thermo Fisher Scientific, MA, USA), TB Green® Premix Ex Taq™ II (Tli RNaseH Plus), ROX plus (product number RR82LR, Takara Bio, Shiga, Japan) according to the method described (Aizawa et al. 2019), and the reaction conditions were 45 cycles of 95°C for 30 seconds, 95°C for 5 seconds, and 60°C for 30 seconds. *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) was used as an endogenous control,

and the level of mRNA expression relative to *GAPDH* was determined by the $2^{-\Delta\Delta Ct}$ method. The following primer sequences were used *ACTA2* (*αSMA*: *alpha-smooth muscle actin*): forward 5′-TAGAACACGGCATCATCA-3′ and reverse 5′-CCAGAGTCCAGCACAATA-3′, *TNC* (*tenascin-C*): forward 5′-CACAGCCACGACAGAGGC-3’ and reverse 5’-AAAGGCATTCTCCGATGCCA-3′, *CDH1* (*E-cadherin*): forward 5′-TTTGTACAGATGGGGTCT TGC-3 and reverse 5′-CAAGCCCACTTTTCATAGTTCC-3′, *PDPN* (*podoplanin*): forward 5′-TCCACGGAGAAAGTGGA TGG-3′ and reverse 5′-CCTTCCCGACATTTTTCGCA-3′, GAPDH: forward 5′-GCACCGTCAAGGCTGAGAAC-3′ and reverse 5′-TGGTGAAGACGCCAGTGGA-3′.

Gene silencing by siRNA

Two types of specific siRNA (Stealth siRNA) (Thermo Fisher Scientific, MA, USA) against podoplanin (*PDPN*) were used [HSS116395 (si1), HSS173792 (si2)]. Silencer™ select Negative Control No.1 siRNA (Thermo Fisher Scientific, MA, USA) was used as a negative control (siNC). Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, MA, USA) was used for siRNA transfection. The method was performed according to the method noted (Wang et al. 2021).

3.5×10^5 cells of each sample were plated on a 6-well plate, and the cell density was confirmed to be 60-80% on the next day. A solution of 150 μL Opti-MEM (Thermo Fisher Scientific, MA, USA), 9 μL Lipofectamine RNAiMAX and a solution of 150 μL Opti-MEM, and 30 pmol siRNA were mixed and allowed to react for 5 minutes at room temperature. The mixture was added to the medium of the sample and transfection was performed. Experiments were performed after 24 to 48 hours.

Twenty-four to 48 hours after transfection of siNC, si1 and si2 siRNAs to CAF, samples were washed with PBS and isolated with 0.25% trypsin. A migration assay was performed as described above to investigate the effect of inhibition of *PDPN* expression on the migration ability of fibroblasts. In this procedure, a sample cultured in a 100 mm dish was divided and distributed on a 6-well plate, and transfection and migration assay were performed as a series of operations.

Immunohistochemistry and clinical analysis

Immunohistochemical staining of histopathological specimens for tenascin-C and podoplanin were performed in the cases that underwent resection for gallbladder cancer at our institution from 2005 to 2019.

For slide preparation, 4-μm thick paraffin-embedded sections of tumor material were cut at the sites with deepest tumor invasion. The sections were deparaffinized in xylene, rehydrated and treated with 0.3% H_2O_2. Podoplanin staining was performed using a Ventana BenchMark ULTRA automated immunohistochemistry/in situ hybridization slide staining system (Roche, Basel, Switzerland) with D2-40 (Roche Diagnostics, Rotkreuz, Switzerland) as the primary antibody. Tenascin-C staining was performed by autoclaving (121°C, 5 minutes) with citrate buffer (pH 6.0) for antigen retrieval and, after blocking, the sections were treated with tenascin-C antibody (Novus Biologicals, CO, USA) diluted 400-fold and allowed to react overnight at 4°C. After washing with PBS, they were reacted with a secondary antibody (goat biotin-labeled anti-rabbit IgG antibody, Nichirei Bioscience Inc., Tokyo, Japan) for 30 minutes at room temperature. After rinsing with PBS, peroxidase-labeled streptavidin (Nichirei Biosciences Inc.) was reacted for 30 minutes at room temperature. Color development was performed with 3,3′-diaminobenzidine (DAB) and the slides were counterstained with haematoxylin.

The staining intensity was analyzed using HistoQuest according to the reference (Derricott et al. 2016). The stained slides of each case were observed with a light microscope (BZ-X800, KEYENCE, Osaka, Japan), and a representative field reflecting the degree of staining of the case was photographed with a 20x objective. First, all cases were observed and the hue of multiple sites typically stained with DAB was set as a positive hue in HistoQuest using a color picker. Next, from the photographed images of each case, an arbitrary region of interest (ROI) of 0.02 mm^2 containing mainly stromal cells was identified at a site with DAB staining. As tumor cells and stromal cells intermingled it was difficult to completely exclude tumor cells from the ROI, and small amounts of tumor cells were also included in the assessment target. A positive area was determined based on the previously set color tone, and the sum of positive signal values in the ROI was measured and defined as the positive intensity. It was visually confirmed that the unstained area had not been misidentified.

Patient factors included sex, age, T-factor, N-factor, TNM staging, and recurrence type. For prognostic analysis, recurrence-free survival and overall survival were analyzed by *TNC* and *PDPN* expression levels using Kaplan-Meier curves with recurrence and death as events. Univariate analysis and multivariate analysis using the COX proportional hazards model were performed to clarify whether each item of sex (male), age (≧ 65), TNM stage (≧ III), *TNC* expression, *PDPN* expression affected DFS and OS.

Statistical analysis

Data are expressed as mean ± standard error. Each experiment was carried out in triplicate. Comparative tests of experimental data were performed as follows. Welch’s t-test was used to compare the total NF group and the total CAF group. Dunnett’s test was used to compare each sample with the control. Correlation analysis was performed using Pearson’s correlation coefficient. Survival curves were constructed using the Kaplan-Meier method, and significant differences were examined using the log-rank test. Immunohistochemistry and analysis of patient background factors were analyzed using Fisher’s exact test. Statistical analysis was performed using JMP pro 14 (SAS Institute Inc., NC, USA), and p-value < 0.05 was considered statisti-

cally significant. An asterisk (*) indicates $p < 0.05$, and a double asterisk (**) indicates $p < 0.01$.

Results

Primary culture of cancer-associated fibroblasts and normal fibroblasts

There were 11 cases of gallbladder cancer surgery between May 2018 and August 2020, named in order from CAF1 to CAF11, as shown in Supplementary Table S1. CAFs were collected in 10 cases, excluding CAF3. However, for CAF5 and CAF7, most of the cells did not adhere to the culture flask when thawed after cryopreservation and the cell culture could not be continued. Therefore, the CM collected before freezing could be used for the experiment, but the CAF migration test could not be performed. As for the normal gallbladder, NF was successfully collected and preserved from all control samples (NF1-4).

All of these cells exhibited a spindle-shaped morphology (Supplementary Fig. S1A), and no clear morphological difference was observed between NFs and CAFs. Gene expression of αSMA as a fibroblast marker was quantified by RT-PCR and compared with that of the gallbladder cancer cell line TGBC2TKB (Supplementary Fig. S1B). αSMA was barely expressed in TGBC2TKB, but was expressed in NFs and CAFs. No significant difference in the expression levels was observed between NF and CAF. We also quantified E-cadherin as an epithelial cell marker and found expression in TGBC2TKB, but little expression in NF and CAF (Supplementary Fig. S1C).

CAF and NF cell migration assay

A transwell migration assay was performed to compare the cell migration ability of fibroblasts. Fig. 1A shows the results of the migration assay. All NFs, CAF1 and CAF4-10 showed almost no migration to the lower membrane layer, but in CAF2 and CAF11 many cells migrated to the lower membrane layer (Fig. 1B). Comparing the total NF group and the total CAF group, the CAF group showed significantly increased migratory activity (NF group vs. CAF group, $*p < 0.05$). When comparing the total NF group and each CAF, CAF1, 4, 6, 8, 9, 10 showed no significant difference compared to the NF group, and CAF2, 11 significantly increased the number of migratory cells compared to the NF group. ($**p < 0.01$).

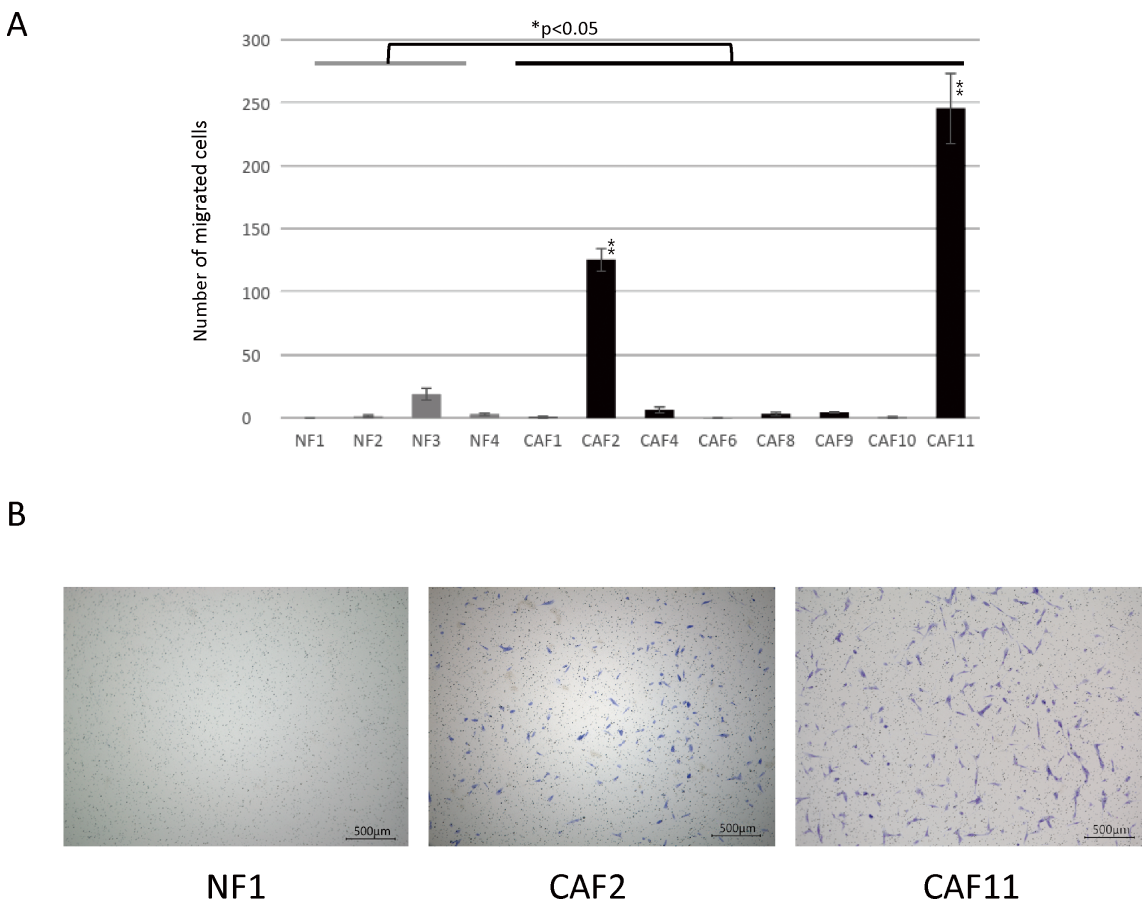

Fig. 1. Examination of CAF and NF mobility.
The migration ability of each sample was compared by transwell migration assay. CAF2 and CAF11 showed cell migration to the lower layer of the membrane, but almost no cell migration was observed in other samples. Compared to the NF group, CAF2 and CAF11 showed significantly increased migration ability (A, B).

Investigation of the effect of CM on cell proliferation of gallbladder cancer cell lines

The proliferative effect of CM derived from CAF and NF on gallbladder cancer cell lines was analyzed by MTS assay. Hereafter, CM derived from NF will be referred to as NF-CM and CM derived from CAF1 will be referred to as CAF1-CM.

When OCUG1 was cultured in each CM for 96 hours, no significant difference in proliferation was observed between NF-CM group and CAF-CM group (NF group vs. CAF group, $p = 0.83$) (Supplementary Fig. S2A). The NF-CM group and each CAF-CM were compared individually, but no significant difference was observed in any CAF-CM.

On the other hand, the proliferation of TGBC2TKB after culturing in each CM for 96 hours was significantly enhanced in the CAF-CM group compared to the NF-CM group (NF-CM group vs. CAF-CM group, $^{**}p < 0.01$) (Supplementary Fig. S2B). When comparing the NF-CM group and each individual CAF-CM, CAF10-CM significantly enhanced the proliferation of TGBC2TKB compared to the NF-CM group ($^{**}p < 0.01$). No significant difference in proliferative capacity was observed in the other CAF-CMs.

Investigation of the effect of CM on the cell migration ability of gallbladder cancer cell lines

The effects of CM on the cell migration ability of gallbladder cancer cell lines were investigated using the scratch assay.

CAF-CM significantly enhanced the migration ability of OCUG1 (NF-CM group vs. CAF-CM group, mean:

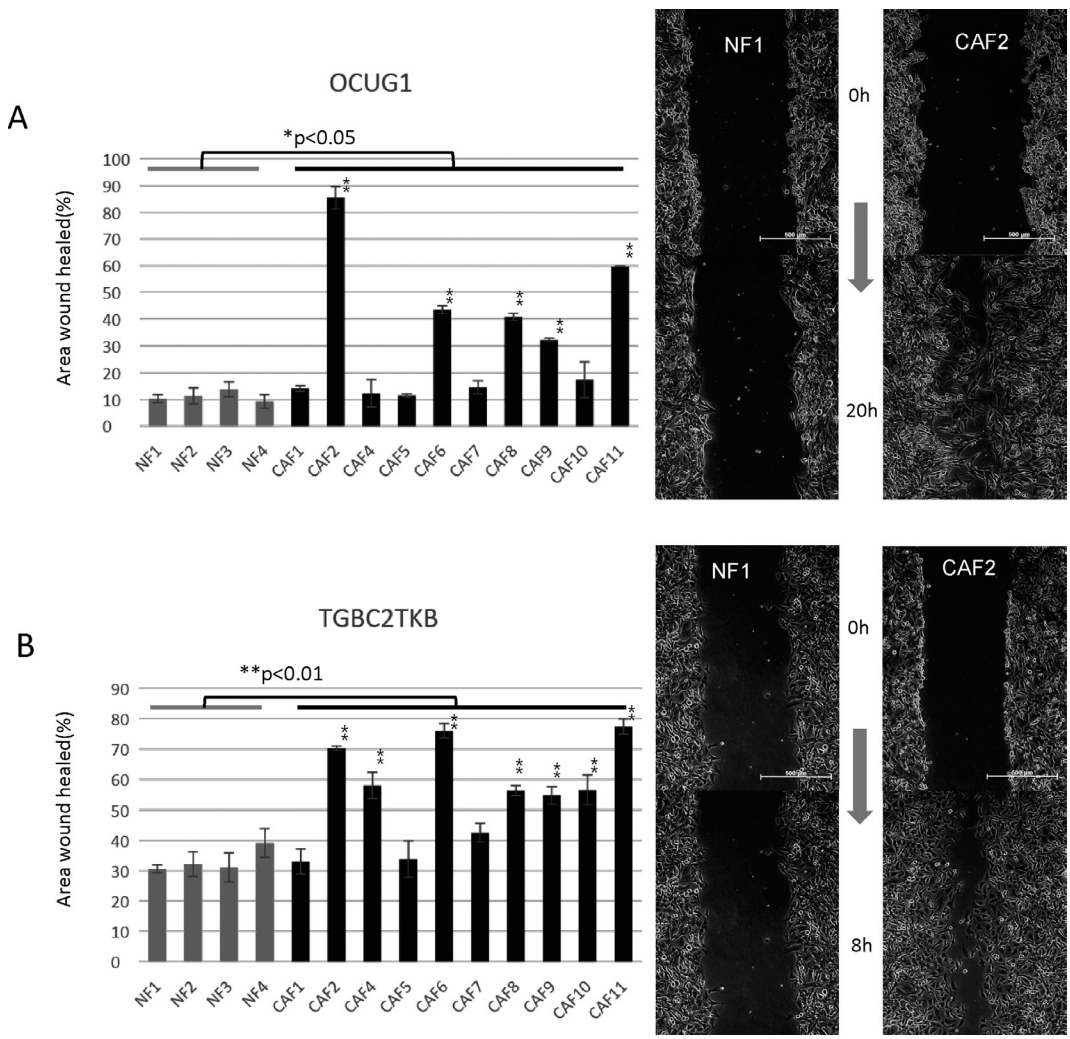

Fig. 2. Wound healing test of gallbladder cancer cell lines using CM.
A wound healing test using CM was performed on each sample to compare the migration ability of the gallbladder cancer cell lines. The CAF-CM group significantly improved the migration ability of OCUG1 compared to the NF-CM group. Among the CAF-CM groups, there was variation among the samples, and CAF2, 6, 8, 9 and 11 CM significantly enhanced the migration ability of OCUG1 compared to the NF-CM group (A). The CAF-CM group significantly increased the migration activity of TGBC2TKB compared to the NF-CM group. CAF2, 4, 6, 8, 9, 10 and 11 CM significantly enhanced the migration ability of TGBC2TKB compared to the NF-CM group (B).

11.1% vs. 33.1%, *p < 0.05). The effect of CM on cancer cell migration varied among CAFs. Comparing the NF-CM group and each CAF-CM individually, CAF1, 4, 5, 7, 10 CM had no significant difference in migration ability compared to the NF-CM group, and CAF2, 6, 8, 9 and 11 CM significantly enhanced OCUG1 migration compared to the NF-CM group (Fig. 2A).

CAF-CM also significantly enhanced TGBC2TKB migration compared to the NF-CM (NF-CM group vs. CAF-CM group, mean 33.2% vs. 55.9%, **p < 0.01). Similar to the results of OCUG1, variations in CAF were observed among CAFs, and although there was no significant difference in CM for CAF1, 5 and 7 compared to the NF-CM, CAF2, 4, 6, 8, 9 and 10, 11 CM significantly enhanced the migration of TGBC2TKB compared to the NF-CM (Fig. 2B).

Investigation of the effect of CM on the invasion ability of gallbladder cancer cell lines

Based on the results of the above migration ability test, CM from CAF2 and CAF11, which strongly enhance cancer cell migration, were used for the invasion assay.

Experiments with OCUG1 showed that CM from both CAF2 and CAF11 significantly enhanced OCUG1 infiltration compared to the control NF-CM (Fig. 3A).

Similarly, experiments with TGBC2TKB showed that CM from both CAF2 and CAF11 significantly enhanced TGBC2TKB invasion compared to the control (Fig. 3B).

Comprehensive analysis of CAF gene expression

Thus far, it has become clear that the properties of CAFs differ among each CAF. To comprehensively analyze gene expression in CAFs, microarrays targeting NF, CAF1 and CAF2 were performed. CAF1 represents CAFs with a low progression effect on cancer, and CAF2 represents those with a high progression effect.

We performed a clustering analysis of the genes expressed in CAF, and the results are shown in Supplementary Fig. S3A. Although the expression patterns of each sample were similar, CAF1 and NF1 in particular were classified as groups with closer expression patterns, while CAF2 was further away from both groups. This showed that CAF2 had a different gene expression pattern compared to NF1 and CAF1.

Next, we searched candidate genes that influenced the results of the functional analysis. First, 15 genes (Shiga et al. 2015; Kobayashi et al. 2019; Chen et al. 2020) known as CAF markers from previous reports were listed (Supplementary Table S2A). Then, 16 of the 225 genes whose expression was found to be five times higher in CAF2 than in NF1 were identified, which related to cell migration (Supplementary Table S2B). *tenascin-C* (*TNC*) and *Podoplanin* (*PDPN*) were identified as genes common to these 15 CAF markers and 16 migration-related genes. Those two genes were used as target genes in this study (Supplementary Fig. S3B).

Gene expression analysis by RT-PCR

RT-PCR was performed to examine the expression levels of *TNC* and *PDPN* in CAF and NF.

Regarding the expression level of *TNC*, the expression was significantly increased in the CAF group compared to the NF group. Comparing the expression level of each CAF with the NF group, CAF1, 4, 5, 6, 7 and 9 showed no

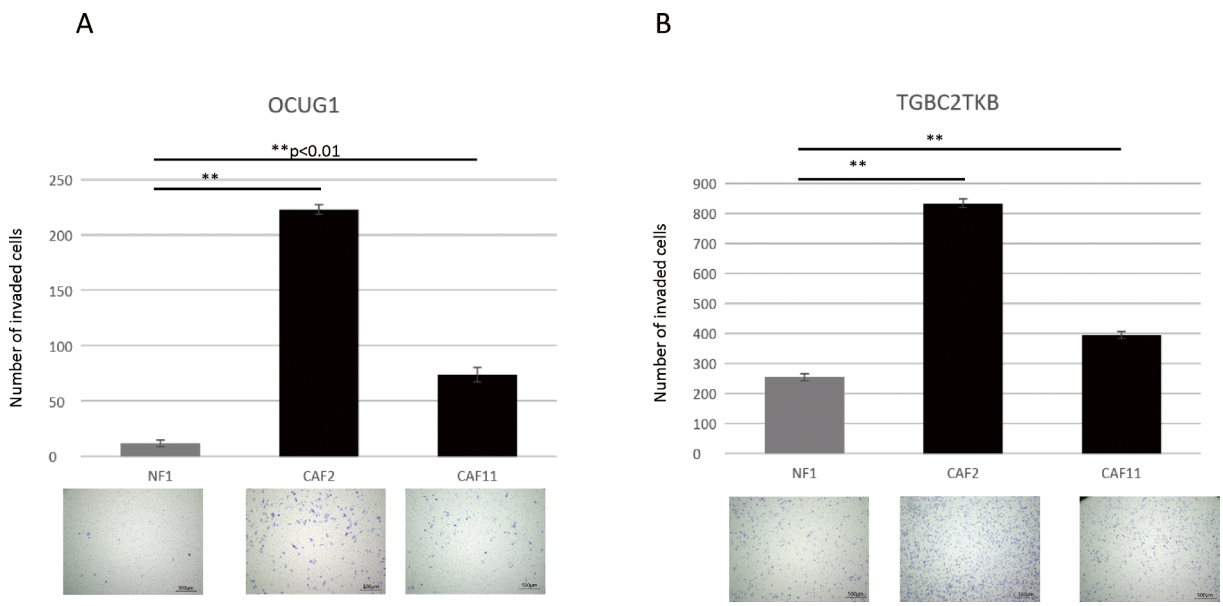

Fig. 3. Invasion assay of gallbladder cancer cell lines using CM.
To compare the invasion ability of gallbladder cancer cell lines, a Matrigel invasion assay was performed using CM from each sample. CAF2-CM and CAF11-CM significantly enhanced the invasive ability of OCUG1 compared to NF1-CM (A). CAF2-CM and CAF11-CM significantly increased the invasive ability of TGBC2TKB compared to NF1-CM (B).

significant difference from the NF group. On the other hand, CAF2, 8, 10 and 11 showed significantly higher expression compared to the NF group (Fig. 4A). When comparing the expression levels of the NF group and the gallbladder cancer cell lines, TGBC2TKB showed significantly higher expression than the NF group.

Regarding the expression level of *PDPN*, there was no significant difference in the expression level when comparing the whole NF group with the whole CAF group. When comparing the expression level of each CAF with the NF group, CAF1, 4, 5, 6, 7, 8, 9, 10 showed no significant difference with the NF group, but CAF2, 11 showed no significant difference with the NF group. In comparison, a significantly higher expression was shown (Fig. 4B). Comparing the expression levels of the NF group and each gallbladder cancer cell line, OCUG1 and TGBC2TKB showed significantly reduced *PDPN* expression.

Correlation between expression levels of TNC and PDPN in CAF and migratory ability

We analyzed the correlation between the expression levels of *TNC* and *PDPN* in relation to the migratory ability of CAFs and NFs, and the effect of CM on the migratory ability of cancer cell lines. The expression levels of *TNC* and *PDPN* were positively correlated with the migration of CAF and NF, respectively (Supplementary Fig. S4A,B $^{**}p < 0.01$), the correlation coefficient with *TNC* was 0.84 and the correlation coefficient with *PDPN* was 0.98.

A positive correlation was observed between the migration ability of OCUG1 using CM and the expression level of *TNC* ($^{**}p < 0.01$, correlation coefficient: 0.86) (Supplementary Fig. S4C), and there was also a positive correlation with the expression level of *PDPN* ($^{**}p < 0.01$, correlation coefficient: 0.68) (Supplementary Fig. S4D). A positive correlation was also observed between the migration ability of TGBC2TKB and the expression level of *TNC* ($^{*}p < 0.05$, correlation coefficient: 0.65) (Supplementary Fig. S4E), and a positive correlation was also observed with the expression level of *TNC* ($^{*}p < 0.05$, correlation coefficient: 0.62) (Supplementary Fig. S4F).

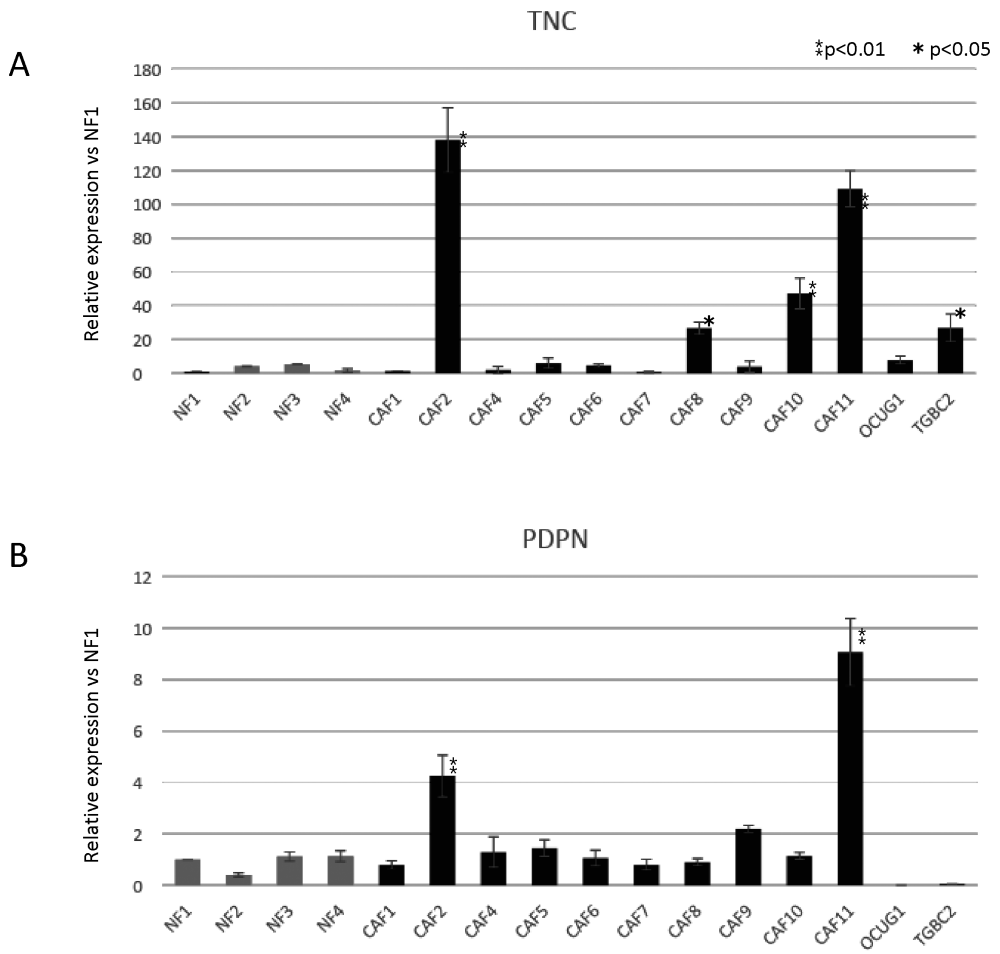

Fig. 4. Expression of *TNC* and *PDPN* in NF, CAF and gallbladder cancer cell lines.
The expression levels of *TNC* and *PDPN* in each sample were compared by RT-PCR. For *TNC*, CAF2, 8, 10 and 11 showed significantly higher expression compared to the NF group. TGBC2TKB also showed significantly high expression (A). For *PDPN*, CAF2 and 11 showed significantly higher expression compared to the NF group. In addition, almost no expression was observed in the gallbladder cancer cell lines (B).

Functional analysis of PDPN

To investigate the effect of *PDPN* on the migration ability of CAF itself and cancer cell migration by CM, *PDPN* expression was suppressed by siRNA and a functional analysis was performed.

Negative control siRNA (siNC) and siRNA (si1, si2) against *PDPN* were transfected to CAF2 and CAF11, and the suppressed expression of *PDPN* was confirmed (Fig. 5A,B).

First, a migration assay was performed to compare the ability of CAFs to migrate. For both CAF2 and CAF11, knockdown of *PDPN* by si1 and si2 significantly reduced the migration compared to siNC (**p < 0.01) (Fig. 5C,D).

A comparative study of the effect of CM collected from *PDPN* knockdown CAFs on the migration ability of cancer cell lines was then performed (Fig. 5E,F).

CM from *PDPN* suppression in CAF2 and CAF11, showed a slight decreasing effect on the migration ability of gallbladder cancer.

Analysis of immunohistochemical results and clinical prognosis

We performed immunohistochemistry of TNC and PDPN on the 56 clinical specimens of gallbladder cancer, which were removed at our institute from 2005 to 2019, and investigated the expression level and their clinical course.

The positive cut-off values for TNC and PDPN were

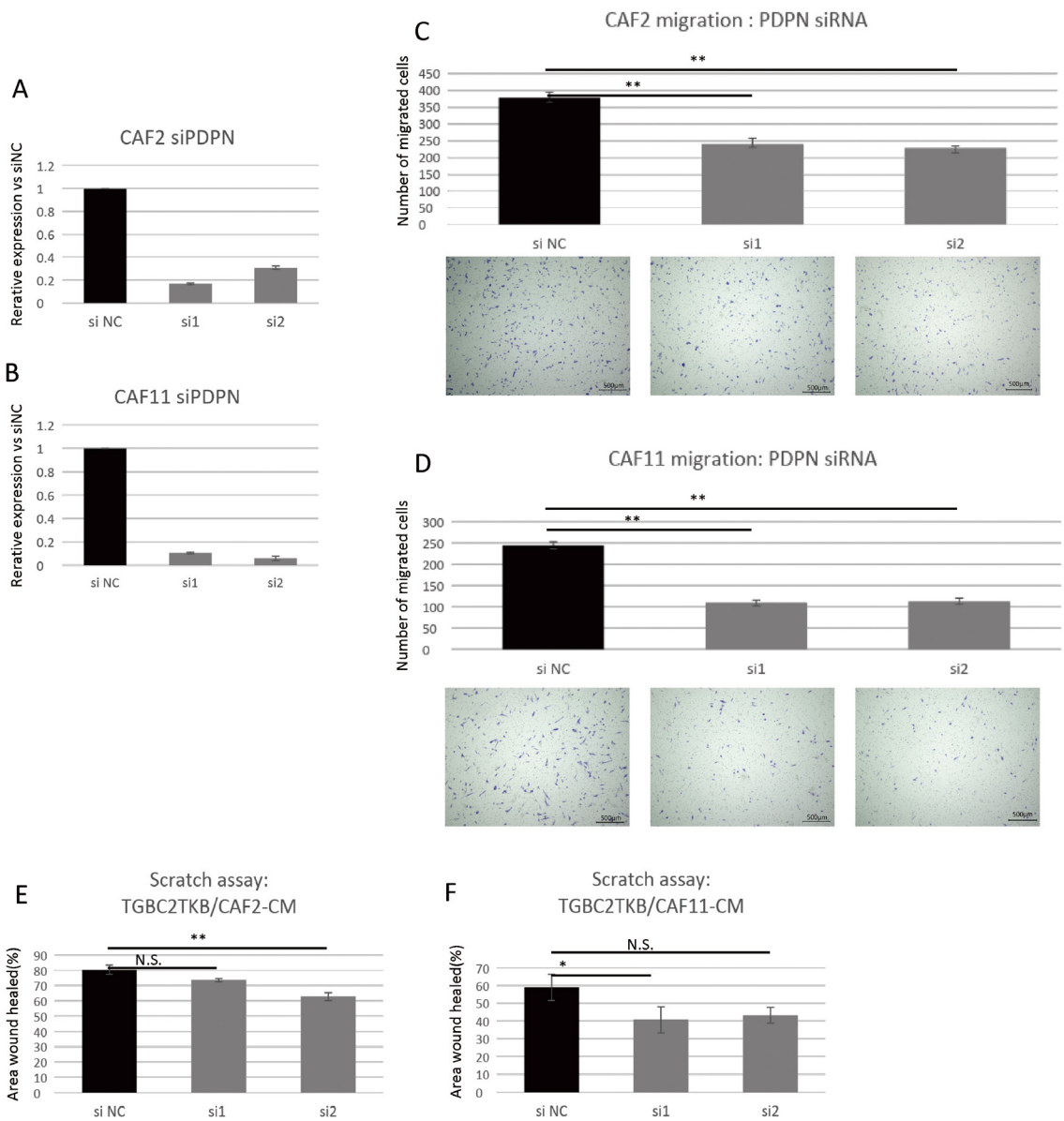

Fig. 5. *PDPN* knockdown by siRNA and effect on CAF migration ability.
In both CAF2 and CAF11, *PDPN* si1 and si2 showed decreased expression of *PDPN* compared to siNC (A, B). In both CAF2 and CAF11, si1 and si2 showed significantly reduced migration ability compared to siNC (C, D). Suppression of *PDPN* in CAF had little effect on the CM-mediated migration of cancer cells (E, F).

determined using receiver operator characteristic (ROC) curves for TNC and PDPN with overall survival of 12 months or less as the event. The cut-off values for TNC and PDPN were 7.16 and 6.35, respectively. Cases with this value or higher were classified as a high expression (positive) group, and cases with a lower value were classified as a low expression (negative) group.

Microscopic images of high and low expression for TNC and PDPN are shown in Figs. 6A and 7A, respectively. TNC was predominantly stained in the stroma, but weak staining was also observed in tumor cells. PDPN did not stain tumor cells, but staining was observed in the stroma.

Kaplan-Meier curves were constructed for TNC and PDPN with recurrence and death as events in each group, and disease-free survival (DFS) and overall survival (OS) were analyzed.

DFS and OS were significantly shorter in the TNC positive group than in the negative group (DFS: $^{**}p < 0.01$, OS: $^{**}p < 0.01$). The 5-year relapse-free rate was 0.26 in the positive group and 0.67 in the negative group, and the 5-year overall survival rate was 0.23 in the positive group and 0.60 in the negative group (Fig. 6B).

Analysis of the background factors showed that the pathological TNM stage and local recurrence rate were significantly worse in the TNC positive group (TNM, v: $^{**}p < 0.01$, local recurrence: $^{*}p < 0.05$).

In the PDPN positive group, DFS and OS were significantly shorter than in the negative group (DFS: $^{**}p < 0.01$, OS: $^{**}p < 0.01$) (Table 1A). The 5-year relapse-free rate was 0.40 in the positive group and 0.85 in the negative group, and the 5-year overall survival rate was 0.34 in the positive group and 0.81 in the negative group (Fig. 7B).

Analysis of the background factors showed that the pathological TNM stage, T-factor, and distant recurrence were significantly worse in the PDPN positive group (TNM, T-factor, distant recurrence: $^{**}p < 0.01$, INF: $^{*}p < 0.05$) (Table 1B).

COX proportional hazard analysis was performed using DFS or OS as the event and sex, age, TNM stage, TNC expression and PDPN expression as dependent factors. The results showed that the TNM stage and TNC expression were significantly associated with OS, and the TNM stage, TNC expression, and PDPN expression were associated with OS (Table 2).

Discussion

In this study, we performed functional analysis and gene expression analysis for CAF and NF using primary cultured cells to elucidate the characteristics of CAF in

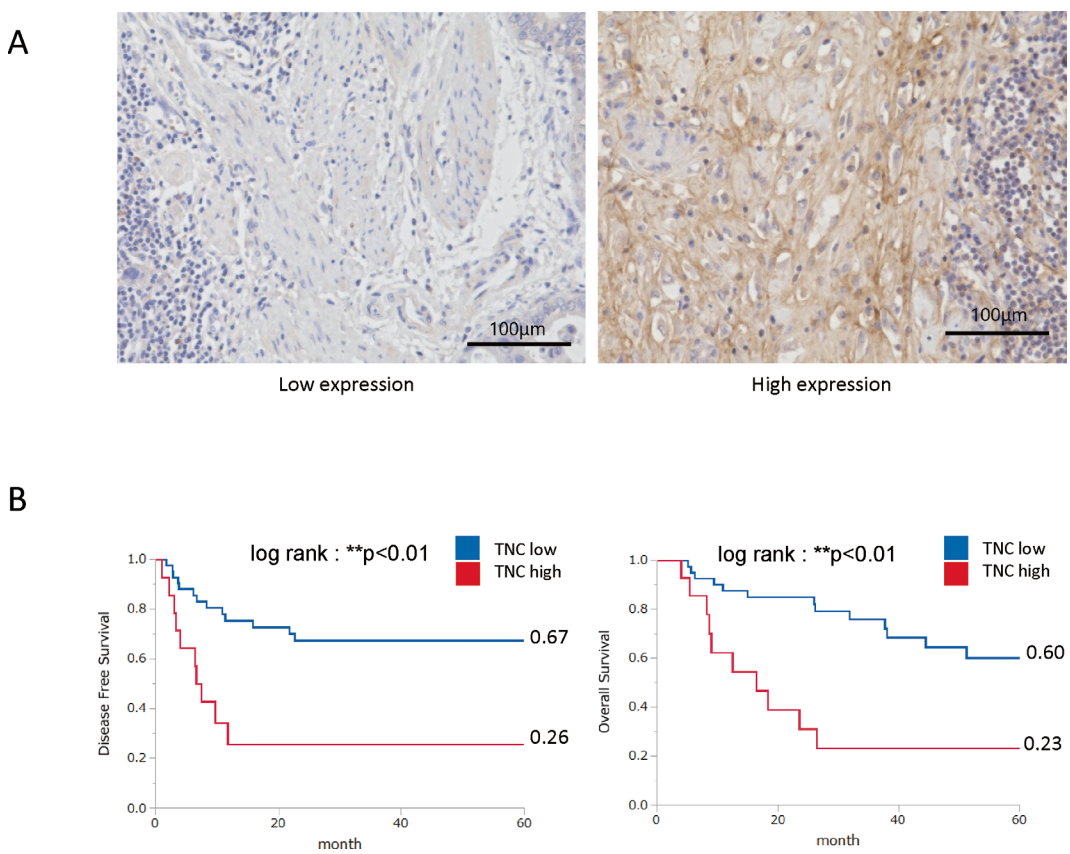

Fig. 6. TNC immunohistochemistry and analysis of clinical prognosis.
The cut-off value determined from the ROC curve is 7.16. A microscopic image showed staining predominantly in the stroma (A). Kaplan-Meier curves for DFS and OS in each TNC group are shown (B). The high expression group showed significantly worse DFS and OS than the low expression group.

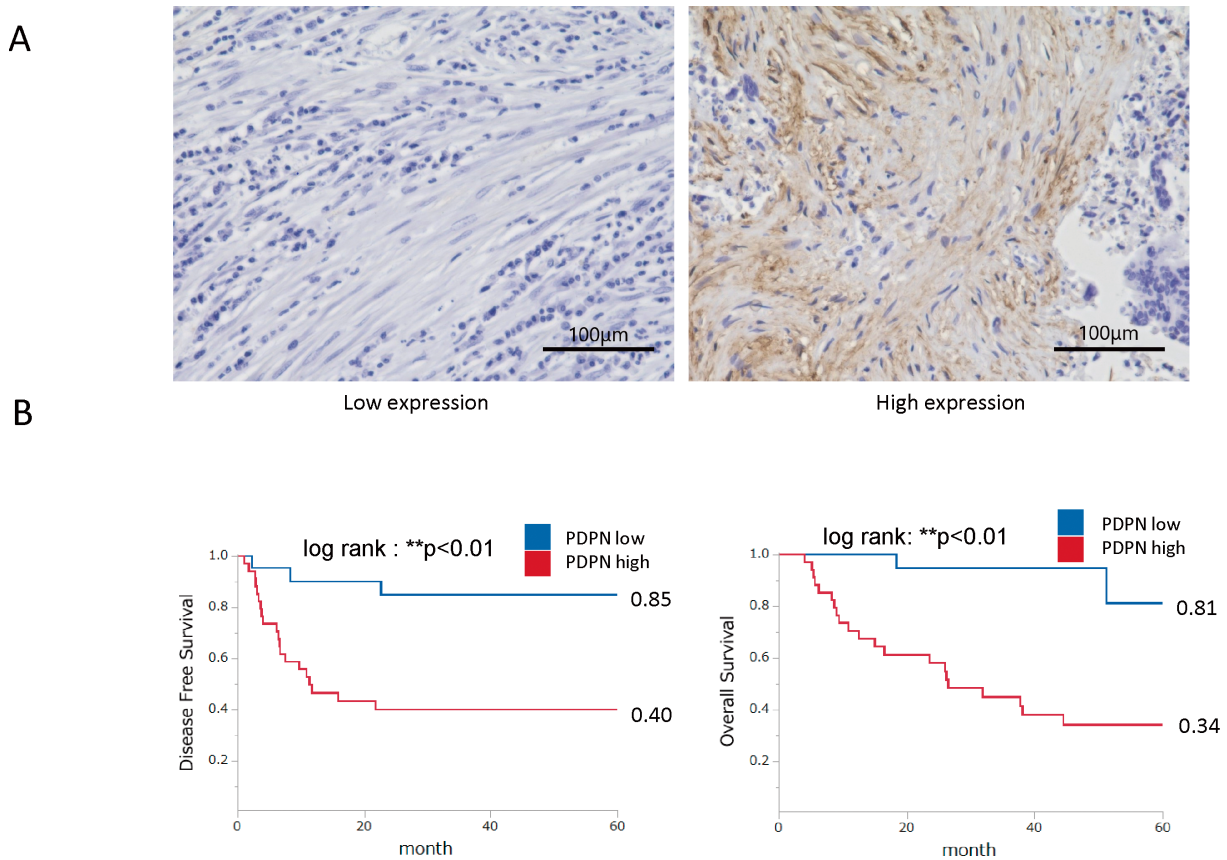

Fig. 7. Immunohistochemistry of PDPN and analysis of clinical prognosis.
The cut-off value determined from the ROC curve is 6.35. In the microscopic image, almost no staining was observed in the cancer cells and staining was observed in the stroma (A). Kaplan-Meier curves for DFS and OS in each PDPN group are shown (B). The high expression group showed significantly worse DFS and OS than the low expression group.

gallbladder cancer and the mechanism of interaction with cancer cells. Our results showed that the expression of *TNC* and *PDPN* in CAF is related to the clinical prognosis in gallbladder cancer.

It has been reported that CAF modifies the malignant characteristics in various types of cancer (Shiga et al. 2015), and five reports have been published concerning gallbladder cancer (Wang et al. 2019; Chen et al. 2020; Pan et al. 2020; Shi et al. 2021; Wang et al. 2021). *NADPH oxidase 4* (*NOX4*) (Pan et al. 2020), *angiopoietin-like protein 4* (*ANGPTL4*) (Wang et al. 2021), *thrombospondin 4* (*TSP4*) (Shi et al. 2021), *Interleukin-8* (*IL-8*) (Chen et al. 2020), and *NADPH oxidase 1* (*NOX1*) (Wang et al. 2019) have been reported to be correlated with gallbladder cancer. In this study, similar to Pan et al. (2020) and Wang et al. (2019), we searched for genes that promote the malignancy of gallbladder cancer by a comprehensive analysis between CAF and NS. In addition, we performed functional analysis of the established CAFs, differentiating CAFs according to the malignant potential such as migration and invasion, and then searched for genes with differential expression among them. This approach is novel and our research findings can be considered meaningful.

In general, CAFs are considered to be "fibroblasts present in cancer tissues" (Öhlund et al. 2014). In addition, although αSMA was used as a marker for fibroblasts in this study, αSMA is sometimes used as a marker for CAF. Aizawa et al. (2019) reported that CAF and NF could not be distinguished by the amount of αSMA expression and, in the present study, there was no difference in the expression of αSMA in CAF from NF, which is the control, and the results were similar to those of Aizawa et al. (2019) (Supplementary Fig. S1B).

When examining the migration ability of CAFs, the results of the transwell migration assay showed that more CAF2 and CAF11 cells migrated to the lower layer of the membrane compared to NF (Fig. 1). However, other CAFs showed similar migration ability to NF, suggesting that there are differences in the functions of CAFs. In general, CAFs are thought to differentiate when cancer cells exert some stimuli on NFs (Webber et al. 2010; Shiga et al. 2015), and it has been suggested that the degree of changes in their phenotypes differs in each case of gallbladder cancer.

Regarding the importance of CAF in cancer invasion, Gaggioli et al. (2007) reported that the squamous cell carcinoma cell line SCC12 does not invade collagen gel alone, and SCC12 can invade the gel only in co-culture with CAF.

Table 1. Cancer characteristics by gene expression of TNC and PDPN.

A. TNC		Low	High	p value
n		42	14	
age (mean)		67.4	69.3	0.759
sex	male	22	6	
	female	20	8	0.752
TNM	0	3	0	
	I	5	3	
	II	10	1	
	III	19	2	
	IV	5	8	**0.005
T factor	Tis	2	0	
	1	7	3	
	2	20	3	
	3	10	3	
	4	3	5	0.090
N factor	0	26	7	
	1	16	7	0.535
local recurrence		2	4	*0.029
distant recurrence		14	6	0.536
PDPN	low	18	4	
	high	24	10	0.529

B. PDPN		Low	High	p value
n		22	34	
age		70.3	66.2	0.064
sex	male	14	14	
	female	8	20	0.171
TNM	0	3	0	
	I	6	2	
	II	5	6	
	III	6	15	
	IV	2	11	**0.009
T factor	Tis	2	0	
	1	7	3	
	2	10	13	
	3	3	10	
	4	0	8	**0.005
N factor	0	16	17	
	1	6	17	0.105
local recurrence		0	6	0.071
distant recurrence		3	17	**0.009
TNC	low	18	24	
	high	4	10	0.529

Table 2. Survival analysis with TNC and PDPN.

A. COX hazard analysis with DFS as an event					
	Univariate HR (95%CI)	p value	Multivariate HR (95%CI)		p value
sex (male)	0.64 (0.28-1.48)	0.30	-	-	-
age (≥ 65)	0.68 (0.27-1.59)	0.37	-	-	-
stage (≥ III)	21.68 (2.91-161.4)	** < 0.01	17.8	(2.31-137.3)	** < 0.01
TNC	3.27 (1.40-7.63)	** < 0.01	3.24	(1.30-8.06)	*0.01
PDPN	5.77 (1.71-19.48)	** < 0.01	2.78	(0.78-9.95)	0.17

B. COX hazard analysis with OS as an event					
	Univariate HR (95%CI)	p value	Multivariate HR (95%CI)		p value
sex (male)	0.71 (0.31-1.59)	0.40	-	-	-
age (≥ 65)	0.88 (0.37-2.14)	0.79	-	-	-
stage (≥ III)	19.37 (2.61-143.6)	** < 0.01	17.01	(2.26-128.3)	** < 0.01
TNC	3.43 (1.46-8.07)	** < 0.01	3.82	(1.49-9.77)	** < 0.01
PDPN	5.66 (1.68-19)	** < 0.01	3.82	(1.01-13.33)	*0.04

Gaggioli et al. (2007) studied this mode of invasion in detail and found that CAFs, not cancer cells, were present at the tip of the invasion during co-culture, and that cancer cells migrated into the remodeled region created by the invasion of CAFs. They called this type of invasion “CAF-led invasion”. They also found that CAFs infiltrated gels even when CAFs were cultured alone (Gaggioli et al. 2007). It has also been reported that CAFs are present at

the leading edge of infiltration and induce cancer cell invasion in lung and gastric cancers (Neri et al. 2015; Satoyoshi et al. 2015). If a similar invasion pattern occurs in gallbladder cancer, the migration and invasion ability of CAFs could be of great significance in the invasion of cancer cells.

In addition to the mechanism of "CAF-led invasion", in which CAF itself precedes invasion, the secreted proteins from CAF enhance the malignancy of cancer cells (Yamamura et al. 2015). There is also a report that CM of CAF enhances cancer cell proliferation (Yoshida et al. 2015), but to clarify this also in gallbladder cancer, a cell proliferation test by MTS assay was performed in this study. As a result, CAF10-derived CM significantly enhanced the proliferative ability of TGBC2TKB compared to the NF group. However, CAF10-CM did not enhance the proliferation of OCUG1. No other CAF showed superiority over NF. Therefore, it is suggested that the effect of CM on cancer cell proliferation differs depending on the cancer cells or CAFs (Supplementary Fig. S2A,B).

The migration ability test by wound healing assay and the invasion ability test by Matrigel transwell invasion assay showed that some CAF-derived CMs significantly enhanced their migration and invasion ability in gallbladder cancer cell lines compared to NF-derived CM (Figs. 2A,B and 3A,B). In CAF-CM as well as the result of the migration ability of CAF itself, the effect of enhancing the mobility ability in gallbladder cancer is different for each CAF.

In this study, a comprehensive gene expression analysis was performed using "high-grade" CAF, which has high migratory ability and enhances the migratory and invasive ability of gallbladder cancer through CM. As a result, we identified *TNC* and *PDPN* as some of the genes upregulated in high-grade CAFs that have a promoting effect on gallbladder cancer (Fig. 4A,B). As a result, we identified two genes, *TNC* and *PDPN*, highly expressed in "high-grade" CAF.

TNC is an extracellular matrix glycoprotein involved in migration and epithelial-mesenchymal transition (EMT). It is found in malignant tumors of the mouth, throat, lung, liver, pancreas, kidney and bladder (Orend and Chiquet-Ehrismann 2006). Stroma-deposited *TNC* induces EMT through integrin receptors expressed on the cell surface and regulates cell migration. *TNC* has been shown to promote migration and invasion in pancreatic and ovarian cancer (Cai et al. 2017; Steitz et al. 2020). The expression of *TNC* in CAF is considered a poor prognostic factor and it has been reported that the high expression group of other cancers has a high degree of progression and poor OS (Ni et al. 2017; Yang et al. 2017). In our study, immunohistochemistry and prognostic analysis showed significantly worse DFS and OS in the high *TNC* group, which suggests that *TNC* enhances the migration and invasion of gallbladder cancer and affects its progression.

PDPN (*podoplanin*) is a mucin-type glycoprotein that activates the Rho-ROCK pathway by phosphorylating proteins such as ezrin, radixin and moesin on its intracellular domain and regulates cell adhesion and migration (Krishnan et al. 2018; Sikorska et al. 2019). *PDPN* has been shown to be expressed in squamous cell carcinoma of the tongue, lung and esophagus and has been reported to enhance cell migration and invasion (Kato et al. 2005; Martín-Villar et al. 2010; Saigusa et al. 2011). In CAFs, its expression has been confirmed in cholangiocarcinoma, pancreatic cancer, lung adenocarcinoma, etc., and is often reported as a poor prognostic factor (Aishima et al. 2008; Kawase et al. 2008; Shindo et al. 2013). Neri et al. (2015) showed that knockdown of *PDPN* in CAFs reduced the invasion of both CAFs and cancer cells in co-cultures of lung adenocarcinoma and CAFs, and also showed that *PDPN*-positive CAFs were present at the onset of tumor invasion *in vivo*, and pleural infiltration was frequently observed. In our study, immunohistochemistry and prognostic analysis showed that the PDPN high-expression group had significantly shorter DFS and OS.

Treatments targeting *TNC* (Lingasamy et al. 2020) and *PDPN* (Shiina et al. 2016) are currently being developed, and they may become a CAF-mediated treatment for gallbladder cancer.

The limitations of this study are that the numbers of investigated CAF are small, and that there should be other genes that enhance gallbladder cancer malignancy than *TNC* and *PDPN*. To elucidate the characteristics of CAF in gallbladder cancer and the mechanism of interaction with cancer cells, further comprehensive analysis with a larger number of samples will be necessary.

This study demonstrated that CAF promotes gallbladder cancer migration and invasion through CM and through a mechanism related to CAF migration and invasion, and identified the factor involved in this mechanism. It was shown that *TNC* and *PDPN* are strongly related to the prognosis.

Funding

This work was supported by JSPS KAKENHI Grant Number JP126750110.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Files

Please find supplementary file(s);
https://doi.org/10.1620/tjem.2024.J126