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RESEARCH

Cancer-associated fibroblasts promote pro-tumor functions of neutrophils in pancreatic cancer via IL-8: potential suppression by pirfenidone

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Abstract

Background The mechanisms by which neutrophils acquire pro-tumor properties remain poorly understood. In pancreatic cancer, cancer-associated fibroblasts (CAFs) may interact with neutrophils, directing them to promote tumor progression.

Methods To validate the association between CAFs and neutrophils, the localization of neutrophils was examined in clinically resected pancreatic cancer specimens. CAFs were produced by culturing in cancer-conditioned media, and the effects of these CAFs on neutrophils were examined. In vitro migration and invasion assays assess the effect of CAF-activated neutrophils on cancer cells. The factors secreted by the activated neutrophils were also explored. Finally, pirfenidone (PFD) was tested to determine whether it could suppress the pro-tumor functions of activated neutrophils.

Results In pancreatic cancer specimens, neutrophils tended to co-localize with IL-6-positive CAFs. Neutrophils co-cultured with CAFs increased migratory capacity and prolonged life span. CAF-affected neutrophils enhance the migratory and invasive activities of pancreatic cancer cells. IL-8 is the most upregulated cytokine secreted by the neutrophils. PFD suppresses IL-8 secretion from CAF-stimulated neutrophils and mitigates the malignant traits of pancreatic cancer cells.

Conclusion CAFs activate neutrophils and enhance the malignant phenotype of pancreatic cancer. The interactions between cancer cells, CAFs, and neutrophils can be disrupted by PFD, highlighting a potential therapeutic approach.

Keywords Cancer-associated fibroblasts · Neutrophil · Anti-fibrotic agent · Pirfenidone

Abbreviations

Abs	Antibodies
αSMA	α-Smooth muscle actin
CAF	Cancer-associated fibroblast
CM	Conditioned medium
FBS	Fetal bovine serum
iCAF	Inflammatory CAF
IL-6	Interleukin-6
IL-8	Interleukin-8
MPO	Myeloperoxidase
myCAF	Myofibroblastic CAF
NF-κB	Nuclear factor-κB
PFD	Pirfenidone
P/S	100 U/mL penicillin and 100 µg/mL streptomycin
TME	Tumor microenvironment

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Introduction

Pancreatic cancer is characterized by desmoplasia as a histopathological feature [1], and the tumor microenvironment (TME) is believed to contribute to its biological malignancy [2, 3]. Cancer cells interact with surrounding cells to form

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TME [4–7], which is essential for pancreatic cancer [8]. Among the components of the TME, cancer-associated fibroblasts (CAFs) are considered crucial [9], and various mechanisms through which they influence tumor biology have been proposed [10, 11]. However, a comprehensive understanding of the TME is still lacking, and the relationship between CAF and other components of the TME remains unclear [12].

Neutrophils are a significant component of TME [13], but their role has not been well studied owing to their short life span [14, 15]. Recent studies have revealed that neutrophils play a variable role within the TME [16] and significantly impact tumor progression and treatment outcomes [17–19]. Neutrophils secrete various factors and interact with other TME components [20]. Reports are emerging on the interactions between tumors and neutrophils [21] as well as between CAFs and neutrophils [22]. The role of neutrophils influenced by CAFs in tumor dynamics is gaining attention [23], and many aspects remain unknown and require further investigation.

We hypothesized that CAF-influenced neutrophils may be involved in promoting the malignancy of pancreatic cancer cells and investigated the interaction between tumors, CAF, and neutrophils. Our findings indicate that CAF-activated neutrophils enhance the malignant phenotype of cancer cells. Furthermore, pirfenidone (PFD), a drug used to treat idiopathic pulmonary fibrosis, suppresses CAF-induced activation of neutrophils, thereby reducing the malignant traits of pancreatic cancer cells.

Material and methods

Cell culture and reagents

The human pancreatic cancer cell lines, MIA PaCa-2 and KP4, were obtained from the American type culture collection. The human fibroblast cell line WI38 was obtained from the Japanese Collection of Research Bioresources. The cells were cultured in RPMI-1640, DMEM, or EMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (P/S) (168–23,191 FUJIFILM). The cultures were maintained at 37 °C in a humidified incubator with 20% O_2 and 5% CO_2.

Isolation of neutrophils

Neutrophils were isolated from healthy volunteers. Whole blood was collected by venipuncture into blood collection tubes coated with EDTA 2 K (#365,900; Becton, Dickinson and Company). Five milliliters of whole blood was layered over 5 mL of Polymorphprep (#114,683, Abbott Diagnostics Technologies AS) in a 15-mL tube and centrifuged at 500 g for 35 min at room temperature. The lower leukocyte band, containing neutrophils, was collected and washed twice with PBS. Neutrophils were resuspended in RPMI-1640 medium without FBS.

Conditioned media preparation

Conditioned media (CM) from MIA PaCa-2 (MIA PaCa-2-CM) or KP4 (KP4-CM) cells was collected after 48 h of incubation in serum-free RPMI-1640 at 100% confluence. After centrifugation, the supernatant was used as the cancer CM and stored at − 80 °C. Conditioned media from CAFs (CAF-CM) was prepared in the same manner.

Generation of CAF

WI38 cells were cultured until they reached confluency. The culture medium was then changed to a cancer-conditioned medium (CM) with 10% FBS, and the medium was replaced daily for 4 days. The resulting WI38 cells were designated CAFs.

Immunofluorescence staining

Paraffin-embedded tissue samples were deparaffinised and rehydrated. Non-specific binding was blocked using 10% donkey serum (Abcam, ab7475) for 30 min at room temperature. To detect neutrophils, iCAFs, and myCAFs, the samples were incubated overnight at 4 °C with anti-myeloperoxidase (MPO) (1:500, RSD, AF3667), anti-IL-6 (1:100, Abcam, ab6672), and anti-αSMA (1:320, Cell Signaling, D4K9N) antibodies, respectively. The tissues were then incubated with secondary antibodies, Alexa Fluor 488 (1:1000, Abcam, ab150129) and Alexa Fluor 647 (1:1000, Abcam, ab150075), in 2% FBS for 1 h at room temperature. Images were obtained using an IX83 microscope (Olympus).

Flow cytometry

The antibodies (Abs) used for flow cytometry were anti-IL-8, anti-αSMA (Cell Signaling Technology), and anti-rabbit IgG Alexa Fluor 647 (Abcam). Cultured cells were collected, washed twice, and resuspended in 100 µL PBS containing 2% FBS. The cells were fixed in 4% paraformaldehyde solution and stained with specific antibodies. Analyses were performed using a FACScan flow cytometer and the CellQuest software (BD Biosciences).

Cancer cells migration and invasion assay

In vitro migration and invasion assays were performed in chambers of 8-µm transwell inserts with or without Matrigel (BD FalconTM), respectively. After trypsinization and

centrifugation, the cancer cells were placed in the upper chamber of each well insert with serum-free medium, and RPMI-1640 was added to the lower chamber. After incubation for 24 h at 37 °C, the non-invading cancer cells were wiped off, and the cells on the bottom side of the upper chamber were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Stained cells were counted under a light microscope at × 200.

Neutrophil migration assay

The in vitro migration assay was performed using 3-μm Transwell inserts (BD Falcon™). Isolated neutrophils labeled with CellTracker Red (1 μM, Thermo Fisher) were added to serum-free medium in the upper chamber of each insert. Induction factors were added to the lower chamber. After a 3-h incubation at 37 °C, the number of cells that migrated to the lower chamber was determined using an IX83 microscope (Olympus, Tokyo, Japan) at a magnification of × 200.

Time lapse confocal microscopy

For all video microscopy experiments, time lapse videos were captured using an FV10i confocal microscope (Olympus) equipped with a cell culture chamber. For the neutrophil life span assay, neutrophils were stained with CellTracker Red (1 μM, Thermo Fisher Scientific) and incubated with 10% FBS for 24 h. SYTOX Green (1 μg/mL, Thermo Fisher Scientific) was added to the medium with or without CAF-CM. Time lapse videos were recorded every 30 min for 24 h. The areas of the stained neutrophils were measured.

Cytokine array

To detect 42 human cytokines in neutrophils, culture supernatants were assayed using a human cytokine antibody array membrane (Abcam, ab133997), according to the manufacturer's instructions.

Cell Proliferation assay

MIA PaCa-2 cells were cultured in 24-well plates, and the culture medium was changed (RPMI-1640, non-stimulated neutrophil secretion, CAF-CM-stimulated neutrophil secretion, and CAF-CM-stimulated neutrophil secretion + PFD) after 24 h. After 24 and 48 h, the cells were harvested using trypsin and counted using a cell counter.

ELISA assay

To evaluate IL-8 levels in neutrophil secretions, culture supernatants were assayed using a human IL-8 enzyme-linked immunosorbent assay (ELISA) kit (Proteintech, KE00006) according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism 10 software. The significance of differences between groups was analyzed using Student's t-test or one-way ANOVA. Statistical significance was set at $P < 0.05$. All experiments were repeated at least thrice.

Result

Neutrophils are present with iCAF in the TME of pancreatic cancer

To investigate the presence and localization of neutrophils in the TME of pancreatic cancer, clinical specimens were observed with HE staining, revealing neutrophils with segmented nuclei. Neutrophils were more ubiquitously distributed in the stroma than in cancer cell nests (Fig. 1A). Using fluorescent immunostaining of MPO-positive neutrophils, their localization in cancer cells and stromal tissue areas was compared. Neutrophils were significantly more abundant in the stromal areas than in the cancerous areas (Fig. 1B). These findings indicate that neutrophils are present in pancreatic cancer tissues but tend to localize to fibroblasts rather than cancer cells.

The stromal tissue of pancreatic cancer is predominantly formed of fibroblast [24], and recent studies have proposed that fibroblasts can be categorized into myofibroblasts (myCAFs) and inflammatory fibroblasts (iCAFs) [25–27]. It has been reported that myCAFs are present in direct proximity to cancer cells, whereas iCAFs are located more distantly from cancer cells within the dense tumor stroma [26]. To investigate which type of fibroblasts co-localized with neutrophils, fibroblasts were labeled with αSMA, a marker of myCAFs, and IL-6, a marker for iCAFs, and the localization of neutrophils was examined in pancreatic cancer specimens. αSMA-positive myCAFs tended to be located near the cancer region, whereas IL-6-positive iCAFs tended to be distant from the cancer region (Fig. 1C, D). Interestingly, neutrophils identified as MPO-positive cells tended to co-localize more prominently with iCAFs than with myCAFs (Fig. 1C, D).

iCAFs activate neutrophils and prolong their life span

To examine the effect of CAFs on neutrophils in vitro, WI38 human fibroblasts were changed into CAFs using conditioned medium (CM) from MIA PaCa-2 human pancreatic

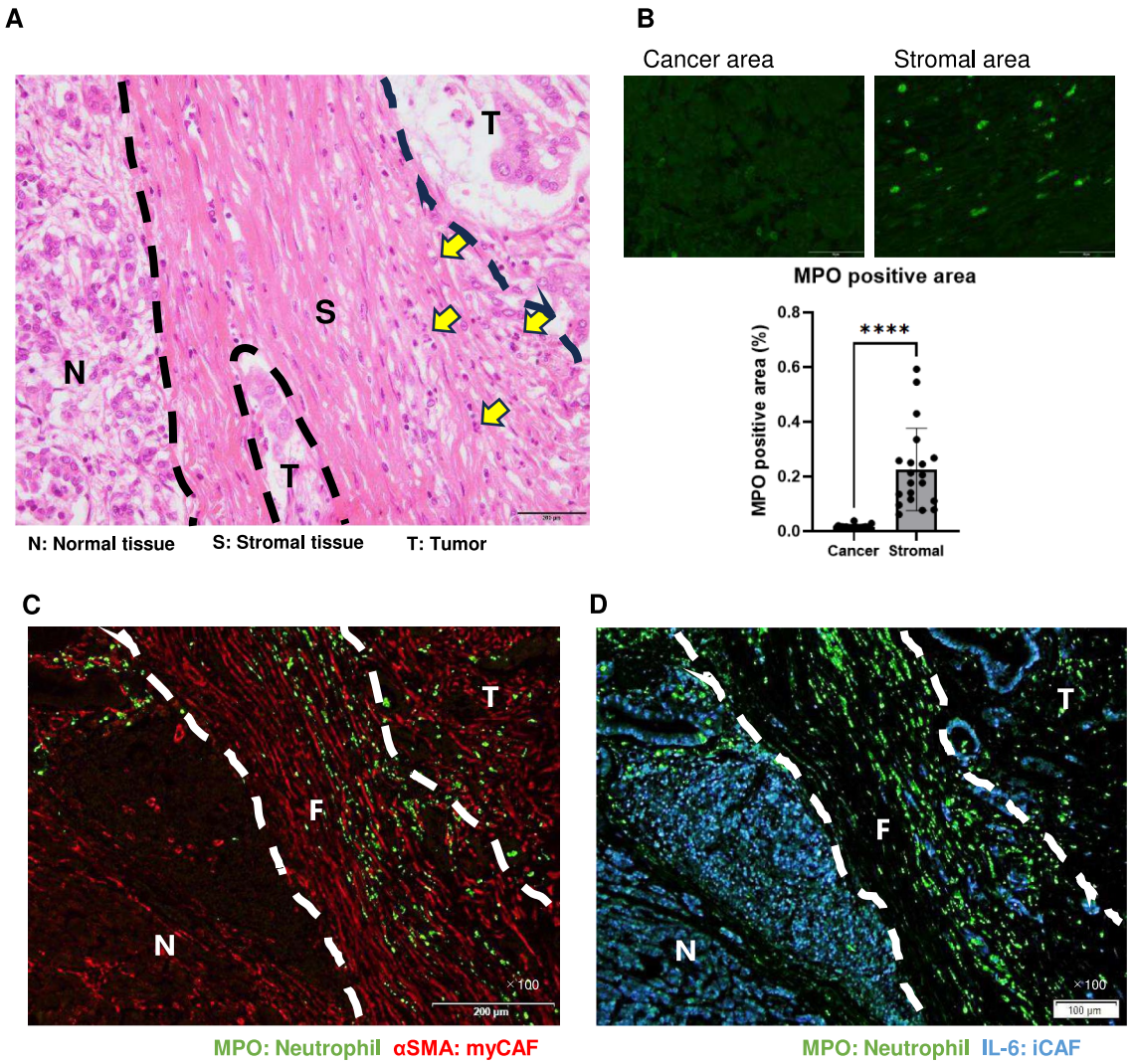

Fig. 1 Neutrophils are present with iCAFs in the TME of pancreatic cancer. **A** HE staining of human pancreatic cancer specimens shows neutrophils predominantly in stromal tissue areas. Yellow arrows indicate neutrophils. T: Tumor, S: Stromal tissue, N: Normal tissue. Image taken at 100×magnification. **B** Comparison of neutrophil presence in cancer cell areas and stromal tissue. Neutrophils were stained with an anti-MPO antibody using fluorescent immunostaining. MPO-positive areas (%) in 20 random fields were compared between cancer cell and stromal tissue areas. Green: MPO-positive neutrophils. Mean ± SD is shown. Each dot represents the MPO-positive area (%) per field (×400). Student's t-test was used. ****, $P < 0.0001$. **C** Representative immunofluorescent images showing neutrophils (MPO) and myCAFs (αSMA) in a human pancreatic cancer specimen. Green: Neutrophils; Red: myCAFs. T: Tumor, S: Stromal tissue, N: Normal tissue. Image taken at 100×magnification. **D** Representative immunofluorescent images showing neutrophils (MPO) and iCAFs (IL-6) in a human pancreatic cancer specimen. Neutrophils tend to co-localize with iCAFs in the stroma. Green: Neutrophils; Blue: iCAFs. T: Tumor, S: Stromal tissue, N: Normal tissue. Image taken at 100×magnification

cancer cells (Fig. 2A). WI38 cells were cultured in MIA PaCa-2-CM for four days and then subjected to FACS analysis to assess the expression of αSMA, a marker for myCAFs, and IL-8, a marker for iCAFs [28]. The results showed that CAFs induced by MIA PaCa-2-CM were αSMA-negative and IL-8-positive, indicating that these fibroblasts had more iCAF properties (Fig. 2B). Similarly, treatment of WI38 cells with KP4-CM induced IL-8-positive iCAFs (Fig. S1).

We hypothesized that CAFs induced by pancreatic cancer cells might affect the phenotype of neutrophils. To assess the effect of CM from CAFs (CAF-CM) on neutrophils, we first investigated whether CAF-CM enhances the migratory ability of neutrophils. Compared with MIA PaCa-2-CM and unstimulated WI38-CM, MIA PaCa-2-induced CAF-CM significantly enhanced neutrophil migration (Fig. 2C). Since IL-8 expression is a marker of neutrophil activation, we investigated whether neutrophils were activated by treatment with CAF-CM by examining intracellular IL-8 in neutrophils using FACS. Compared to untreated neutrophils, those treated with MIA PaCa-2-CAF-CM for 24 h

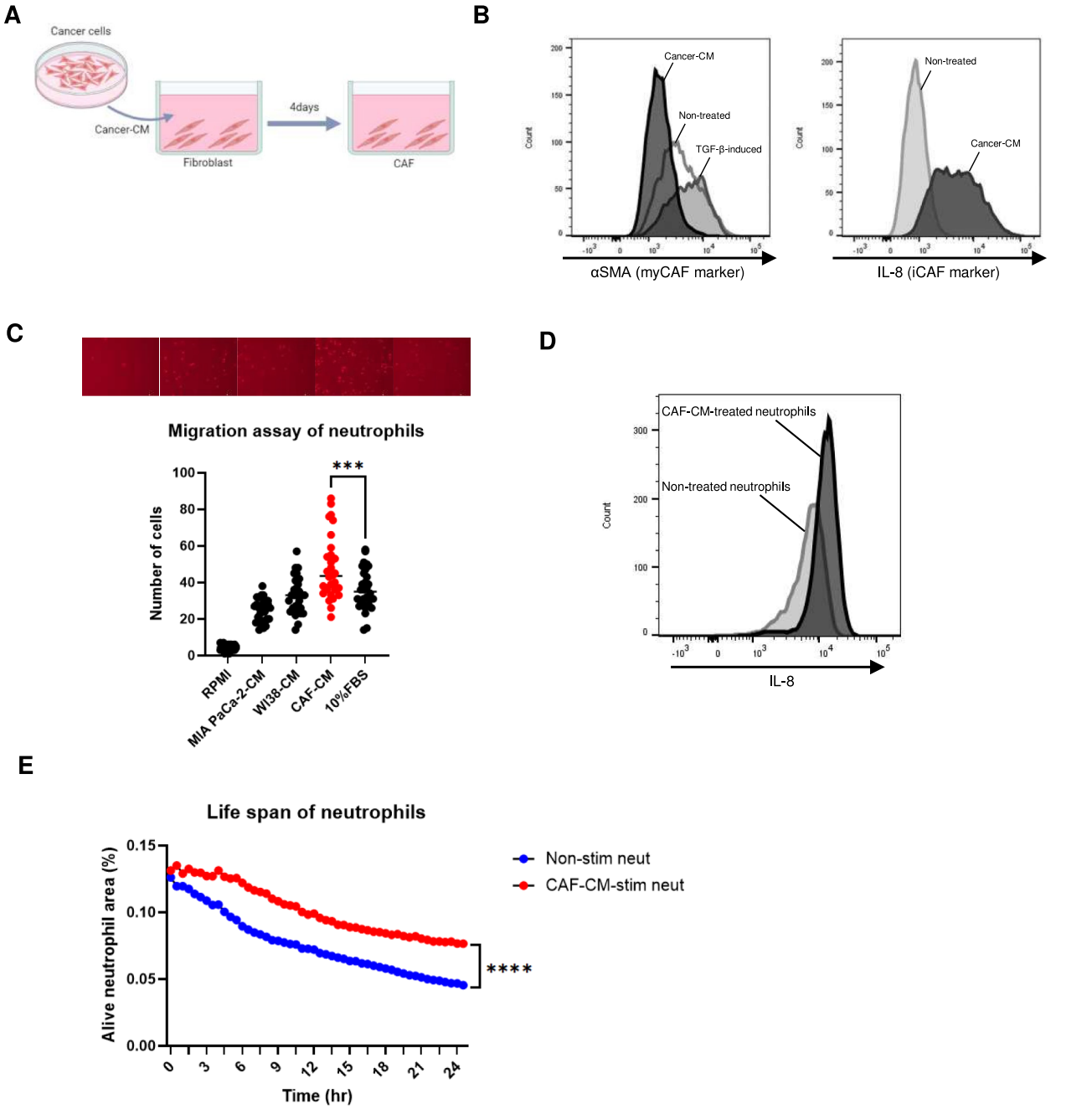

Fig. 2 iCAFs increase the migratory ability of neutrophils, activating them, and prolonging their life span. **A** WI38 cells were cultured in cancer CM for 4 days to produce CAFs. **B** αSMA and IL-8 are representative markers of myCAF and iCAF, respectively. Left: αSMA expression within CAFs induced by MIA PaCa-2-CM was lower than TGF-β-treated CAFs and non-treated control WI38 cells. Right: IL-8 expression within MIA PaCa-2-CM-induced CAFs was higher than in non-treated WI38 cells. **C** Migration assay of neutrophils. Image taken at 100×magnification and the number of migrated cells per field was counted. Neutrophil migration was significantly increased in the CAF-CM group compared to the FBS-treated positive control. Mean ± SD is shown. Each dot represents the number of cells per field (×100). One-way ANOVA with Tukey's test was used. ***, $P = 0.0004$. **D** Compared to non-stimulated neutrophils, those treated with CAF-CM for 24 h showed increased intracellular IL-8. E. Comparing the area of living neutrophils in 9 random fields of view after 24 h, CAF-CM-stimulated neutrophils had a significantly larger area. Student's t-test was used. ****, $P < 0.0001$

showed elevated IL-8 levels (Fig. 2D). Next, we investigated whether CAF-CM affected the life span of neutrophils. Time lapse observations of MIA PaCa-2-CAF-CM-stimulated neutrophils (CAF-CM-stim neut) showed a significantly longer life span than that of non-stimulated neutrophils (non-stim neut) (Fig. 2E, Supplementary video). These results

indicated that CAFs activate neutrophils and prolong their life span.

Neutrophils stimulated by CAF-CM increase IL-8 secretion, enhancing the malignant trait of cancer cells

To investigate whether CAF-CM-stimulated neutrophils affected the malignant traits of cancer cells, their effects on cancer cell migration and invasion were examined using migration and invasion assays (Fig. 3A). Both the migration and invasion of pancreatic cancer cells were significantly enhanced when co-cultured with neutrophils stimulated by CAF-CM, compared to untreated neutrophils (Fig. 3B).

To identify factors that enhance the migration and invasion of pancreatic cancer cells, secreted factors from neutrophils were analyzed using cytokine arrays (Fig. 3C, S2). Among these, IL-8 was the most significantly increased cytokine in neutrophils stimulated by CAF-CM compared with that in untreated neutrophils (Fig. 3C). IL-8 enhanced the migration and invasion of pancreatic cancer cells, and the migratory ability of these cells was potentiated in an IL-8 concentration-dependent manner (Fig. 3D, S3A). Additionally, IL-8, a chemokine for neutrophils [29], significantly increased the migratory ability of neutrophils (Fig. S3B).

Thus, the results indicate that neutrophils stimulated by CAF-CM increased IL-8 secretion, thereby enhancing the malignant traits of pancreatic cancer cells.

PFD suppresses IL-8 secretion from neutrophils, thereby suppressing the malignant traits of cancer cells enhanced by neutrophils

As CAFs and neutrophils cooperate to increase cancer malignancy, targeting CAFs could break this malignant cycle. The anti-fibrotic agent pirfenidone (PFD) was tested to determine its effect on tumor-promoting interactions between CAFs, neutrophils, and cancer cells. PFD itself was not cytotoxic to neutrophils and did not alter their morphology (Fig. S4A). Furthermore, PFD did not inhibit the induction of CAFs, as the expression of IL-8 in CAFs was unaffected by PFD treatment during CAF induction by pancreatic cancer CM (Fig. S4B).

Next, we investigated whether PFD suppressed the migration and invasion of pancreatic cancer cells promoted by activated neutrophils (Fig. 4A, B). When PFD was added after neutrophil activation, the migration and invasion of pancreatic cancer cells were not suppressed (Fig. 4C). However, when PFD was administered during neutrophil activation, migration and invasion of pancreatic cancer cells were significantly suppressed (Fig. 4D). Furthermore, when PFD was added during CAF-CM production, the ability of CAF-CM to promote neutrophil-mediated migration and invasion of pancreatic cancer cells was abolished (Fig. 4E).

Although IL-8 levels in neutrophil secretions were elevated by CAF-CM stimulation, PFD administration during neutrophil activation significantly decreased IL-8 secretion by neutrophils (Fig. 5A). Although the presence of neutrophils significantly increased MIA PaCa-2 cell proliferation (Fig. 5B), PFD did not suppress this neutrophil-promoted cancer cell proliferation, indicating that PFD does not directly affect cancer cell growth.

These results indicated that PFD can act at the point where neutrophils are stimulated by CAF-CM, suppressing the production of IL-8 by neutrophils. Therefore, PFD potentially suppresses the malignant traits of pancreatic cancer cells by targeting the CAF–neutrophil axis.

Discussion

This study investigated how CAF-stimulated neutrophils affect cancer cells and demonstrated that they enhance the malignant traits of cancer cells by increasing IL-8 secretion. In addition, PFD suppressed IL-8 secretion from neutrophils, thereby mitigating the malignant traits of cancer cells (Fig. 5C).

Neutrophil infiltration in various cancer tissues has recently been reported to be associated with a poor prognosis [30], including pancreatic cancer [31, 32]. Clinically, the neutrophil-to-lymphocyte ratio (NLR) has been proposed as a prognostic marker for many cancers [33–35]. The role and importance of neutrophils in cancer have become increasingly apparent, supporting the notion that neutrophils promote cancer progression, although not universally [36]. Neutrophils in cancer retain functional plasticity and can undergo alternative activation when exposed to various cues in the TME [37, 38]. Thus, differences in the localization of neutrophils within tumors may alter their function and clinical implications [36]. However, the mechanisms by which neutrophils are reprogrammed into a tumor-promoting phenotype are not yet clear, and are likely to vary among different cancers.

CAFs are classified into iCAFs and myCAFs, with myCAFs located near the tumor and iCAFs in the surrounding outer area [26]. As previously reported, myCAFs were observed in the proximity of the tumor, and iCAFs were also observed in the outer surrounding area. Interestingly, neutrophils in the TME tended to co-localize with iCAFs. As for the markers for iCAFs, Ohland et al. used IL-6 as a marker for iCAFs when they first defined those distinct fibroblast populations in pancreatic cancer [26]. Subsequently, single-cell analysis of pancreatic ductal adenocarcinoma by Elyada et al. in the same group revealed that iCAFs also express IL-8 [28]. Therefore, IL-8 was

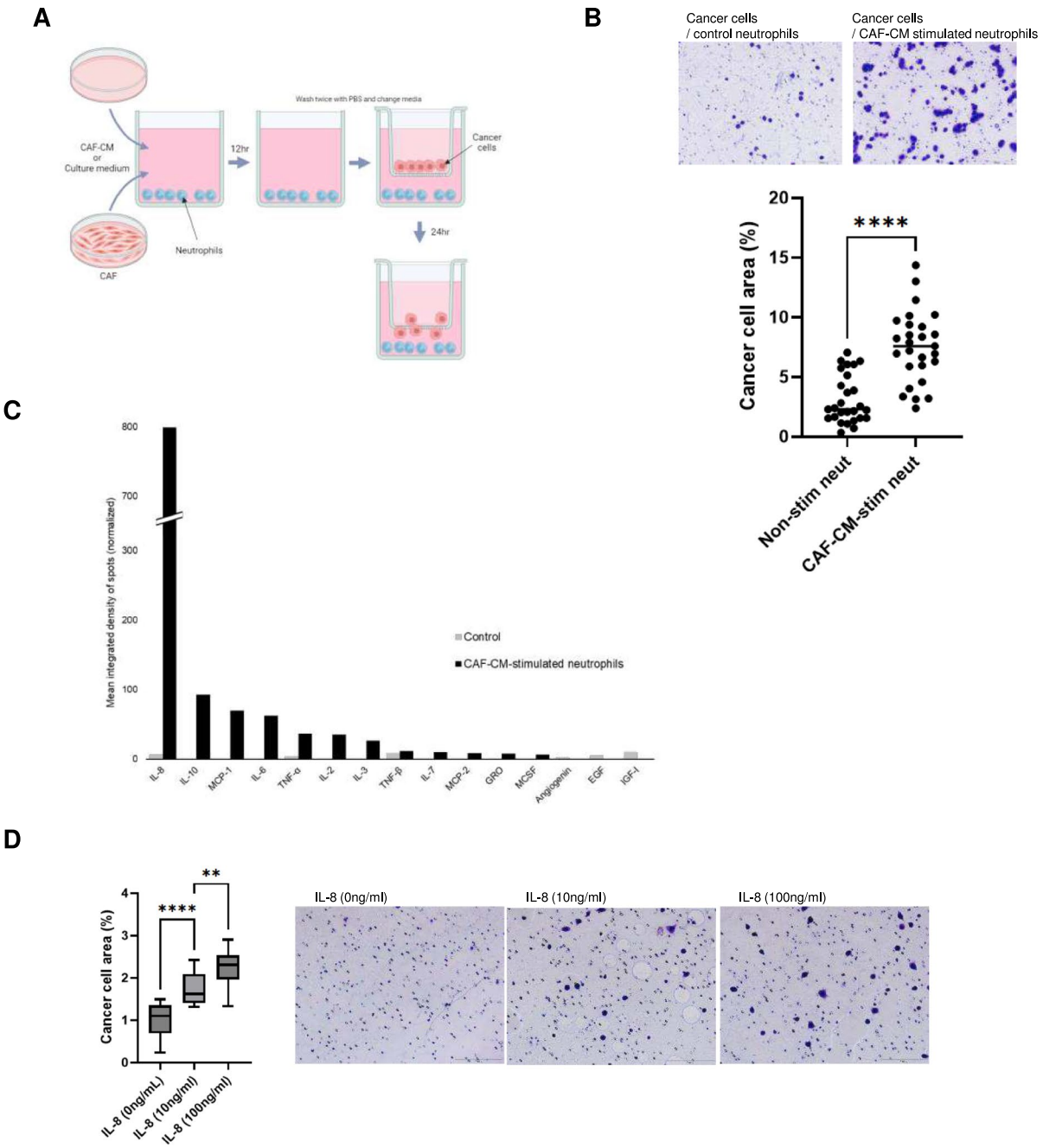

Fig. 3 IL-8 is secreted from CAF-CM-activated neutrophils. **A** Schema of the migration and invasion assays of human pancreatic cancer cells co-cultured with neutrophils. Migration and invasion assays of cancer cells were performed using neutrophils as inducers. These neutrophils were cultured in either culture medium or CAF-CM for 12 h and then washed twice with PBS before the assay. **B** CAF-CM-stimulated neutrophils significantly enhanced the migration of cancer cells compared to non-stimulated neutrophils. Mean ± SD is shown. Each dot represents the cancer cell area (%) per field (×100). Student's t-test was used. ****, $P < 0.0001$. **C** Cytokine array of neutrophil secretions. Elevated levels of IL-8, IL-10, IL-6, IL-7, and MCP-1 were found in the supernatants of CAF-CM-stimulated neutrophils, with IL-8 being the most increased. **D** Migration assay of MIA PaCa-2 cells using IL-8 as an inducer. Cancer cell migration was enhanced in a concentration-dependent manner by IL-8. Mean ± SD is shown. Each dot represents the number of cells per field (×100). One-way ANOVA with Tukey's test was used. **, $P < 0.005$, ***, $P = 0.0004$

adopted as an alternative marker for iCAFs in flow cytometry. Regarding the association between CAFs and neutrophils, this study showed that CAFs enhance neutrophil migration and that IL-8 is a chemokine for neutrophils [19]. Additionally, our data showed that CAFs prolong the life span of neutrophils, which would otherwise be

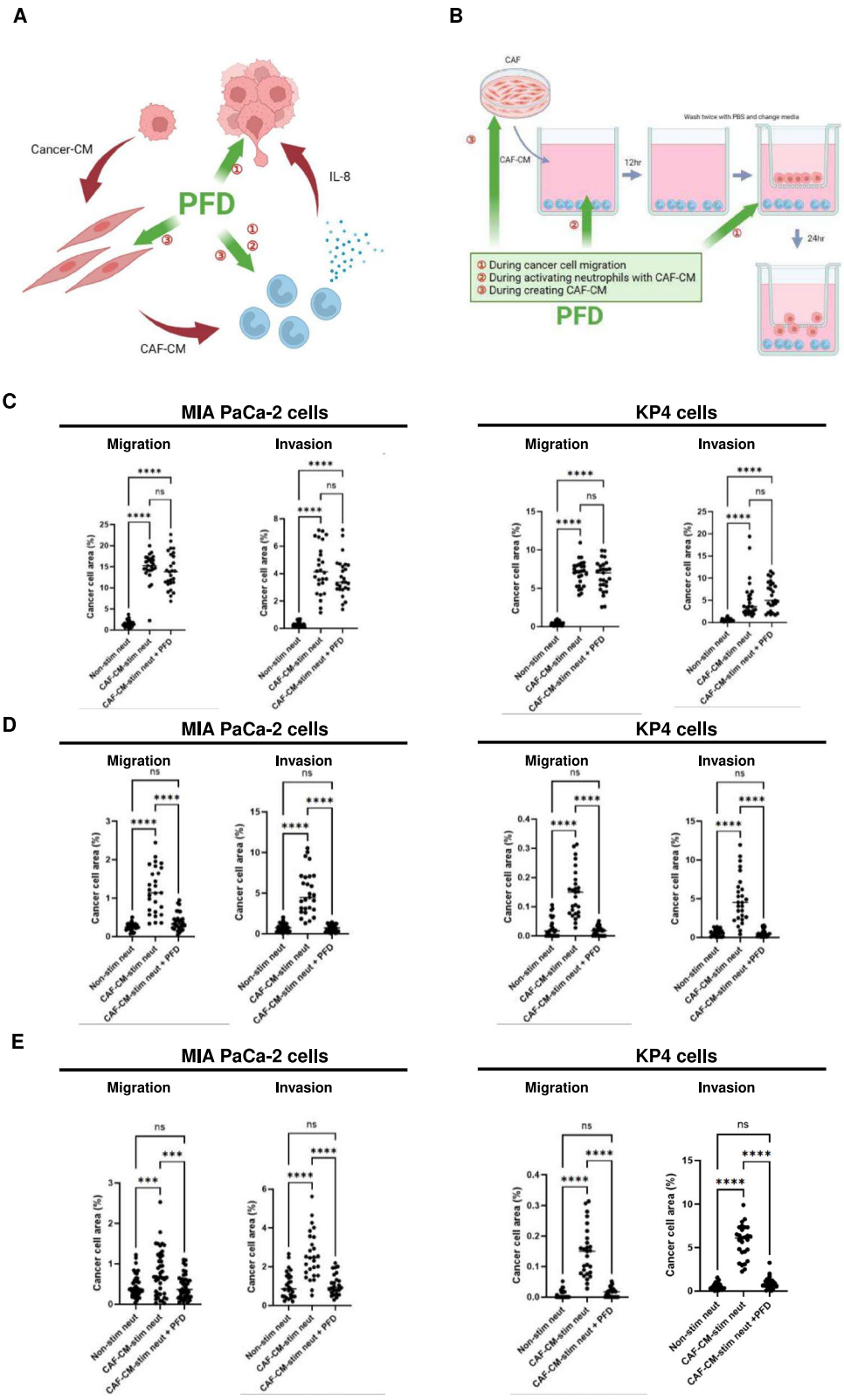

Fig. 4 Migration and invasion of human pancreatic cancer cells are enhanced by neutrophils, which are inhibited by PFD. **A** PFD was administered at three different points in the migration or invasion assay of cancer cells using neutrophils as inducers: during cancer cell migration or invasion, during neutrophil activation with CAF-CM, and during CAF-CM creation. **B** Schema of experimental procedure. **C** Administration of PFD during pancreatic cancer cell migration and invasion did not suppress the increased migration and invasion induced by CAF-CM-stimulated neutrophils. Mean ± SD is shown. Each dot represents the cancer cell area (%) per field (× 100). One-way ANOVA with Tukey's test was used. ****, $P < 0.0001$. **D** Administration of PFD during neutrophil activation with CAF-CM suppressed the increased migration and invasion of cancer cells induced by CAF-CM-stimulated neutrophils. Mean ± SD is shown. Each dot represents the cancer cell area (%) per field (× 100). One-way ANOVA with Tukey's test was used. ****, $P < 0.0001$. **E** Administration of PFD during CAF-CM creation from CAFs suppressed the increased migration and invasion of cancer cells by CAF-CM-stimulated neutrophils. Mean ± SD is shown. Each dot represents the cancer cell area (%) per field (× 100). One-way ANOVA with Tukey's test was used. ***, $P < 0.001$, ****, $P < 0.0001$

short-lived [39, 40]. Based on these findings, IL-8-expressing iCAFs may attract more neutrophils than myCAFs, and these activated neutrophils can survive longer, resulting in the co-localization of neutrophils with iCAFs in the TME. Other researchers have also reported that CAFs enhance neutrophil migration, suppress apoptosis, and activate

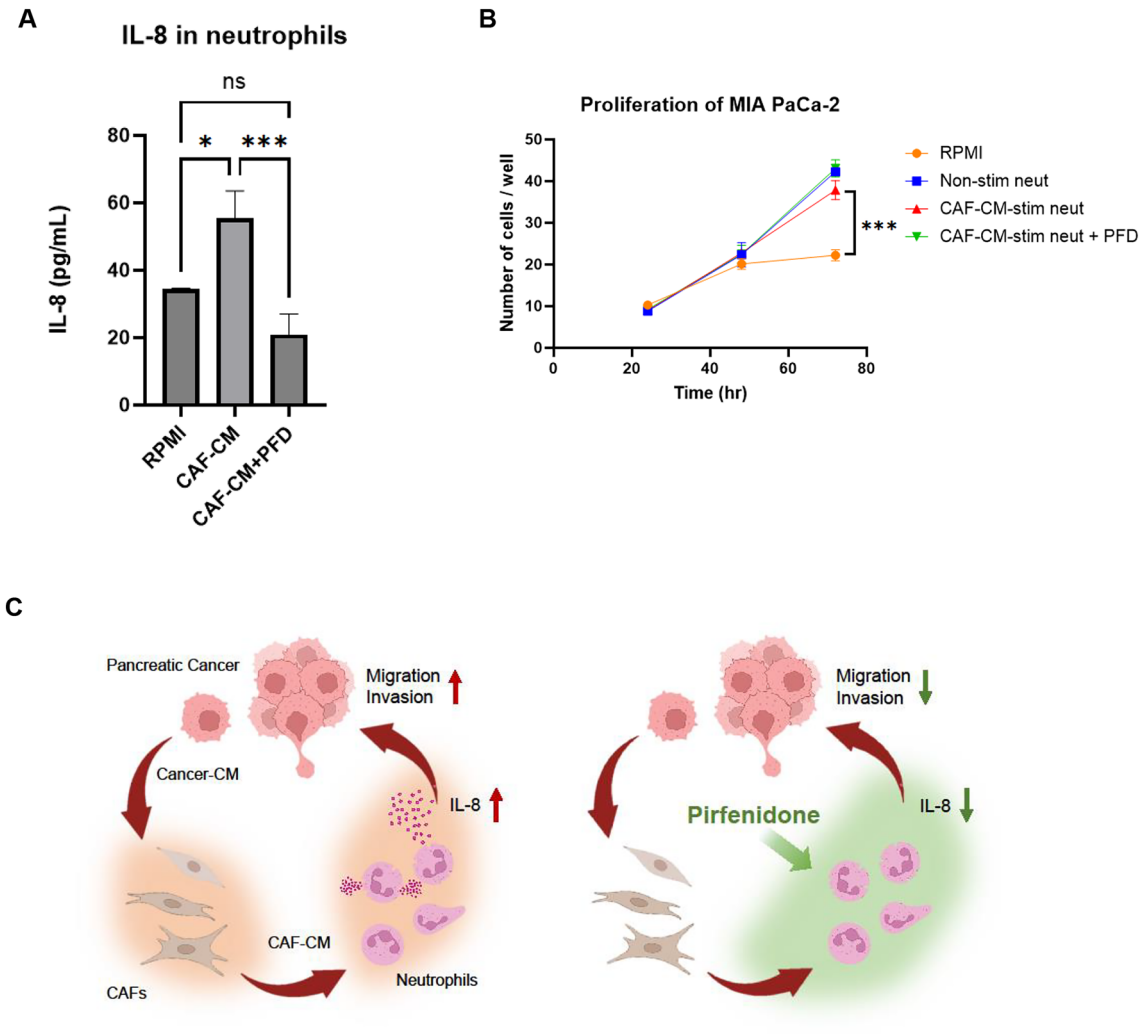

Fig. 5 IL-8 secreted by activated neutrophils is suppressed by PFD. **A** IL-8 secretion by neutrophils measured by ELISA. IL-8 secretion was significantly increased in neutrophils stimulated with CAF-CM compared to non-stimulated neutrophils. PFD administration during neutrophil activation with CAF-CM significantly suppressed IL-8 secretion. **B** Effect of secretions from activated neutrophils on cancer cell proliferation. The presence of neutrophils significantly increased MIA PaCa-2 proliferation. Mean ± SD is shown. Each dot represents the number of cells per well. Student's t-test was used. ***, $P < 0.001$. **C** Pancreatic cancer cells induce fibroblasts to become iCAFs, and iCAF-CM activates neutrophils, increasing their IL-8 secretion and enhancing the malignant traits of pancreatic cancer cells. Conversely, PFD acts on activated neutrophils, suppressing the malignant traits of pancreatic cancer cells. This figure was created with BioRender.com

neutrophils [23]. Neutrophils tend to coexist more with CAFs in cancer tissues, creating an environment where they can cooperatively influence cancer cells.

There are few reports on the function of CAF-affected neutrophils in cancer cells [22]. Our findings indicate that CAF-affected neutrophils enhance the malignancy of cancer cells. Each component of the cancer microenvironment is thought to be intricately intertwined, and numerous studies have shown the pro-tumor effects of CAFs [41]. Our data provide a novel insight that neutrophils affected by CAFs also directly exert pro-tumor effects on cancer cells. Further research is necessary to elucidate the precise mechanisms by which neutrophils affected by CAFs act on cancer cells and the action of CAFs on neutrophils.

To explore strategies for suppressing the pro-tumor effects of activated neutrophils, we examined the effect of PFD on neutrophils. Although PFD has already been clinically applied for treating idiopathic pulmonary fibrosis [42], it has been reported to inhibit desmoplasia in pancreatic cancer in preclinical models [26, 43]. Furthermore, neutrophils have been shown to increase IL-8 secretion when stimulated with LPS and PFD can suppress IL-8 secretion by neutrophils [44]. These data support our results that administering PFD to neutrophils activated by CAF-CM suppresses IL-8

secretion from neutrophils. Although the detailed mechanism has not been verified, it has been shown that in a rat model of prostatitis, PFD downregulated IL-8 by suppressing the phosphorylation of NF-κB [45]. Further research is needed to understand the mechanism by which PFD suppressed IL-8 secretion by neutrophils in our models.

A limitation of this study is the use of WI38 cells, which are fibroblasts derived from human fetal lung tissue rather than from pancreatic cancer. As such, these cells may not fully represent the CAFs present in the pancreatic tumor microenvironment. Therefore, the interpretation of the results presented in this study should consider this limitation.

Although our in vitro study demonstrated that targeting the CAF–neutrophil axis may have implications for cancer therapy, the feasibility of this approach in vivo remains to be verified. It is also possible that similar mechanisms operate in other tumor microenvironments beyond pancreatic cancer. Extending this research to other cancer types may reveal new therapeutic strategies applicable to various malignancies.

In conclusion, our results revealed the function of CAF-affected neutrophils in cancer cells within the cancer microenvironment, which has been largely unexplored. Considering the development of novel strategies to target the cancer microenvironment, anti-fibroblast agents such as PFD could be a potential approach to inhibit the tumor-promoting properties of activated neutrophils via the suppression of CAFs.

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Authors' contribution TY was involved in conceptualization, methodology, investigation, and writing—original draft. SKa was responsible for conceptualization, methodology, writing—reviewing and editing, supervision, and funding acquisition. SN, AT, MY, KS, YN, and SF conducted investigation. SKu, SKi, YK, and FT took part in supervision. KT assisted with resources. TO carried out pathological investigation. HT helped with methodology and supervision. TF participated in conceptualization, writing—reviewing and editing, and supervision.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Consent for publication Not applicable.

Ethics approval The study using clinical samples from patients with pancreatic cancer was approved by the institutional review board of Okayama University (No. 2406–025).

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