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Vimentin Expression in Tumor Microenvironment Predicts Survival in Pancreatic Ductal

Adenocarcinoma: Heterogeneity in Fibroblast Population

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Running head: CAF functional heterogeneity in PDAC

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Synopsis: The functional roles of cancer-associated fibroblast markers in pancreatic ductal adenocarcinoma remain unclear. We showed that these fibroblasts possess molecular and functional heterogeneity and their expression of vimentin without α -smooth muscle actin is associated with poor patient survival.

Abstract

Background: The tumor microenvironment, including cancer-associated fibroblasts (CAFs), plays various clinical roles in cancer growth. CAFs are a heterogeneous population and express a variety of mesenchymal markers. However, the clinical roles for CAFs expressing different markers in pancreatic ductal adenocarcinoma (PDAC) remain unknown.

Methods: We reviewed 67 resected PDAC patients who had not received preoperative therapy. Each primary tumor was analyzed for vimentin and α -smooth muscle actin (α -SMA) expression by immunohistochemical and dual immunofluorescence staining.

Results: There was no correlation between the percentage of cells expressing vimentin and α -SMA in the tumor stroma (Pearson's correlation coefficient: $r = 0.171$). Higher vimentin expression ($p = 0.018$) was associated with significantly shorter overall survival in PDAC patients. Using dual immunofluorescence staining, vimentin-positive CAFs were divided into two subpopulations: co-expression of α -SMA, and no co-expression of α -SMA. In PDAC, the level of co-expression had no effect on survival using univariate analysis (median survival time, 33.3 months for low co-expression vs. 18.2 months for high co-expression; log-rank, $p = 0.143$). However, multivariate analysis clarified that CAFs expressing vimentin alone was an independent predictor of poor survival ($p = 0.014$; hazard ratio, 2.305; 95% confidence interval, 1.181–4.497).

Conclusions: Vimentin-positive CAFs without co-expression of α -SMA were associated with poor survival in PDAC, and CAFs possessed molecular and functional heterogeneity in this disease.

Introduction

Cancer cells cannot survive without the coexistence of various types of stromal cells.¹ The tumor microenvironment is formed by cancer cells and stromal cells, which interact by direct contact or paracrine mechanisms via various cytokines and chemokines.²

Pancreatic ductal adenocarcinoma (PDAC), which has a poor prognosis and is one of the most lethal tumors³, generally possesses an intense stromal histopathology surrounding the cancer cells, which is called the desmoplastic stroma.⁴ The desmoplastic stroma occupies up to 80% of the entire cancer nodule in PDAC⁴ and fibroblasts are its major cellular component.⁵

Collectively, the term “cancer-associated fibroblasts” (CAFs) is used to describe all fibroblasts within a tumor that show certain morphological and functional features.⁶ CAFs represent a heterogeneous population.^{7,8} They express various mesenchymal markers, such as vimentin and α -smooth muscle actin (α -SMA)⁹, and they consist of multiple cell types, including resident fibroblasts, tumor cells that have undergone epithelial to mesenchymal transition, adipocytes, bone marrow-derived mesenchymal cells, and pancreatic stellate cells.^{10, 11} Therefore, a specific marker has not yet been identified for CAFs. It is well known that CAFs play important roles in many steps during tumor development and progression in various tumors and metastatic lymph nodes.^{12, 13} However, the functional differences in each CAF subpopulation in PDAC, as assessed by marker expression, remain unclear.

In the tumor, fibroblasts can exert physiological functions, altering the status of the cancer cells

through paracrine effects and direct contact.¹⁴ We hypothesized that subpopulations of CAFs, with their distinct marker expression representing CAF heterogeneity, harbor different roles in PDAC progression. The present study aimed to answer two questions by determining the degree of expression of vimentin and α -SMA in the tumor stroma of clinical samples: 1) how heterogeneous is the expression of vimentin and α -SMA, which are expressed in CAFs, in the tumor stroma of PDAC?, and 2) what CAF markers are associated with patient survival?

Methods

Patient selection

Between January 2009 and December 2016, we retrospectively searched the electronic medical records of Shiga University of Medical Science Hospital to identify patients who underwent pancreatectomy for PDAC. Patients receiving preoperative chemotherapy or radiotherapy were excluded from the study. Sixty-seven patients were enrolled in the study. Postoperative adjuvant chemotherapy was performed in 67% (45/67) of the patients. Clinical and pathological reports were reviewed for age, sex, tumor size, histological differentiation, invasion depth (pT), nodal status (pN), and distant metastasis (pM). The follow-up period was a minimum of 2 years or until death. The median follow-up period was 25.8 months (range 1.6–103.9 months) and 28 patients (41.8%) died during this period. The pTNM classification was applied according to the 7th TNM classification of the Union for International Cancer Control.¹⁵

The protocol of this study was approved by the ethics committee of Shiga University of Medical Science (registration No. 29-171). We provided the patients the opportunity to opt out; however, the need for obtaining informed consent was waived because of the study's retrospective design.

Pathological specimens

Surgical tissue blocks of the 67 PDAC patients were obtained. The specimens had previously been fixed in 10% formalin and embedded in paraffin. The tissue blocks were sliced into 4- μ m-thick sections and mounted on glass slides. Several 4- μ m-thick sections were cut from each paraffin block; one was stained with hematoxylin and eosin and examined by an experienced pathologist (K.M.) to verify the histopathological diagnosis. The others were subjected to immunohistochemical (IHC) staining and dual immunofluorescence (IF) staining for vimentin and α -SMA.

IHC staining procedure

For IHC staining, the slides were deparaffinized by xylene treatment, rehydrated in a graded ethanol series, and then heated in an electric kettle at 98°C with antigen retrieval solution (Immunosaver[®], Nisshin EM, Tokyo, Japan) for 45 min. Endogenous peroxidases were blocked by immersing sections in 3% H₂O₂ in 100% methanol for 10 min at 25°C, and the sections were subsequently incubated with a blocking reagent (Blocking One[®], Nacalai Tesque, Kyoto, Japan) at 25°C for 20 min. The tissue sections were incubated overnight at 4°C with an anti-vimentin antibody

(1:100, #5741S, Cell Signaling Technology, Inc., Danvers, MA, USA) or anti- α -SMA antibody (Clone 1A4, Dako, CA, USA). The following day, the slides were incubated with a secondary antibody (Simple Stain MAX PO[®], NICHIREI BIOSCIENCES INC., Tokyo, Japan) for 30 min at 25°C, and the antigen was visualized by 3,3'-diaminobenzidine staining (DAB[®], Dako, California, USA) for 15 min.

Dual IF staining procedure

Four-micrometer-thick sections were processed using the IHC protocol as above. Vimentin was detected with an anti-vimentin antibody (1:100, #5741S, Cell Signaling Technology, Inc.), and α -SMA was detected with an anti- α SMA antibody (1:100, #ab7817, Abcam plc, Cambridge, UK). The secondary antibodies employed were Alexa Fluor[®] 488 goat anti-mouse (A-11029) and Alexa Fluor[®] 594 goat anti-rabbit (A-11012) (Molecular Probes, Invitrogen, Carlsbad, CA, USA) antibodies at dilutions of 1:200. The tissues were incubated with secondary antibodies for 60 min. ProLong[®] Diamond Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (P36962, Molecular probes, Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) was used as the mounting agent. A BZ-X800 microscope (Keyence, Osaka, Japan) was used for image analysis.

Evaluation of immunostaining

Blinded microscopic evaluation of the slides was performed by an experienced pathologist (K.M.).

The pathologist, in consultation with the first author (H.M.), demarcated three areas in the central part of the tumor at a magnification of 200× for analyses. We selected the stromal area that did not contain cancer cells, but which had ductal structures, and also stained positive for hematoxylin and eosin. The percentage of stained stromal cells was assessed using Image J (National Institutes of Health, Bethesda, MD, USA) for the IHC staining. Furthermore, the images were captured in three different stromal areas which were randomly selected in the central part of the tumor at 200× magnification for the IF staining on microscope with BZ-X800 (Keyence, Osaka, Japan), and the quantification was performed using Hybrid Cell Count BZ-H4C analyzer software (Keyence, Osaka, Japan). The percentage of stained stromal cells area and the stromal cell number was calculated. Then, the average percentage of stained stromal cells and the average stromal cell number per tumor area (mm²) was calculated. The average stromal cell number in the tumor was calculated as [the average stromal cell number per tumor area (count/mm²)] × [maximum tumor area (mm²)].

Statistical analysis

To determine a suitable cut-off value for the expression of each marker, we used a receiver operating characteristic (ROC) curve analysis based on patient death at the median follow-up time, according to a previous report.¹⁶ A suitable cut-off value for the intensity of staining was defined as the point on the ROC curve closest to the (0,1) point (Supplementary figure 1). Then, this cut-off was used for the analysis of overall survival (OS) and recurrent free survival (RFS).

Categorical variables are expressed as numbers and percentages, whereas continuous variables are expressed as medians with interquartile ranges. Fisher's exact tests (for categorical variables) and Mann-Whitney U tests (for continuous variables) were used to compare factors. OS was calculated from the date of surgery to the date of the last follow-up or patient death. RFS was the time from curative surgery to the time of first tumor recurrence or the final follow-up date. Univariate survival analysis was performed according to the Kaplan-Meier method, and survival was compared using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards regression model. The level for significance was $p < 0.05$ and confidence intervals (CIs) were determined at the 95% level. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (version 2.13.0; The Foundation for Statistical Computing, Vienna, Austria).¹⁷

Results

Differential expression of CAF mesenchymal markers in PDAC patients

Figure 1a shows hematoxylin and eosin staining of a representative tumor and Figure 1b shows vimentin and α -SMA expression in the tumor stroma by IHC staining. The median vimentin expression in the tumor stroma was 16.4% (ranging from 5% to 28%), and the median α -SMA expression was 15.2% (ranging from 4% to 29%) (Figure 1c, 1d). There was no correlation between vimentin and α -SMA expression in the tumor stroma (Pearson's correlation coefficient: $r = 0.171$;

Figure 1e).

We performed dual IF staining for vimentin and α -SMA to confirm their co-expression or separate expression in individual fibroblasts. Figure 1f shows the co-expression of vimentin and α -SMA in individual cells, and 5.9% of cells (median, ranging from 1.3% to 11.8%) expressed both markers in the tumor stroma (Figure 1h). Figure 1g shows the fibroblasts expressing either vimentin (singleVim) or α -SMA (singleSMA). The median expression of singleVim and singleSMA was 9.3% (ranging from 1.9% to 23.0%) and 8.4% (ranging from 2.2% to 22.8%) of cells in the tumor stroma, respectively (Figure 1i, 1j). Considering only fibroblasts, 24.5% (median, ranging from 6.7% to 44.8%) co-expressed vimentin and α -SMA. In addition, the median CAF number in the tumor was 2,805,673 cells (ranging from 135,356 to 15,691,980 cells), and the median CAF number per tumor area was 4140 cells/mm² (ranging from 1,725 to 6,796 cells/mm²).

Vimentin expression in the PDAC stroma is associated with poor survival

The patients were classified according to the cut-off value of vimentin expression determined by ROC curve analysis. Forty-three (64.2%) and 24 (35.8%) patients were categorized in the low vimentin expression (Vim^{low}) and high vimentin expression (Vim^{high}) groups, respectively. Table 1 shows the characteristics and clinicopathological features of each group. There were no significant differences in tumor markers (Carcinoembryonic antigen, Carbohydrate antigen 19-9 (CA19-9), and Dupan-2), tumor size, TNM classification, and histological differentiation. With respect to RFS,

there was no significant difference between the Vim^{high} and Vim^{low} groups (median RFS time, 9.9 months vs. 13.9 months; log-rank, $p = 0.072$) (Figure 2a). In contrast, the OS of the Vim^{high} group was significantly shorter than that of the Vim^{low} group (median survival time (MST), 15.0 months vs. 33.3 months; log-rank, $p = 0.018$) (Figure 2b).

In contrast, there was no significant difference between the high and low α -SMA expression groups with respect to RFS (median RFS time, 17.5 months vs. 10.2 months; log-rank, $p = 0.138$) (Figure 2c). However, the OS of the high α -SMA expression group was significantly longer than that of the low α -SMA expression group (MST, 33.0 months vs. 19.0 months; log-rank, $p = 0.048$) (Figure 2d).

Furthermore, as to CAF number in the tumor, a high number group was associated with significantly shorter overall survival (OS) than a low number group (MST, 15.0 months vs. 35.8 months; log-rank, $p = 0.002$), and was associated with a shorter recurrent-free survival (RFS) (median RFS time, 10.1 months vs. 18.0 months; log-rank, $p = 0.077$). However, in regard to CAF number per tumor area, there was no significant difference between high and low number groups in OS (MST, 19.0 months vs. 29.9 months; log-rank, $p = 0.332$) and RFS (median RFS time, 10.2 months vs. 12.0 months; log-rank, $p = 0.312$) (Supplementary figure 2).

The subpopulation of CAFs co-expressing vimentin and α -SMA does not affect PDAC survival

Regarding CAFs with co-expression, 38 patients (56.7%) were categorized in the low

co-expression group, and 29 patients (43.3%) were assigned to the high co-expression group. There were no significant differences in patient characteristics between the two groups (Table 2). As shown in Figure 3a and 3b, there was no significant difference between the low and high co-expression group in RFS (median RFS time, 12.5 months vs. 11.1 months; log-rank, $p = 0.511$) and OS (MST, 33.3 months vs. 18.2 months; log-rank, $p = 0.143$).

As for single α -SMA expression, 33 patients (49.3%) were assigned to the low singleSMA expression (singleSMA^{low}) group, and 34 patients (50.7%) to the high singleSMA expression (singleSMA^{high}) group. Body mass index was significantly lower in the singleSMA^{low} group (20.7kg/m² vs. 22.4kg/m², $p = 0.045$), and platelet count was significantly lower (17.7/ μ l vs. 20.7/ μ l, $p = 0.019$) in the singleSMA^{low} group. There were no significant differences in tumor markers (CEA, CA19-9, and Dupan-2), tumor size, TNM classification, and histological differentiation. With regard to recurrent-free survival, there was no significant difference between the two groups (log-rank $p = 0.222$). However, the singleSMA^{low} group had a shorter overall survival compared to the singleSMA^{high} group (log-rank $p = 0.065$) (Supplementary figure 3).

Expression of vimentin without α -SMA in CAFs indicates poor survival in PDAC

Next, we analyzed another subpopulation, namely CAFs with singleVim expression. The patients were classified according to the cut-off value of singleVim expression determined by ROC curve analysis. Forty-five patients (67.2%) were categorized in the low singleVim expression

(singleVim^{low}) group and 22 patients (32.8%) were categorized in the high singleVim expression (singleVim^{high}) group. The clinicopathological characteristics of each group are shown in Table 2. CA19-9 ($p = 0.049$) and Dupan-2 ($p = 0.039$) levels were significantly higher in the singleVim^{high} group, and tumor size was significantly larger in the singleVim^{high} group ($p = 0.017$). There were no significant differences in TNM classification and histological differentiation, but more lymphatic invasion presented in the singleVim^{low} group ($p = 0.037$). As shown in Figure 3c and 3d, the singleVim^{high} group had a significantly shorter OS (MST, 15.0 months vs. 33.0 months; log-rank, $p = 0.014$) and RFS (median RFS time, 9.2 months vs. 15.1 months; log-rank, $p = 0.035$) than the singleVim^{low} group did.

Table 3 shows univariate and multivariate analyses of clinicopathological factors and CAF markers determined by dual IF staining. In the univariate analysis, the singleVim^{high} group (log-rank, $p = 0.014$) was significantly associated with poor survival. Multivariate analysis clarified that the singleVim^{high} group was the only independent predictor of poor survival ($p = 0.014$; HR, 2.305; 95% CI, 1.181–4.497).

Discussion

In the present study, we clarified two important clinical findings. First, CAFs constituted a heterogeneous population in human PDAC. There was no correlation between vimentin and α -SMA expression in CAFs, and CAFs not only expressed vimentin and α -SMA separately, but also

co-expressed them. Second, the frequency of CAFs only expressing vimentin was associated with poor survival in PDAC. PDAC is aggressive and lethal, and morbidity due to this disease is increasing worldwide.³ Surgery is the only curative therapy currently available. However, pancreatic resection is an aggressive therapy with a high complication rate.¹⁸⁻²⁰ Recently, two novel, combination chemotherapies have led to a major improvement in the survival of patients with PDAC.^{21, 22} However, these cytotoxic therapies also have severe side effects, and the survival benefits afforded by them are not ideal. PDAC is characterized as a desmoplastic mass, and treatment strategies targeting the tumor stroma are considered to have great potential in PDAC. The present study suggests that CAFs expressing vimentin alone have a tumor-promoting role in human clinical samples. If CAFs can be classified by function, a specific CAF subpopulation associated with poor outcomes might become a new target candidate for PDAC treatments.

First, our study showed that CAFs comprised a heterogeneous population in human PDAC, with cells not only co-expressing vimentin and α -SMA but also expressing vimentin and α -SMA separately. PDAC is accompanied by intense fibrosis of the tumor stroma, and CAFs are the major cellular components of this stroma.⁵ Previous reports have reviewed the multiple sources of CAFs, such as resident fibroblasts, tumor cells converted via epithelial to mesenchymal transition, adipocytes, bone marrow-derived mesenchymal cells, and pancreatic stellate cells^{10, 11}, and their molecular heterogeneity.^{7, 8} However, to the best of our knowledge, there have been no reports investigating the degree of co-expression of each CAF marker in clinical samples. The present study

showed that approximately 25% of CAFs in the tumor stroma co-express vimentin and α -SMA. It is necessary to examine multiple CAF markers to investigate CAF subpopulations and their clinical roles, because each specific marker cannot identify a particular CAF subtype and solitary CAF markers do not reflect all CAFs.^{7, 8}

Second, we demonstrated that the frequency of CAFs expressing vimentin alone was associated with poor survival in PDAC. Previous reports had associated survival with only one specific marker, for example α -SMA²³⁻²⁶ or fibroblast activated protein.^{27, 28} Regarding α -SMA, some reports demonstrated that high α -SMA expression is a poor prognostic factor in PDAC, namely that CAFs with α -SMA expression are tumor-promoting.^{23, 24} In contrast, depletion of α -SMA expression was shown to accelerate PDAC progression with shortened survival, namely that CAFs with α -SMA expression are tumor-suppressive.^{25, 26} Thus, the clinical role of α -SMA-expressing CAFs is still controversial in PDAC. Regarding vimentin expression, to the best of our knowledge, there have been no reports investigating its clinical role in the tumor stroma of PDAC.

Recently, defining the CAF subtype by the expression pattern of multiple markers was proposed.²⁹ However, we believe that we are the first to show the correlation between patient survival and expression of multiple CAF markers in the PDAC stroma. Because of the molecular heterogeneity of CAFs, single-stain IHC cannot evaluate the clinical impact of co-expression or single expression in individual fibroblasts. In the present study, stromal vimentin expression was associated with poor survival in PDAC using IHC single staining. Therefore, we divided the vimentin-expressing

population into two subpopulations determined by dual IF staining, one expressing both vimentin and α -SMA, and one expressing only vimentin. Our findings demonstrated that the subpopulation expressing only vimentin possessed a tumor-promoting role, while the subpopulation co-expressing vimentin and α -SMA was not related to PDAC survival. Recent report proposed classification of subtypes of fibroblast on the basis of function, such as tumor-restraining CAFs, tumor-promoting CAFs, secretory CAFs, and extracellular matrix-remodeling CAFs.⁹ Our results suggested that α -SMA positive CAFs have tumor-restraining role and vimentin positive CAFs have tumor-promoting role. Then, tumor-promoting role is canceled by tumor-restraining role in co-expressing CAFs. Thus, multiple staining is a critical method to clarify the CAF subpopulation. To the best of our knowledge, our study is the first to investigate the clinical role of CAF subpopulations by using human clinical samples of PDAC. However, it is difficult to determine how single vimentin expressing CAFs induce tumor-promoting environment in this study. Therefore, the function of single vimentin expressing CAFs should be investigated by the culture of CAF of human PDAC in the future.

The present study had several limitations. First, this was a retrospective study. Second, the number of enrolled patients was small. Third, previously processed clinical samples were used; hence, inconsistencies in sample processing conditions were possible, including formalin fixation time and time after resection to analysis, which may have influenced the staining results. However, we believe that the premise of association of multiple CAF markers and clinical prognosis, which we

demonstrated in this study, will be helpful for future CAF research.

In conclusion, we observed that CAFs possessed molecular and functional heterogeneity, and that the vimentin-positive CAFs without α -SMA co-expression were associated with poor survival in PDAC. If tumor-promoting CAFs can be identified selectively, these may be useful for the development of new therapeutic strategies for PDAC. Future novel studies are needed to clarify the effects of CAFs and the therapeutic potential of targeting them.

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Figure legends

Figure 1. α -SMA-expressing and vimentin-expressing CAFs are a heterogeneous population. (a) Loupe image of H&E-stained tumor tissue. (b) H&E, vimentin, and α -SMA staining in the tumor stroma. Vimentin and α -SMA are expressed in mesenchymal cells and are stained brown (200 \times magnification). (c, d) Distribution of the percentage of vimentin-expressing (c) and α -SMA-expressing (d) cells in the central part of the tumor. (e) Scatter diagram comparing the relationship between vimentin and α -SMA expression rate. Pearson's correlation coefficient (r) is 0.171. (f, g) Immunofluorescence staining of vimentin and α -SMA in the tumor stroma (400 \times magnification). Vimentin and α -SMA co-expressed in the same fibroblast (f). Fibroblasts expressing only α -SMA (white circle) and only vimentin (white dotted circle) existed in the tumor stroma (g). (h, i, j) Distribution of the percentage of cells co-expressing α -SMA and vimentin (h), vimentin alone (singleVim) (i), and α -SMA alone (singleSMA) (j) in the central part of the tumor over the 67 cases. H&E, hematoxylin and eosin; α -SMA, alpha-smooth muscle actin; CAFs, cancer-associated fibroblasts.

Figure 2. Survival outcome in PDAC with respect to single IHC staining of CAFs. (a, b) Kaplan-Meier analyses of RFS (a) and OS (b) according to vimentin expression in the central part of the tumor stroma. (c, d) Kaplan-Meier analyses of RFS (c) and OS (d) according to α -SMA expression in the central part of the tumor stroma.

CAF, cancer-associated fibroblasts; PDAC, pancreatic ductal adenocarcinoma; IHC,

immunohistochemical; RFS, recurrent free survival; OS, overall survival; α -SMA, alpha-smooth muscle actin.

Figure 3. Prognostic impact in PDAC of the CAF subpopulation determined by dual IF staining. (a, b) Kaplan-Meier analyses of RFS (a) and OS (b) according to the co-expression of vimentin and α -SMA in the central part of the tumor stroma determined by dual IF staining. (c, d) Kaplan-Meier analyses of RFS (c) and OS (d) according to the degree of vimentin single expression in the central part of the tumor stroma determined by dual IF staining.

CAFs, cancer-associated fibroblasts; IF, immunofluorescence; PDAC, pancreatic ductal adenocarcinoma; RFS, recurrent free survival; OS, overall survival; α -SMA, alpha-smooth muscle actin.

Supplementary Figure legends

Supplementary figure 1. Receiver operating characteristic (ROC) curve of Vimentin expression rate (a), co-expression rate of α -SMA and Vimentin (b), single α -SMA expression (c), and single Vimentin expression (d) for alive or dead analysis at the median follow-up time.

The areas under the ROC are 0.604 (95% CI, 0.448–0.760), 0.538 (95% CI, 0.396–0.681), 0.655 (95% CI, 0.524–0.786), and 0.598 (95% CI, 0.452–0.743), respectively.

Supplementary figure 2. Kaplan-Meier analyses of RFS (a) and OS (b), according to the CAF number in the tumor, and of RFS (c) and OS (d), according to the CAF number per tumor area, as determined by dual IF staining.

CAF, cancer-associated fibroblasts; RFS, recurrent-free survival; OS, overall survival; IF, immunofluorescence.

Supplementary figure 3. Kaplan-Meier analyses of RFS (a) and OS (b) according to the degree of α -SMA single expression in the central part of the tumor stroma, as determined by dual IF staining.

RFS, recurrent-free survival; OS, overall survival; α -SMA, alpha-smooth muscle actin; IF, immunofluorescence.

Table 1. Characteristics of the 67 patients with respect to vimentin expression

	Vimentin expression		<i>p</i> -value
	Vim ^{low} group (n=43)	Vim ^{high} group (n=24)	
Background			
Age, years	71.0 (62.0-75.5)	71 (63.8-75.3)	0.927
Gender, male / female	28 (65%) / 15 (35%)	14 (58%) / 10 (42%)	0.608
Body mass index, kg/m ²	22.2 (19.6-23.7)	20.5 (17.6-23.0)	0.166
Diabetes mellitus	11 (26%)	12 (50%)	0.061
Hypertension	15 (35%)	11 (46%)	0.438
Biliary drainage	13 (30%)	8 (33%)	0.791
Adjuvant chemotherapy	30 (70%)	15 (63%)	0.594
Preoperative findings			
Hemoglobin, g/dL	12.4 (11.2-13.8)	12.5 (11.5-13.7)	0.605
White blood cell count, / μ L	5000 (4400-6000)	5700 (4575-7325)	0.102
Platelet count, / μ L	19.4 (17.2-22.7)	18.2 (15.3-22.3)	0.403
Prothrombin activity, %	92.0 (86.5-100.0)	92.0 (78.8-101.0)	0.891
Albumin, g/dL	3.5 (3.4-3.9)	3.8 (3.5-3.9)	0.315
Aspartate aminotransferase, U/L	21 (18-41)	26 (20-46)	0.425
Alanine aminotransferase, U/L	23 (15-48)	30 (19-54)	0.605
Total bilirubin, g/dL	0.79 (0.56-1.27)	0.67 (0.50-1.06)	0.539
Creatinine, mg/dL	0.69 (0.59-0.96)	0.66 (0.58-0.75)	0.239
C-reactive protein, mg/dL	0.09 (0.05-0.31)	0.09 (0.06-0.19)	0.948
Hemoglobin A1c, %	6.0 (5.8-7.4)	7.7 (5.9-9.3)	0.088
Tumor markers			
Carcinoembryonic antigen, mg/dL	4.0 (2.4-6.2)	4.6 (3.8-6.6)	0.252
Carbohydrate antigen 19-9, mg/dL	98.0 (37.5-211.0)	202.0 (44.8-357.0)	0.182
Dupan-2, mg/dL	110.0 (25.0-450.0)	155.0 (57.8-732.5)	0.231
Operative findings			
Operation method			0.470
Pancreaticoduodenectomy	27 (63%)	13 (54%)	
Distal pancreatectomy	12 (28%)	6 (25%)	
Total pancreatectomy	4 (9%)	5 (21%)	

Operation time, min	488 (386-596)	508 (382-540)	0.761
Blood loss, mL	945 (603-1716)	928 (713-1566)	0.891
Histopathological findings			
Tumor size, mm	23.0 (20.5-29.5)	30.0 (24.0-36.3)	0.050
Histological differentiation			0.283
Well	13 (30%)	8 (33%)	
Moderately	22 (51%)	15 (63%)	
Poorly	8 (19%)	1 (4%)	
pT classification			0.788
pT1	2 (5%)	1 (4%)	
pT2	2 (5%)	0 (0%)	
pT3	39 (90%)	23 (96%)	
pT4	0 (0%)	0 (0%)	
pN classification			0.799
pN0	18 (42%)	9 (38%)	
pN1	25 (58%)	15 (62%)	
pM classification			0.533
pM0	41 (95%)	24 (100%)	
pM1	2 (5%)	0 (0%)	
pStage (UICC 7 th)			0.816
Stage IA	1 (%)	0 (0%)	
Stage IB	2 (%)	0 (0%)	
Stage IIA	14 (%)	9 (%)	
Stage IIB	24 (%)	15 (%)	
Stage III	0 (0%)	0 (0%)	
Stage IV	2 (%)	0 (0%)	
Lymphatic invasion			0.052
Negative	1 (2%)	4 (17%)	
Positive	42 (98%)	20 (83%)	
Venous invasion			1.000
Negative	2 (5%)	1 (4%)	
Positive	41 (95%)	23 (96%)	
Neural invasion			0.614

Negative	2 (5%)	2 (8%)
Positive	41 (95%)	22 (92%)

Data are expressed as medians with interquartile ranges or as numbers with percentages.

pT, invasion depth; pN, nodal status; pM, distant metastasis; UICC 7th, 7th TNM classification of the Union for International Cancer Control

Table 2. Characteristics of the 67 patients with respect to co-expression of vimentin and α -SMA, and single expression of vimentin

	Co-expression of vimentin and α -SMA			Single expression of vimentin		
	Low co-expression	High co-expression	<i>p</i> -value	singleVim ^{low}	singleVim ^{high}	<i>p</i> -value
	group (n=38)	group (n=29)		group (n=45)	group (n=22)	
Background						
Age, years	70 (63-76)	71 (62-75)	0.990	71 (62-75)	71 (65-76)	0.878
Gender, male / female	24 / 14 (63% / 37%)	18 / 11 (62% / 38%)	1.000	29 / 16 (64% / 36%)	13 / 9 (59% / 41%)	0.789
Body mass index, kg/m ²	22.2 (19.4-23.5)	20.9 (17.3-23.4)	0.519	21.8 (19.1-23.6)	21.0 (17.8-23.4)	0.435
Diabetes mellitus	12 (32%)	11 (38%)	0.613	14 (31%)	9 (41%)	0.584
Hypertension	13 (34%)	13 (45%)	0.452	16 (36%)	10 (45%)	0.594
Biliary drainage	10 (26%)	11 (38%)	0.426	13 (29%)	8 (36%)	0.582
Adjuvant chemotherapy	26 (68%)	19 (66%)	1.000	32 (71%)	13 (59%)	0.409
Preoperative findings						
Hemoglobin, g/dL	12.5 (11.2-13.7)	12.3 (11.3-14.0)	0.635	12.6 (11.3-13.8)	12.0 (11.2-13.2)	0.310
White blood cell count, / μ L	5100 (4600-6100)	5000 (4300-6700)	0.608	5000 (4400-6100)	5450 (4525-7375)	0.262
Platelet count, / μ L	18.8 (16.8-22.4)	19.7 (15.5-23.6)	0.995	19.4 (17.3-22.4)	18.2 (15.0-23.2)	0.431
Prothrombin activity, %	89.5 (83.3-98.0)	93.0 (87.0-103.0)	0.142	92.0 (87.0-101.0)	91.5 (78.3-100.8)	0.466
Albumin, g/dL	3.6 (3.4-4.0)	3.7 (3.4-3.9)	1.000	3.6 (3.4-3.9)	3.6 (3.4-3.9)	0.763
Aspartate aminotransferase, U/L	20.5 (17.3-42.0)	27.0 (19.0-45.0)	0.382	21.0 (16.0-37.0)	29.5 (20.5-47.3)	0.092
Alanine aminotransferase, U/L	23.0 (14.3-47.3)	34.0 (20.0-53.0)	0.285	22.0 (14.0-45.0)	32.5 (22.3-62.5)	0.118
Total bilirubin, g/dL	0.73 (0.52-1.38)	0.83 (0.58-1.10)	0.864	0.76 (0.53-1.11)	0.86 (0.59-1.25)	0.669

Creatinine, mg/dL	0.70 (0.59-0.90)	0.64 (0.56-0.81)	0.362	0.66 (0.58-0.90)	0.69 (0.58-0.81)	0.616
C-reactive protein, mg/dL	0.09 (0.05-0.29)	0.09 (0.05-0.30)	0.914	0.09 (0.05-0.30)	0.09 (0.06-0.20)	0.910
Hemoglobin A1c, %	6.3 (5.8-7.6)	7.0 (5.8-8.7)	0.434	6.3 (5.8-7.7)	6.7 (5.8-8.9)	0.596
Tumor markers						
Carcinoembryonic antigen, mg/dL	4.2 (2.6-6.6)	3.9 (3.4-6.0)	0.995	4.0 (2.5-6.0)	4.1 (3.8-7.2)	0.094
Carbohydrate antigen 19-9, mg/dL	102 (37-262)	99 (39-346)	0.737	94 (37-176)	258 (67-379)	0.049
Dupan-2, mg/dL	65 (25-750)	135 (60-425)	0.311	69 (25-230)	360 (71-905)	0.039
Operative findings						
Operation method			0.733			0.321
Pancreaticoduodenectomy	23 (61%)	17 (59%)		28 (62%)	12 (54%)	
Distal pancreatectomy	11 (29%)	7 (24%)		13 (29%)	5 (23%)	
Total pancreatectomy	4 (10%)	5 (17%)		4 (9%)	5 (23%)	
Operation time, min	491 (385-549)	500 (382-602)	0.826	484 (378-586)	516 (431-573)	0.556
Blood loss, mL	928 (383-1538)	948 (781-1805)	0.161	931 (736-1645)	994 (736-1595)	0.551
Histopathological findings						
Tumor size, mm	23.0 (19.0-31.0)	28.0 (24.0-36.0)	0.046	24.0 (21.0-28.0)	31.0 (23.0-40.0)	0.017
Histological differentiation			0.613			0.622
Well	10 (26%)	11 (38%)		15 (33%)	6 (27%)	
Moderately	22 (58%)	15 (52%)		23 (51%)	14 (64%)	
Poorly	6 (16%)	3 (10%)		7 (16%)	2 (9%)	
pT classification			1.000			1.000
pT1	2 (5%)	1 (3%)		2 (4%)	1 (5%)	

pT2	2 (5%)	0 (0%)		2 (4%)	0 (0%)
pT3	34 (90%)	28 (97%)		41 (92%)	21 (95%)
pT4	0 (0%)	0 (0%)		0 (0%)	0 (0%)
pN classification			0.804		0.428
pN0	16 (42%)	11 (38%)		20 (44%)	7 (32%)
pN1	22 (58%)	18 (62%)		25 (56%)	15 (68%)
pM classification			0.502		1.000
pM0	36 (95%)	29 (100%)		43 (96%)	22 (100%)
pM1	2 (5%)	0 (0%)		2 (4%)	0 (0%)
pStage (UICC 7 th)			0.580		0.730
Stage IA	1 (3%)	0 (0%)		1 (2%)	0 (0%)
Stage IB	2 (5%)	0 (0%)		2 (4%)	0 (0%)
Stage IIA	12 (32%)	11 (38%)		16 (36%)	7 (32%)
Stage IIB	21 (55%)	18 (62%)		24 (54%)	15 (68%)
Stage III	0 (0%)	0 (0%)		0 (0%)	0 (0%)
Stage IV	2 (5%)	0 (0%)		2 (4%)	0 (0%)
Lymphatic invasion			0.645		0.037
Negative	2 (5%)	3 (10%)		1 (2%)	4 (18%)
Positive	36 (95%)	26 (90%)		44 (98%)	18 (82%)
Venous invasion			1.000		1.000
Negative	2 (5%)	1 (3%)		2 (4%)	1 (5%)
Positive	36 (95%)	28 (97%)		43 (96%)	21 (95%)

Neural invasion			0.628		0.593
Negative	3 (8%)	1 (3%)		2 (4%)	2 (9%)
Positive	35 (92%)	28 (97%)		43 (96%)	20 (91%)

Data are expressed as medians with interquartile ranges or as numbers with percentages.

pT, invasion depth; pN, nodal status; pM, distant metastasis; UICC 7th, 7th TNM classification of the Union for International Cancer Control

Table 3. Univariate and multivariate analyses of conventional prognostic factors and expression of CAF markers determined by dual IF staining

Characteristics	Univariate analysis				Multivariate analysis		
	No. Cases	MST, months	2-year survival rate, %	<i>p</i> -value	Relative risk	95% confidence interval	<i>p</i> -value
Expression of CAF markers							
singleVim ^{low} group	45	33.0	63.1	0.014	1		
singleVim ^{high} group	22	15.0	30.3		2.305	1.181-4.497	0.014
Expression of CAF markers							
Low co-expression group	38	33.3	58.4	0.143			
High co-expression group	29	18.2	44.3				
Age							
< 65 years old	21	35.5	72.4	0.019	1		
≥ 65 years old	46	19.6	42.2		1.803	0.870-3.738	0.113
BMI							
< 22 kg/m ²	35	18.2	42	0.172			
≥ 22 kg/m ²	32	33.3	63				
Adjuvant chemotherapy							
Yes	45	26.0	56.1	0.129			
No	22	13.7	42				
Tumor size							
< 20 mm	13	NA	70	0.037	1		
≥ 20 mm	54	22.1	48.1		1.900	0.653-5.529	0.239
pT classification							
pT1 / pT2	5	48.2	100	0.117			
pT3 / pT4	62	20.2	47.3				
pN classification							
pN0	27	35.5	66.2	0.131			
pN1	40	19.6	41.5				
pM classification							
pM0	65	25.8	51.8	1.000			
pM1	2	NA	100.0				

Histological differentiation

well	21	29.9	57.4	0.871			
moderately	37	26.0	52.0				
poorly	9	16.6	37.5				
lymphatic invasion							
positive	62	25.9	53.1	0.834			
negative	5	15.2	40.0				
venous invasion							
positive	64	22.1	49.3	0.323			
negative	3	29.9	100.0				
neural invasion							
negative	4	NA	100.0	0.027	1		
positive	63	22.1	48.3		7.58E+07	0.000-NA	0.996

CAF, cancer-associated fibroblast; IF, immunofluorescence; MST, median survival time; NA, not available; Vim, vimentin; BMI, body mass index; pT, invasion depth; pN, nodal status; pM, distant metastasis
