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Research Paper

Stromal SPARC expression and patient survival after chemoradiation for non-resectable pancreatic adenocarcinoma

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Abbreviations: α-SMA, α-smooth muscle actin; CI, confidence interval; CRT, chemoradiation; CTGF, connective tissue growth factor; Gy, gray; IHC, immunohistochemistry; LAPC, locally advanced pancreatic cancer; OS, overall survival; PDAC, pancreatic ductal adenocarcinoma; PSC, pancreatic stellate cells; PTV, planning target volume; SPARC, secreted protein acidic and rich in cysteine; TGFβ, transforming growth factor-β

Key words: chemoradiation, pancreatic cancer, locally advanced, stroma, SPARC, pancreatic stellate cells

Purpose: Pancreatic stellate cells (PSC) drive desmoplasia in pancreatic cancer. Our study analyzed both tumor and PSC, since interaction of these cell types may promote tumor progression.

Results: SPARC was expressed predominantly in the peritumoral and distal stroma. SPARC in distal stroma correlated inversely with overall survival of the patients with LAPC (p = 0.013) with a relative hazard of 2.23 (95% CI, 1.05 to 4.72; p = 0.036). TGFβ1 in the tumor was also a negative prognostic factor (p = 0.03). Within the tumor cells, phospho-Akt correlated with TGFβ1, SPARC and survivin. Tumor phospho-Akt correlated with stroma phospho-Akt, tumor TGFβ1 correlated with stroma TGFβ1 and α-SMA, tumor survivin correlated with stroma survivin and distal SPARC. Within the stroma, SPARC and TGFβ1 correlated with α-SMA. Peritumoral SPARC correlated with distal SPARC. In vitro, SPARC was highly expressed in hPSC but not in Panc-1 cells. Exogenous SPARC did not change radiation resistance but increased the invasion of Panc-1 cells both in monoculture and in coculture with hPSC.

Experimental design: Immunohistochemical expression of SPARC, CTGF, TGFβ1, phospho-Akt, survivin and α-SMA was analyzed prior to chemoradiation in 58 locally advanced pancreatic cancer (LAPC) biopsy specimens. Fisher's exact test served to detect associations between tumor and PSC expression of markers. Kaplan-Meier analysis and multivariate analysis were used to evaluate the association of marker expression with overall survival. SPARC expression was analyzed in human pancreatic cancer cells (Panc-1) and in human PSC (hPSC) and the effect of SPARC on the invasion of Panc-1 cells was measured in monoculture or in coculture with hPSC.

Conclusions: Our hypothesis of a detrimental effect of PSC on patient survival in LAPC after chemoradiation is supported by the inverse correlation of SPARC in distal stromal cells with patients survival. Furthermore in vitro data indicate that paracrine SPARC from PSC increases the invasion of pancreatic cancer cells.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) ranks fourth among cancer related death in the United States and other industrialized countries. Resection is feasible in 15–20% of all patients and remains the only potentially curative treatment option.[1] Chemoradiation plays an important role in the treatment of patients with non-metastatic disease and is the most effective treatment of locally advanced pancreatic cancer (LAPC) (reviewed in ref. 2). Chemoradiation is also given in adjuvant situations and is currently being tested for the neoadjuvant approach.

Pancreatic ductal adenocarcinoma is characterized by a strong desmoplastic reaction.[3] Radiation to the pancreas increases the desmoplastic reaction of the organ as reported after neoadjuvant therapy.[4] Therefore, the response of the tumor to radiation might be determined by both the reaction of cancer cells and of stromal cells. Interactions between cancer cells and the surrounding stromal fibroblasts have been suggested to play a critical role in tumor invasion and metastasis (reviewed in ref. 5). To date very little is known about this tumor stoma interaction and whether the desmoplastic reaction is an obstacle or supportive for tumor cure. A number of reports suggest that desmoplastic reaction in pancreatic carcinomas promotes the malignant phenotype of cancer cells to the detriment to the host.[6-8] Pancreatic stellate cells (PSC) have recently been described as a stromal component in the pancreas and were identified to be responsible for the development of pancreatic fibrosis after various kinds of pancreatic insults.[9] Quiescent PSC are distinguished from normal fibroblasts in that they contain vitamin A storing fat

droplets. Upon activation the fat droplets disappear as the cells differentiates into a α-SMA expressing myofibroblast like phenotype. Activated PSC synthesize extracellular matrix proteins and are involved in repair of pancreatic injury, however prolonged PSC activation as seen in chronic pancreatitis and in pancreatic cancer is implicated in the generation of pancreatic fibrosis and tumor desmoplasia.[10,11] However, data on the significance of fibrosis to tumor progression are conflicting since some reports suggested that the effect of fibrosis could restrict tumor growth.[4,12]

The cellular and molecular events initiating and driving desmoplasia in PDAC are incompletely understood at the moment.[13] In this study we used biopsies from patients who had chemoradiation for LAPC to investigate a number of markers, which are known to play a role in the radiation response of tumors and/or normal tissue:

(1) SPARC (secreted protein acidic and rich in cysteine, or osteonectin/BM40) is a member of the family of matricellular glycoproteins (SPARC, thrombospondin 1/2, tenascin C/X and osteopontin), which modulate interactions between cells and the extracellular matrix.[14] The pathways involved in SPARC signaling are not well established, however SPARC has been demonstrated to be a marker of poor prognosis in different cancer types (reviewed in ref. 15) including pancreatic cancer.[7,16,17] In vitro SPARC has been shown to increase growth and to promote survival and invasion of cancer cells.[18-20] However, the data on the significance of SPARC on tumor growth are conflicting as xenograft and orthotopic tumors in $SPARC^{-/-}$ mice have been demonstrated to grow faster than in the wildtype animals.[21,22]

SPARC is produced at high levels in many types of cancers, especially by cells associated with tumor stroma and vasculature. In pancreatic cancer SPARC is highly expressed at the interface between the tumor and the stroma, which makes it particularly interesting in studies of the tumor stroma interaction.[7,23] SPARC expression has recently been associated with shortened survival after pancreatic cancer resection.[7] However to date, there are no reports on the significance of SPARC in patients with conservative treatment.

(2) The phosphoinositide 3-kinase (PI3-K)/Akt pathway is implicated in increased radiation survival in a number of human malignancies.[24-26] In addition, many reports on PSC and the closely related hepatic stellate cells have described the significance of Akt[27-31] and a number of activators of Akt in stellate cells.[32] Therefore, activated Akt could represent a potential common target.

(3) The antiapoptotic protein survivin plays an important role in the malignant progression of many tumor types and cooperates with Akt.[24] Survivin mediates radiation resistance[33] and is differentially expressed in activated hepatic stellate cells.[34]

(4) TGFβ1 has been closely correlated with both radiation induced fibrosis (reviewed in ref. 35) and the desmoplastic reaction in pancreatic carcinoma. Moreover, the TGFβ pathway is frequently altered in pancreatic carcinoma through mutations or methylation of DPC4/smad4 or overexpression of the TGFβ ligand.[36]

(5) CTGF (also termed CCN2) is a downstream molecule of TGFβ1, which is involved in cell growth, migration and survival and is mainly detected in the stromal cells of pancreatic cancer.[12,37,38]

The aim of this study was to investigate the prognostic significance of molecular markers with a reported or suspected function in the tumor and the stroma in patients with LAPC who received CRT. Furthermore, the expression of these markers in the respective compartments was assessed in order to detect associations with clinical and other molecular factors. To detect potential mechanisms that lead to the clinical influence of stromal SPARC expression, we analyzed the biological properties of this matricellular protein and detected SPARC mediated invasion predominantly in cocultures of pancreatic cancer cells with pancreatic stellate cells in comparison to pancreatic cancer cells in monoculture. However, SPARC did not influence radiation sensitivity in vitro.

Table 1 **Primary antibodies used for immunohistochemical analysis**

Antigen specificity	Type	Dilution	Antigen retrieval	Vendor
CK8	M monoclonal	1:200	MW	Immunotech
Vimentin	M monoclonal	1:200	MW	DakoCytomation
α-SMA	M monoclonal	1:300	S	DakoCytomation
p-Akt Ser473	R monoclonal	1:50	MW	Cell Signaling
CTGF	R monoclonal	1:250	CSA	(Dr. Brigstock)
SPARC	M monoclonal	1:250	S	Zymed
TGFβ1	M monoclonal	1:10	Trypsin	Abcam
Survivin	R polyclonal	1:250	S	R&D Systems

CK8, cytokeratin 8; M, mouse; R, rabbit; CSA, catalyzed-signal-amplification^{TM} (Dako, Hamburg Germany); MW, microwave heating; S, steamer heating.

Table 2 **Patient characteristics of 49 patients without resection after CRT**

cT	cN	Grading	Gender
T1: 1 (2%)	N0: 22 (45%)	G1: 1 (2%)	m: 27 (55%)
T2: 8 (16%)	N1: 27 (55%)	G2: 34 (69%)	f: 22 (45%)
T3: 14 (29%)		G3: 14 (29%)	
T4: 26 (53%)		G4: -	

cN, clinical N category; cT, clinical T category, f, female; m, male.

Results

Patient characteristics. Sufficient paraffinized tissue samples for immuno-histochemical evaluation were available from 58 patients. All of these samples were used for expression analysis. However, nine patients subsequently underwent complete resection after CRT and these patients therefore were excluded from survival analysis. The median survival of the 49 patients who did not undergo resection after CRT was 10 months and all patients of this group had died at the time of analysis. Overall survival (OS) was similar to previous survival figures reported for patients after chemoradiation.[2] The distribution of patient characteristics is shown in Table 2.

Immunophenotype of the tumors. Typical ductal structures showed strong expression of CK8 (Fig. 1A) and stromal cells showed strong expression of the typical mesenchymal marker vimentin (Fig. 1B). This facilitated the discrimination of the tumor and stroma compartments. As expected, α-SMA was exclusively expressed in stromal cells and high expression levels were observed in the majority of the stroma of all tumor specimens (Fig. 1C and D and Table 3). This demonstrates the presence of activated stellate cells in these tumor samples.

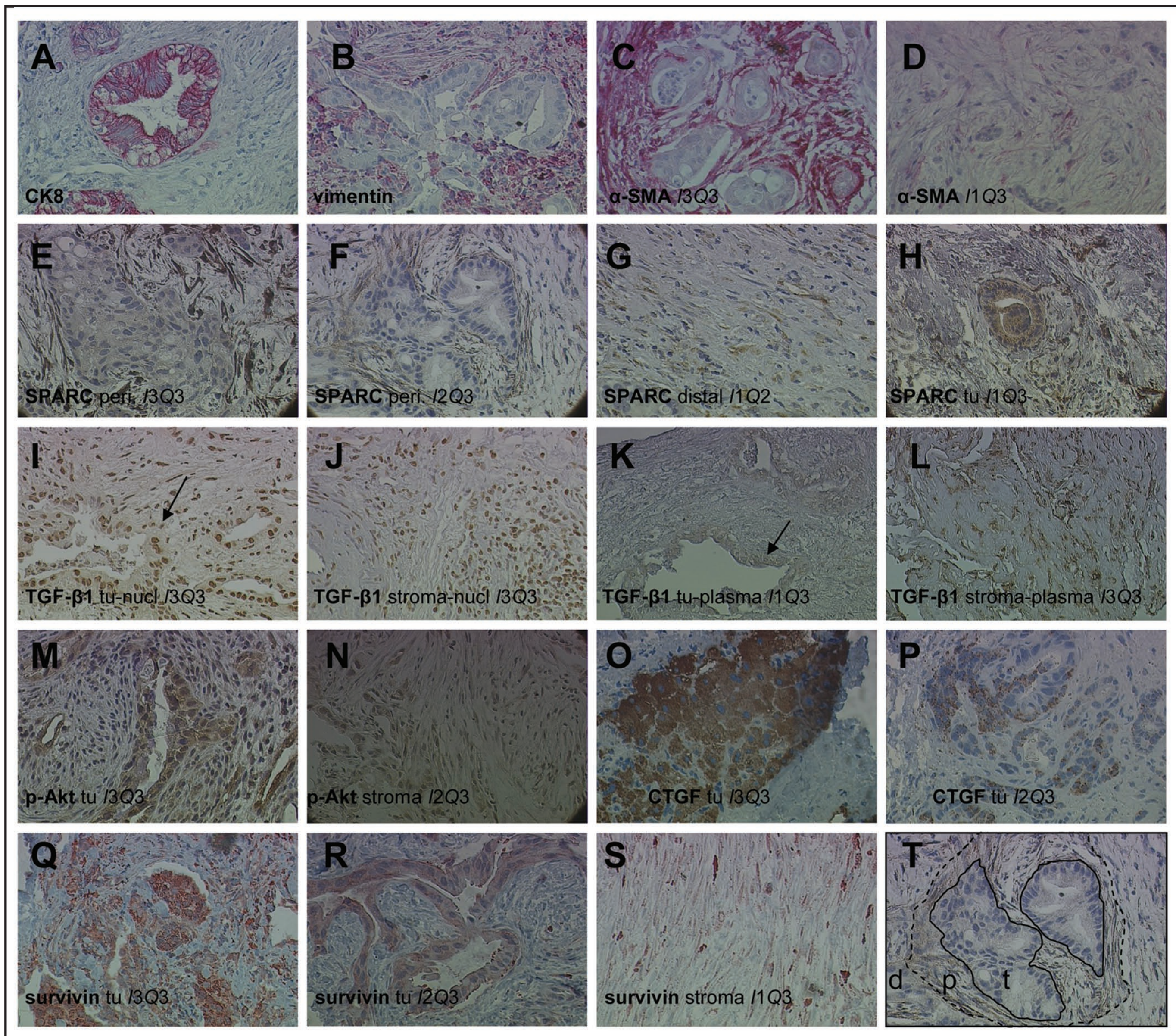

Figure 1. (A–S) Immunostaining of pancreatic tumors with the indicated antibodies and scoring values for intensities (I) and quantities (Q). (T) Schematic illustration of the tumor (t), the peritumoral stroma (p) and of the distal stroma (d). The pictures are x200 magnifications. Abbreviations: α-SMA, α-smooth muscle actin; CK8, cytokeratin 8; CTGF, connective tissue growth factor; distal, distal stroma; nucl, nuclear; p-Akt, phospho-Akt; peri., peritumoral stroma; plasma, cytoplasmic; TGFβ1, tumor growth factor β1; tu, tumor.

Correlation of markers in the tumor (Table 4A). Activated Akt (phospho-Akt) was observed in 84% of the tumor cells (Fig. 1M) and correlated (r_s = Spearman's rank correlation coefficient) with the profibrotic markers TGFβ1 (Fig. 1K, $r_s = 0.468$) and SPARC in tumor cells (Fig. 1H, $r_s = 0.211$). Phospho-Akt also correlated with survivin in the tumor (Fig. 1Q and R).

Correlation of tumor markers with stroma markers (Table 4B). Phospho-Akt was observed in α-SMA positive stroma cells (Fig. 1N) only if it was also positive in tumor cells (Fig. 1M). Stroma and tumor survivin were equally related (Fig. 1Q–S, $r_s = 0.491$). All patients with cytoplasmic tumor TGFβ1 expression (Fig. 1K) also had cytoplasmic stromal TGFβ expression in α-SMA positive cells (Fig. 1L, $r_s = 0.221$). Additionally, cytoplasmic TGFβ1 expression in tumor cells correlated significantly with stroma α-SMA ($r_s = 0.172$) and phospho-Akt ($r_s = 0.392$).

Correlation of markers in the stroma (Table 4C). Fusiform stroma cells (stellate cells) that were positive for α-SMA correlated significantly with TGFβ1 ($r_s = 0.176$) and SPARC (Fig. 1E–H, $r_s = 0.305$) in the stroma. In the stellate cells SPARC expression was seen both in peritumoral and distal regions ($r_s = 0.424$). Expression of SPARC was most intensive in the cytoplasm of peritumoral cells (Fig. 1E, F and T). Two thirds of the distal stellate cells also expressed SPARC (Fig. 1G and T). Distal SPARC was correlated with cytoplasmic survivin staining in the stroma (Fig. 1S, $r_s = 0.341$). SPARC expression status was only associated inversely with T-stage ($r_s = -0.459$) and no other prognostic clinical parameters (N status, grading, age; Table 4B).

Table 3 **Summary of immunohistochemical quantification in the entire cohort of 58 patients**

Antigen specificity	Tumor		Stroma	
	Intensity	Quantity	Intensity	Quantity
α-SMA	-	-	2.15 (0.62)	2.03 (0.77)
IxQ		-	4.58 (2.45)	
phospho-Akt	1.23 (0.78)	2.13 (1.1)	0.29 (0.64)	0.35 (0.80)
IxQ	3.02 (2.16)		0.53 (1.27)	
CTGF	0.80 (0.92)	1 (1.1)	0.25 (0.58)	0.21 (0.49)
IxQ	1.61 (2.33)		0.32 (0.86)	
SPARC	0.17 (0.38)	0.41 (0.94)	Perit. 1.43 (1.06) Distal 0.41 (0.50)	Perit. 1.33 (1.10) Distal 0.80 (1.10)
IxQ	0.41 (0.94)		Perit.: 2.85 (2.83) Distal: 0.78 (1.10)	
Survivin	1.40 (0.98)	2.27 (1.19)	0.62 (0.87)	(0.85)
IxQ	3.92 (2.96)		1.00 (1.70)	
TGFβ1 cytoplasmic	0.33 (0.51)	0.72 (1.16)	0.30 (0.60)	0.37 (0.73)
IxQ	0.76 (1.23)		0.54 (1.33)	
TGFβ1 nuclear	0.89 (1.04)	1.09 (1.28)	1.35 (0.97)	1.57 (1.22)
IxQ	2.15 (2.86)		3.13 (2.89)	

Abbreviations: perit.= peritumoral stroma. Numbers indicate means (standard deviations in brackets) of scores of intensities, quantities and products of intensities x quantities (score range 0–3).

Factors associated with prognosis in univariate and multivariate analysis. None of the classical risk factors had statistical significance for overall survival (T-stage, N-stage, Grading, age) except resection after CRT (p = 0.005) and resected patients (n = 9) were excluded for further survival analysis to avoid bias as a consequence of therapy. Patients whose fibroblasts distal to the tumor cells expressed SPARC had a significantly worse prognosis than those whose tumor stroma did not express SPARC (p = 0.013; Fig. 2A). Median OS time (10.2 months) as well as 10- and 15-month OS rates (52% and 35%, respectively) for patients without stromal SPARC expression were increased compared to patients whose stroma did express SPARC (OS 7.6 months, 29% and 12% survival at 10 and 15 months, respectively). Peritumoral SPARC expression was not of prognostic significance for OS in this study (p = 0.161). High cytoplasmic expression of TGFβ1 in the tumor was also a significant negative prognostic factor (p = 0.03, Fig. 2D). No significance was observed for the rest of the included factors but distinct trends for shorter survival were found for high phospho-Akt in the tumor (p = 0.057, Fig. 2B) and for low CTGF in the tumor (p = 0.055, Fig. 2C). Kaplan-Meier plots of molecular markers with a strong prognostic trend are shown in Figure 2B and C. The variables as shown in Figure 2A–D were included into the multivariate analysis. The adjusted Cox proportional hazards regression for patients whose distal cancer stroma expressed SPARC was 2.23 (95% confidence interval [CI], 1.05 to 4.72; p = 0.036) compared with patients whose distal stroma did not express SPARC.

SPARC has no influence on radiation sensitivity but promotes the invasiveness of pancreatic tumor cells in vitro. In order to test the influence of SPARC on pancreatic cancer cells we first analyzed the basal expression of SPARC in different human pancreatic cancer cell lines and in human pancreatic stellate cells (hPSC). The expression of SPARC was low in Panc-1 cells and below Western blot detection in PSN-1 and MiaPaCa-2, in comparison the high expression seen in hPSC (Fig. 3A). Furthermore, irradiation with 4 Gy did not alter the SPARC expression in either of the cell lines (Fig. 3A). The effect of endogenously expressed stromal SPARC on tumor cells was investigated by directly coculturing hPSC and Panc-1 cells. SPARC expression in stellate cells was knocked-down by siRNA and verified by Western blotting (Fig. 3B, insert) prior to coculture with Panc-1. The hPSC SPARC expression did not alter the radiosensitivity of Panc-1 cells after clinically relevant doses of 2 Gy and 4 Gy in a clonogenic survival assay (Fig. 3B). Likewise, the addition of recombinant SPARC did not modify the clonogenic survival of Panc-1 and PSN-1 cells in monoculture after irradiation with doses from 1–6 Gy (data not shown). We then tested the influence of exogenous SPARC on the invasiveness of Panc-1 cells. Interestingly, recombinant SPARC increased invasion of Panc-1 cocultured with either Panc-1 or with hPSC (Fig. 3C). Furthermore, in the absence of exogenous SPARC, coculture of Panc-1 with the SPARC expressing hPSC cells resulted in markedly increased invasion of Panc-1 cells. Exposure to 4 Gy irradiation did not enhance the invasiveness of Panc-1 cells in response to exogenous SPARC or hPSC coculture (data not shown).

Discussion

We here report SPARC expression in the tumor stroma of non-resectable pancreatic carcinoma as a strong prognostic marker for OS (p = 0.013) of patients who were treated with CRT. The hazard ratio on survival for patients with high compared to low expression of SPARC in fibroblasts of tumor stroma was 2.23 (95% CI, 1.05 to 4.72; p = 0.036). Preclinical analyses showed increased invasion of pancreatic cancer cells after treatment with SPARC in the absence of an effect on radiation resistance. We therefore postulate that increased invasiveness of pancreatic tumors overexpressing SPARC is responsible for its life-shortening effects.

While in this study conventional prognostic markers for resectable patients (e.g., grading, T-category or N-category) had no obvious prognostic influence or trend on OS, three other molecular markers, TGFβ1 (p = 0.03), CTGF (p = 0.055) and phospho-Akt (p = 0.057) portended a distinctive trend on OS. A statistical limitation of this

Table 4 **Association of tumor and stroma variables**

(A)

Tumor/Tumor	p-Akt	CTGF	SPARC	survivin
p-Akt	-	-	0.013 d (0.211)	0.013 d (0.286*)
TGFβ1	0.002 d (0.468**)	-	-	0.008 d (0.277*)
Grade	-	0.018 d (0.179)	-	-
N-stage	0.032 i (-0.224)	-	-	0.011 i (-0.272)

(B)

Tumor/Stroma	α-SMA	p-Akt	CTGF	SPARC peritumoral	SPARC distal	survivin	TGFβ1
p-Akt	-	<0.001 d (0.517**)	-	-	-	-	-
survivin	0.002 d (0.331*)	-	-	-	0.032 d (0.375*)	0.008 d (0.491**)	-
TGFβ1	0.04 d (0.172)	0.006 d (0.392**)	-	-	-	-	0.015 d (0.221)
T-stage	-	-	-	0.013 i (-0.459**)	-	-	-
N-stage	-	-	0.017 i (-0.354**)	-	-	-	-

(C)

Stroma/Stroma	α-SMA	SPARC distal	TGFβ1
α-SMA	-	0.016 d (0.305*)	0.04 d (0.176)
SPARC peritumoral	0.022 d (0.239)	0.008 d (0.424**)	-
Survivin	-	0.034 d (0.341*)	0.019 d (0.409*)

p-values from two-sided Fisher's exact test in the entire cohort of 58 patients. Spearman's rank correlation coefficient in brackets (*= correlation is significant at the 0.05 level or ** at the 0.01 level two-tailed), d = direct correlation, i = inverse correlation.

study is the exploratory nature of the analyses and multiple testing leads to an enhanced rate of type I errors. We chose not to correct the critical value of significance (e.g., Bonferroni correction) in order to avoid a high rate of type II errors. Our finding of the prognostic influence of SPARC is in good congruence with a very recent report on the prognosis of patients with pancreatic carcinoma who had undergone Whipple's procedure.[7] In addition, patients with resected ampullary cancer and stromal overexpression of SPARC lived significantly shorter than those with low expression of SPARC and at the same time had more nodal metastases.[44] Interestingly, the comparison of SPARC mRNA in pancreatic cancer with that in cancer of the papilla of Vater showed overexpression of SPARC in pancreatic carcinoma but not in cancer of the papilla.[17]

Conflicting results from SPARC null mice which displayed enhanced tumor growth of murine lung carcinoma[45] stipulated us to perform preclinical experiments to improve our understanding of the mechanisms of peritumoral SPARC in pancreatic carcinoma after radiation. We were especially interested if SPARC would modulate the radiotherapeutic response as Tai and coworkers reported enhanced sensitivity to radiation and chemotherapy in the presence of SPARC in a colorectal cancer xenograft model.[46] This group reported a higher rate of tumor response after 100 Gy single dose radiation in the SPARC overexpressing tumors. However, there are several limitations to these results as (1) SPARC is overexpressed in the tumor cells themselves and not in stromal cells (2) the radiation dose is far from being clinically relevant and (3) the statistical significance was only reported using a t-test after three weeks of radiation whereas the curves in the Kaplan-Meier plot overlap 10 weeks after radiation. We could not confirm an influence of SPARC on clonogenic radiation survival (Fig. 3B) neither with exogenous SPARC nor after siRNA knock-down of SPARC in hPSC cocultured with pancreatic tumor cells. Therefore we conclude that SPARC has

no influence on radiation survival. However, we observed increased invasiveness of pancreatic tumor cells in the presence of exogenous SPARC without and even more with PSCs. In agreement with out observations, SPARC was shown to promote invasion and migration in pancreatic cancer monoculture,[18] brain tumors, breast cancer and prostate cancer (reviewed in ref. 15). In line with our observation and Infante's in pancreatic carcinoma,[7] the predominant expression in the peritumoral stroma was also described in breast cancer[1], ovarian cancer[2,3] and non-small cell lung cancer.[4] Taken together, the clinical and preclinical observations stress the importance of the tumor stroma in pancreatic cancer, which is a pathohistological hallmark of the disease.[3] Furthermore, our observation of the association of SPARC and α-SMA in the stroma supports the hypothesis that SPARC plays a role in the context of activated PSC.

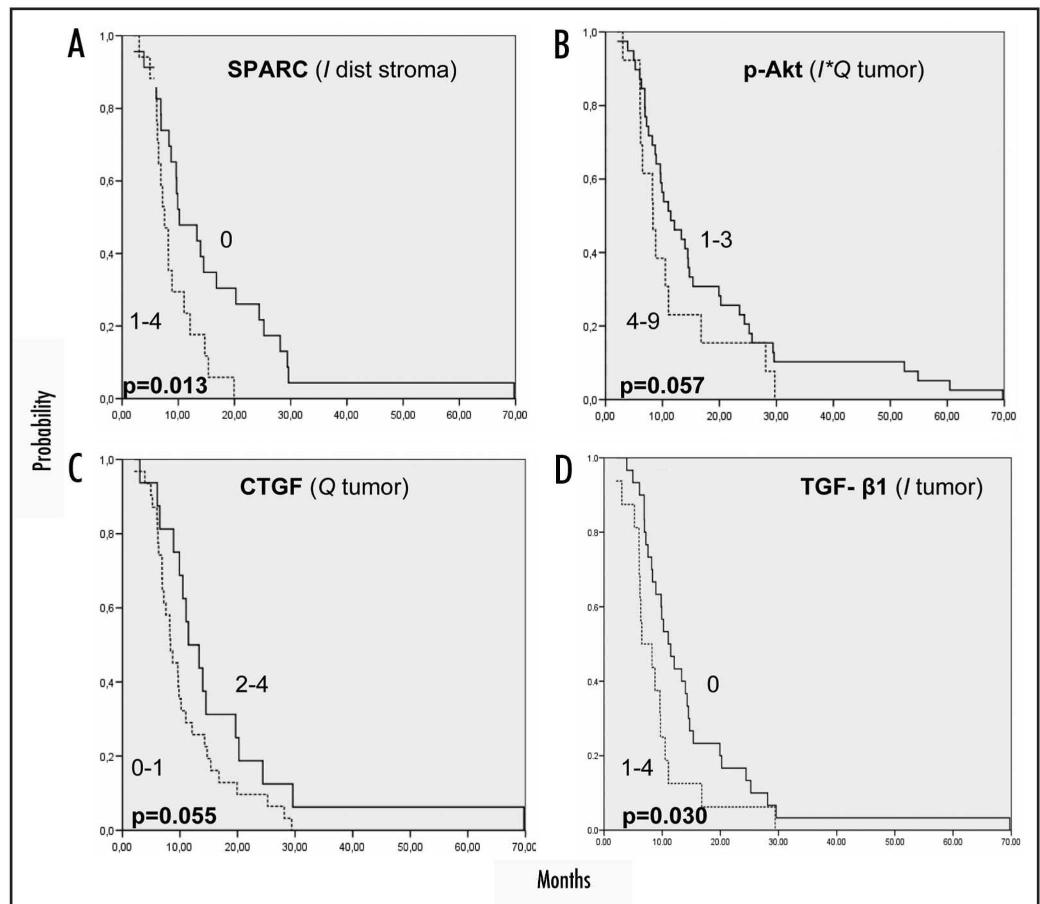

Figure 2. Kaplan-Meier plots showing overall survival in months (x-axis) and the fraction of patients alive (y-axis) with locally advanced pancreatic carcinoma as a function of the expression of (A), SPARC in the stroma distant to the tumor; (B), phospho-Akt in the tumor; (C), CTGF in the tumor and (D), TGFβ1 combined in the tumor and stroma. I = graded intensity of protein expression, Q = graded quantity of protein expression (see materials and methods).

In vitro analysis of SPARC protein expression in cancer cells and stellate cells was in accordance with the immunohistochemistry staining of the tumor samples, as low levels of SPARC was seen in the cancer cells compared to high level in the stroma/stellate cells. Our in vitro data suggests that paracrine SPARC from stellate cells enhance invasion of tumor cells. However, it cannot be precluded that other factors secreted from the stellate cells may also be involved in this effect. Due to the lack of influence on radiation sensitivity in vitro, it is likely that the detrimental effect of SPARC on OS seen in this study is linked to its anti-adhesive and invasion promoting properties.

Our immunohistochemical analysis of biopsy specimens revealed that tumor and stromal phospho-Akt were directly associated as were tumor and stromal TGFβ1. This could be taken as an indication that phospho-Akt and TGFβ1 play a role in both compartments to mediate paracrine communication. This hypothesis is supported by the association of tumor TGFβ1 with stromal α-SMA. This is especially interesting because it could explain the paradox observation of disrupted TGFβ1 signaling in pancreatic carcinoma while at the same time the TGFβ1 ligand is highly overexpressed.[36] Tumor cell TGFβ1 could exert proliferative effects in a paracrine manner on the neighboring PSC rather than on the tumor cells.[47,48] Furthermore, the observed correlation between phospho-Akt and TGFβ1 in tumor cells is in congruence with reports of cooperation between the Ras and the TGFβ1 pathways.[49] The expression of TGFβ1 in the tumor as a negative prognostic marker ($p = 0.03$) found in this study is in good congruence with this hypothesis.

The central role of TGFβ1 in experimental and human pancreas fibrogenesis is well documented (reviewed in ref. 48). TGFβ1 has also been recognized to be responsible for fibrosis of the tumor surrounding stroma via PSC.[47] A recent study demonstrated that the stimulatory effect of the supernatant of pancreatic carcinoma cell lines on PSC was significantly reduced when supernatants were preincubated with neutralizing antibodies against TGFβ1.[10] The negative prognostic influence of TGFβ1 in this study is in concordance with the observed tumor promoting effect of the stroma in preclinical observations.[10] The prognostic significance of TGFβ1 in pancreatic carcinoma has been investigated in a number of other studies (reviewed in ref. 50). Most of these studies described TGFβ1 to have a negative prognostic influence.[36,51,52] A recent study reported high levels of TGFβ2 in the serum to be of negative prognostic significance after resection or palliative treatment of pancreatic cancer.[51] However, in two reports high levels of TGFβ1 expression in resected tumors were correlated with longer survival.[53,54] No prognostic significance could be attributed to nuclear staining of TGFβ1 in LAPC neither in the study of Sears and coworkers[55] nor in our study.

Interestingly, we observed a stronger protein expression of CTGF, a downstream effector of TGFβ1, in the tumor than in PSC and a

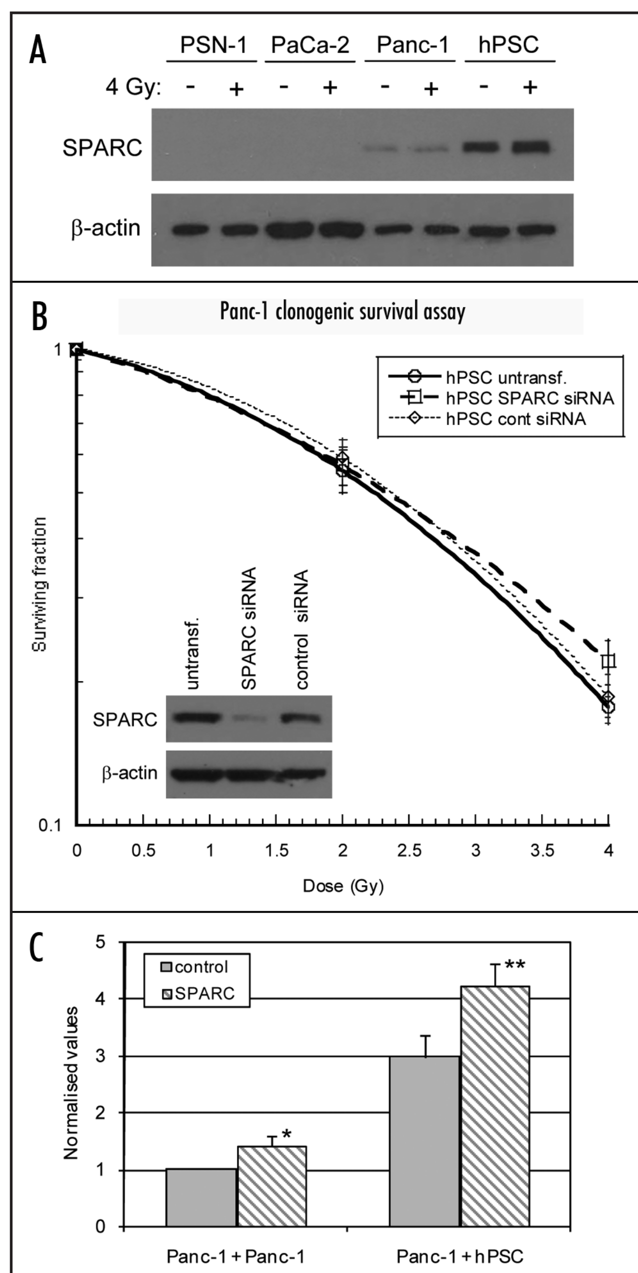

Figure 3. (A) SPARC expression of the pancreatic cancer cell lines PSN-1, MiaPaCa-2 (PaCa-2) and Panc-1 and the stellate cells hPSC was analysed by Western blotting before and one hour after 4 Gy irradiation. β-actin serves as a loading control. (B) Clonogenic survival assay of Panc-1 cells cocultured with hPSC. Panc-1 tumor cells were cocultured directly with hPSC cells, which had been transfected with SPARC or control siRNA 48 hrs earlier. Six hours after Panc-1 and hPSC coculture, cells were irradiated and colonies counted 14 days later. The Western blot inserted demonstrates the siRNA knock-down of SPARC expression in hPSC. (C) Invasion assay with Panc-1 cells grown in coculture with Panc-1 (Panc-1 + Panc-1) or hPSC (Panc-1 + hPSC). SPARC was added to wells only. The graph represents the average values of three independent experiments each performed in triplicates. Error bars denote standard error. *p = 0.15 and **p = 0.001 relative to no-SPARC control.

positive prognostic trend for patients with elevated CTGF expression in tumor cells. Previously it was reported that in pancreatic cancer tissues, fibroblasts were the predominant site of CTGF transcription, whereas the tumor cells appeared to contribute to a lesser extent.[12,37] In contrast to our study, the quoted studies measured the mRNA level and not the protein level of CTGF. Recently, the inhibition of CTGF with a neutralizing CTGF specific monoclonal antibody was reported to abrogate tumor growth and to inhibit lymph node metastases in xenograft pancreatic carcinoma.[56] Both our study and the study of Hartel and coworkers[12] observed a positive prognostic effect of CTGF on patients with pancreatic carcinoma, which is contradictory to a detrimental effect of the stroma. Our observation of high levels of CTGF in the tumor cells is not congruent with the literature. Of note, the study of Hartel and coworkers must be interpreted with caution because of the small sample size comprising only 25 patients who underwent resection.

The short survival of patients with LAPC who have high expression of phospho-Akt in the tumor in our study is in good correlation with the literature on the promotion of survival of tumor cells after radiation.[25,26,57] Furthermore, a study on 61 patients with resected pancreatic tumors also reported short survival for patients with high phospho-Akt expression in the tumor.[58] Interestingly, the expression of Akt2 had no influence on survival even if this specific isoform is upregulated in about 20% in pancreatic cancer. The prognostic influence of phospho-Akt might rather be attributable to the activation of Akt1, which plays the major role in radiation survival.[26] In congruence, the pancreatic cancer cell lines MiaPaCa-2, Panc-1 and Capan-2 are sensitized to radiation after treatment with nelfinavir, a drug that reduces phospho-Akt levels.[59,60] In a trial from Japan, 46% of the patients with resections stained positive for phospho-Akt and in another trial 21% after resection.[61] Compared to the surgical series we observed a rate of 84% of tumors with overexpressed phospho-Akt. We speculate that the more aggressive behaviour of LAPC compared to resectable tumors could be reflected in this high rate. Despite the reported role of Akt in PSC,[27,28] we rarely could observe phospho-Akt expression in the stroma. In vitro studies have shown that in PSC the PI3-kinase pathway is activated by PDGF[27] and that inhibition of Akt almost completely inhibited PSC migration.[28]

Survivin, a member of the family of the inhibitors of apoptosis (IAP), has been linked to Akt and was found to be a negative prognostic factor for patients with resected pancreatic carcinoma who had a survivin expression rate of 68% in tumor cells.[62] We could not observe a prognostic influence of survivin with an overall high rate of 81% of survivin expression in the tumor cells. The most interesting finding in our analysis was that the cytoplasm of the tumor stroma stained positive for survivin in 41%. There is no previous report on stromal staining for survivin, although recently survivin was reported in non-tumor CD34 positive stem cells and in endothelial cells.[63]

In conclusion, our data describe a negative prognostic role of SPARC in the stroma of patients with LAPC who undergo CRT. We propose enhanced tumor cell invasion and migration as a possible mechanism of shortened survival. Additionally, the pattern of correlations of tumor and stroma markers points to an activation of profibrotic proteins by the tumor cells. Taken together, the role of the stroma in the tumor biology of pancreatic carcinoma has been largely neglected and needs to be investigated more thoroughly to be able to identify more effective ways of treatment for this still highly lethal disease.

Materials and Methods

Patients and study design. Between October 1995 and December 2003, a total of 73 patients with biopsy proven previously untreated

locally advanced or borderline resectable pancreatic ductal adenocarcinoma (LAPC) were treated with chemoradiation (CRT) at our institution. Conventionally fractionated, 3-d-conformal irradiation was administered with a total dose of 55.8 Gy (PTV 1) and 50.4 Gy (PTV2), respectively, as described in detail elsewhere.[39] Patients with non-ductal pancreatic neoplasms, with resectable disease and with distant metastasis were excluded from analysis. Histological confirmation was obtained prior to CRT by CT- or ultrasound-guided fine-needle biopsy. The pancreatic mass was targeted using local anesthetic. Biopsies were performed with an 18 G x 10 mm cutting needle with a spring-loaded mechanical gun (Magnum®, Bard, Murray Hill, New Jersey, USA). Selection of specimens with sufficient tumor material was doen by an experienced pathologist (G.N.). The primary outcome of the study was overall survival as determined from date of histological proof of diagnosis to the time of death or last follow-up. Patient follow-up was performed at 3 month intervals for up to 2 years and at 6 month intervals thereafter. This study was conducted as part of a local institutional review board-approved protocol.

Immunohistochemistry. Formalin fixed, paraffin embedded tissue sections were deparaffinized in Xylene (3 x 10 min) and dehydrated in graded ethanol (98%–70%). IHC specific antibodies were used as shown in Table 1 and as described previously for phosphorylated Akt Ser 473,[25] CTGF,[38] TGFβ1,[40] survivin[41] and α-SMA, vimentin and CK8,[42] To confirm the results of nuclear TGFβ1 staining a different primary antibody (Novo-Castra Labs, UK) was used.

Evaluation was performed blinded to the outcome (R.S. and T.B.) separately for tumor and stromal cells. Intensity was scored semiquantitatively: 0 = absent; 1 = weak; 2 = medium; 3 = strong. Quantity was scored 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (>50%) according to the fraction of the cell type stained. A combined score was created: degree of staining = intensity x quantity. Pair wise log-rank test served for comparison of the differences in survival in subgroups of patients.

Cell culture. Panc-1 cells were obtained from ATCC (ATCC, VA) and human pancreatic stellate cells (hPSC) were isolated from the resected normal pancreas of patients who had surgery for pancreatic cancer as described previously (Approved by the Ethics Committee of Tohoku University School of Medicine).[43] These hPSC are regarded to be spontaneously semi-immortalised after growth in culture for a year. Panc-1 and hPSC were cultured in DMEM and Ham's F-12/DMEM, respectively, supplemented with 10% heat-inactivated Foetal Calf Serum (FCS), penicillin sodium and streptomycin sulfate.

Western blotting. Cells were lysed on ice for 20 minutes in lysis buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 1 mM EDTA, 1% Igepal CA-630) containing protease inhibitor cocktail tablets (Boehringer/Roche, UK), phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich, UK) and 1 mM DTT. Concentration of protein extracts was determined by Bradford analysis (BioRad, UK) according to the manufacturer's instructions. Protein extracts were resolved on a NuPAGE 4–12% Bis-Tris mini gel (Invitrogen) and transferred onto a Hybond-C Extra membrane (Amersham Biosciences, UK), which was blocked in 5% low fat milk diluted in Tris Buffered Saline Tween (TBS with 0.1% Tween-20). Membranes were incubated overnight in mouse monoclonal primary antibodies against SPARC (Affinity BioReagents, CO) and β-actin (Sigma-Aldrich, UK). Secondary antibody (peroxidase conjugated rabbit anti-mouse antibody; Pierce Biotechnology, IL) was incubated in 5% milk in TBST for 1 hour. Blots were developed using ECL Western Blotting Detection Reagents (Amersham BioSciences, UK) and Fuji medical X-ray film.

Clonogenic survival assay. For monoculture assays, Panc-1 and PSN-1 cells were plated as single cells four hours prior to irradiation. For coculture assays, hPSC cells were transfected with siRNA (SPARC siGENOME SMART pool or control siRNA, Dharmacon, CO) 48 hours prior to initiation of coculture. hPSC cells were seeded as a monolayer 24 hours before Panc-1 were plated as single cells and irradiated six hours later. After six hours of coculture cells were irradiated in a cesium source irradiator (IBL 637, CIS Bio International, France) at a dose rate of 0.68 Gy/min. Colonies were stained 10–14 days after irradiation and only colonies of more than 50 cells were counted. The surviving fraction was calculated as follows: (Number of colonies formed/number of cells plated) x plating efficiency (unirradiated). Each point on the survival curve represents a mean surviving fraction from three dishes.

Invasion assay. Invasion of the pancreatic cancer cell line Panc-1 was measured by the number of cells invading through Matrigel coated transwell inserts (VWR, UK) in medium containing 10% FBS. Transwell inserts with 8 μm pores were coated with 200 μg Matrigel (BD Biosciences, UK) and 1.5 x 10^4 cells were seeded in upper chamber. Inserts were placed in a 24-well plate seeded with Panc-1 or hPSC cells. SPARC was added to the wells at a final concentration of 2 μg/ml. After 24 hours incubation, inserts were fixed in 70% EtOH for five minutes and stained in hematoxylin (Sigma-Aldrich, UK) for 10 minutes. Non-invading cells from the upper surface of the membrane were removed with a cotton swap. Invaded cells were counted in three randomly selected fields under a light microscope.

Statistical analysis. Descriptive statistics were used to characterize the distribution of patient variables. For categorical variables, frequency and percentage were used. For continuous variables, mean, SD, minimum and maximum were used. For comparisons of baseline characteristics across groups two-sided Fisher's exact tests were used. Spearman's rank correlation coefficient was calculated for the association of varibles in the tumor and the stroma. Survival distribution was estimated by the method of Kaplan and Meier. Survival was defined as months from diagnosis to death because of any cause or last patient contact. Survival was compared between groups of patients by the Log-rank test. All p's quoted were two sided. A p of <0.05 was considered statistically significant. Statistical analyses were performed in SPSS v. 14.0 (SPSS Inc., IL). For significance testing of invasion assays a two-tailed Student's t-test was used.

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