ORIGINAL ARTICLE

The Significance of Histopathological Evaluation of Pancreatic Fibrosis to Estimate Pancreas Cancer Progression

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ABSTRACT

Objective To estimate the importance of the role of pancreatic stellate cells in pancreas cancer progression, their properties were evaluated in relation to clinical details and patient prognosis. **Patients and Methods** Among patients who underwent surgical treatment from 2004 to 2013, the texture of the pancreatic specimens was evaluated by histopathological measurement length of fibrosis, fibrosis grade and the intensity of pancreatic stellate cell activity. **Results** 1. The histopathological measurement length of inter- and intralobular fibrosis increased significantly with progression of FG from grade 0 to 3 (22.0±4.5 *vs.* 23.7±1.9 *vs.* 53.5±6.0 *vs.* 203.7±18.6 and 2.0±0.4 *vs.* 2.7±0.3 *vs.* 8.2±1.0 *vs.* 21.7±3.1, respectively). 2. The histopathological measurement length of inter- and intralobular fibrosis was also significantly longer with the increase in pancreatic stellate cell activity score from 0 to 3 (29.7±5.9 *vs.* 37.4±7.2 *vs.* 68.4±9.5 *vs.* 204.7±20.5 and 3.1±0.5 *vs.* 5.1±1.1 *vs.* 9.7±1.1 *vs.* 21.6±3.4, respectively). 3. The histopathological measurement length of both inter- and intralobular fibrosis correlated with the preoperative level of HbA1c but not with pathological invasion of cancer in lymphatic vessels and the nervous system. 4. Fibrosis grade and pancreatic stellate cell activity did not correlate with pancreas function and clinical factors. 5. Significantly higher rates of positive lymph node metastases were detected in the patients with high fibrosis grade or pancreatic stellate cell activity. 6. There were no significant differences in either fibrosis grade or pancreatic stellate cell activity in whole pancreas cancer cases, and in T3 patients only, 2-year survival rate and median survival term were similar among the different fibrosis grades. However, pancreatic stellate cell activity in T3 patients had a significantly different effect on patient prognosis. 7. Specifically for stage II/III patients, survival rates were similar for different fibrosis grades but clearly different depending on the severity of pancreatic stellate cell activity. When limiting the cancer site to the pancreas head, survival rate and median survival term in T3 patients were clearly poorer as pancreatic stellate cell activity increased but not as fibrosis grade increased. **Conclusion** Fibrotic change as measured by the amount of fibrotic tissue present favors prediction of pancreatic function, whereas pancreatic stellate cell activity favors prediction of cancer progression.

INTRODUCTION

Pancreatic cancer (pancreatic ductal adenocarcinoma) is a lethal disease and is the fourth leading cause of cancer-related deaths worldwide [1]. Late diagnosis and early metastases are the reasons for the critical outcomes, and the 5-year survival rate and median survival period are lower than 5% and 6 months, respectively [2]. Only 20% of patients are deemed suitable for attempted curative surgical resection. Recently developed chemotherapeutic agents including molecular targeting therapy have not produced an acceptable outcome for pancreas cancer compared with that for other solid neoplasms [3, 4]. In spite of the urgent necessity for novel therapy, the complicated biology and unfavorable anatomical character of pancreas cancer hinder these efforts. Among risk factors including age or smoking, the strongest known factor is chronic pancreatitis because inflammation represents a critical key driver for pancreas cancer [5]. In fact, patients with a history of more than 5 years of continuous pancreatitis have a greater than 14-fold risk of developing pancreas cancer [6, 7]. However, the detailed mechanism behind the increased propensity for chronic pancreatitis to develop into pancreas cancer has not yet been fully elucidated, although recent studies have offered suggestions for several signaling pathways related to inflammatory disease [8].

Recently, pancreatic stellate cells (PSCs) have been well studied and have been found to demonstrate desmoplasia production during the progression of pancreatitis [9]. PSCs are located adjacent to the periacinar space and have long cytoplasmic processes that encircle the base of the acinus. They can also be found in perivascular and periductal regions of the pancreas and serve as key participants in the pathobiology of the major disorders of the exocrine pancreas, including pancreatitis and pancreatic cancer. The

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course of chronic pancreatitis is characterized by recurrent episodes of acute pancreatitis with increasing amounts of fibrosis, chronic inflammation, and parenchymal cell loss with each successive episode. In the healthy pancreas, they exhibit abundant vitamin A-containing lipid droplets in the cytoplasm, but during pancreatic injury, acinar cells, inflammatory cells, platelets and endothelial cells produce cytokines or growth factor to activate PSCs. PSCs then start to express α-smooth muscle actin (αSMA) with a transformation of cell shape to a myofibroblast-like phenotype and synthesize extracellular matrix (ECM) components [10]. Pancreas cancer is characterized by dense desmoplastic or fibrotic stroma, and PSCs are central as the principle effector in the production of stroma [11]. PSCs are studied not only for their role in stimulating cancer progression but also in developing angiogenesis [12] and inhibiting apoptosis [13]. Contrastingly, exposure to cancer cells also activates PSCs to increase proliferation, ECM synthesis and migration [13]. The activation of PSCs by various stimulators has been assessed by factors such as αSMA emergence or loss of vitamin A stores [14]. Because sustained activation of PSCs has a role in the fibrosis that is associated with chronic pancreatitis and pancreatic cancer, understanding the biology involved allows the correlation of inflammatory effect with the progression of chronic pancreatitis or pancreas cancer. In the present study, to estimate the importance of the role of PSCs in the progression of pancreas cancer, PSC activation grade was indicated by αSMA expression and their properties were evaluated in relation to clinical details and patient prognosis.

PATIENTS AND METHODS

Patients and Clinical Evaluation

Among the patients who underwent surgical treatment at the Department of Surgical Oncology, Gifu University Hospital between 2004 and 2013, pancreatoduodenectomy (PD) and distal pancreatectomy (DP) were selected as the surgical treatment for 49 and 25 patients with pancreas cancer (mean age= 65.1±13.3 years), respectively, according to the disease site. All patients were operated on and followed by the same team of surgeons who specialized in hepatobiliary and pancreatic surgery. The surgical procedure for reconstruction after PD was performed by separate loop method as described previously [15, 16]. Each patient's condition was evaluated according to clinical features such as preoperative blood data for HbA1c, amylase levels and other common serum factors. This study protocol was approved in accord with the ethical standard of the Declaration of Helsinki for all patients.

Histopathological and Immunochemical Staining

The pancreatic texture was evaluated histopathologically from the specimen at the cut surface of the resected pancreas. Paraffin-embedded tissues were cut into 4-μm serial sections and deparaffinized. The sections were stained with hematoxylin and eosin and Azan for collagen fibers. For immunohistochemistry, the sections were placed in citrate buffer (10 $mmol^{-1}$, pH 6.0) and autoclaved at a chamber temperature of 121°C for 1 minute to retrieve the antigen. They were then rinsed and blocked in 10% H_2O_2 solution with methanol for 10 minutes. Next, the sections were incubated with monoclonal mouse anti-human actin smooth muscle clone 1A4 (Dako, Glostrup, Denmark) at a 1:100 dilution overnight at 4°C. They were then rinsed in PBS and incubated for 30 minutes with a secondary antibody labeled with streptavidin-biotin-peroxidase for mouse monoclonal antibody (Dako LSAB2 System-HRP, Dako). The bound complex was visualized using diaminobenzidine liquid chromogen (SIGMA, Saint Louis, MO) and counterstained with hematoxylin. The immunostaining methods were also described in previous reports [17, 18].

To evaluate the presence of pancreatic fibrosis, the following three histopathological fibrosis parameters were adopted as described previously [19]. They include histopathological measurement length (HML) of the fibrosis, fibrosis grade (FG) as evaluated by fibrotic structural change, and PSC grade as evaluated by the intensity of PSC activity. The HML of actual measurements of collagen fibers for interlobular and intralobular fibrosis was calculated from the average length of 10 different views [19]. The FG in Azan-stained specimens was evaluated using the following four-stage scoring system adopted by Wellner *et al.* [20]: normal pancreas parenchyma and no fibrotic changes, grade 0; mild fibrosis with thickening of periductal fibrosis, grade 1; moderate fibrosis with marked sclerosis of interlobular septa or intralobular sclerosis with no evidence of architectural changes, grade 2; and severe fibrosis with detection of architectural destruction or acinar cell atrophy, grade 3. The immunohistological evaluation of PSC activity was performed using the following four-stage scoring system: no staining except in the periductal tissue, grade 0; weak positive staining that is irregular or sometimes patchy, grade 1; weak positive staining that is always homogenous or diffuse, grade 2; and strong positive staining that is always homogenous and diffuse, grade 3, as described previously [19]. Histological evaluation was performed with the support of two experienced pathologists.

STATISTICAL ANALYSIS

Continuous data are represented as the mean ± standard deviation. Statistical analysis was performed using MedCalc software. A non-parametric Mann-Whitney U test, the Spearman's rank correlation test, chi square test, ANOVA and Kruskal-Wallis test were used. A p value of <0.05 was considered to indicate statistical significance.

RESULTS

Relationships of the Factors

The present measurement of inter- or intralobular fibrosis was evaluated with FG or PSC activity **(Figure 1).** The HML of interlobular fibrosis increased with the progression of FG

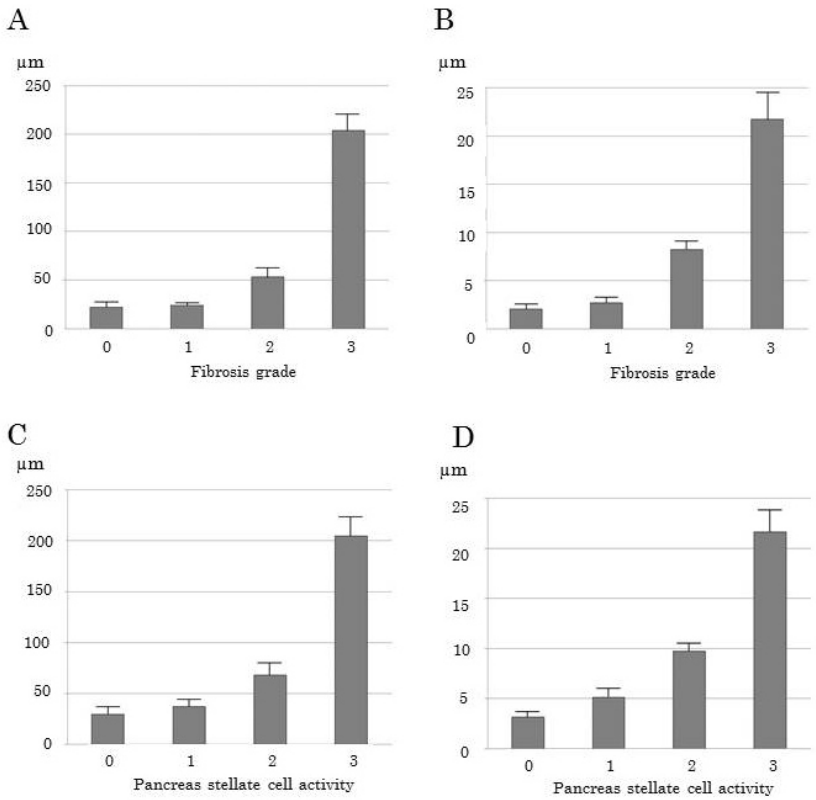

Figure 1: Correlation between measurement of inter- or intra- lobular fibrosis and FG or PSC activity.
The HML of interlobular fibrosis increased with the progression of FG grade **(a.).** and significant differences were detected between each grade except 0-1 (0-1, p=0.6913; 0-2, p=0.0028; 0-3, p<0.0001; 1-2, p<0.0001; 1-3, p<0.0001; and 2-3, p<0.0001). The HML of intralobular sclerosis became significantly longer with the increase in FG **(b.).** (0-3, p<0.0001; 1-2, p<0.0001; 1-3, p<0.0001; and 2-3, p<0.0001). The HML of interlobular fibrosis increased with the increase in PSC activity **(c.).** and significant differences were detected for each (0-1, p=0.4550; 0-2, p=0.0006; 0-3, p<0.0001; 1-2, p=0.0165; 1-3, p<0.0001; and 2-3, p<0.0001). The HML of intralobular sclerosis became significantly longer with the increase in PSC activity **(d.).** (0-1, p=0.1698; 0-2, p<0.0001; 0-3, p<0.0001; 1-2, p=0.0131; 1-3, p<0.0001; and 2-3, p<0.0037).

grade (22.0±4.5 *vs.* 23.7±1.9 *vs.* 53.5±6.0 *vs.* 203.7±18.6), and significant differences were detected between each grade (0-1, p=0.6913; 0-2, p=0.0028; 0-3, p<0.0001; 1-2, p<0.0001; 1-3, p<0.0001; and 2-3, p<0.0001). The HML of intralobular sclerosis also became significantly longer with the increase in FG (2.0±0.4 *vs.* 2.7±0.3 *vs.* 8.2±1.0 *vs.* 21.7±3.1; 0-1, p=0.1876; 0-2, p=0.0004; 0-3, p<0.0001; 1-2, p<0.0001; 1-3, p<0.0001; and 2-3, p<0.0001). The HML of interlobular fibrosis also increased with the increase in PSC activity (29.7±5.9 *vs.* 37.4±7.2 *vs.* 68.4±9.5 *vs.* 204.7±20.5 XX), and significant differences were detected for each (0-1, p=0.4550; 0-2, p=0.0006; 0-3, p<0.0001; 1-2, p=0.0165; 1-3, p<0.0001; and 2-3, p<0.0001). The HML of intralobular sclerosis also became significantly longer with the increase in PSC activity (3.1±0.5 *vs.* 5.1±1.1 *vs.* 9.7±1.1 *vs.* 21.6±3.4 XX; 0-1, p=0.1698; 0-2, p<0.0001; 0-3, p<0.0001; 1-2, p=0.0131; 1-3, p<0.0001; and 2-3, p<0.0037). As shown in **Figure 2**, there was significant correlation of HML between intra-and inter-lobular fibrosis (p<0.0001).

Evaluation of Fibrosis Factors in Pancreas Function and Cancer Progression

The preoperative HbA1C level was found to correlate with HMLs of both intralobular and interlobular fibrosis **(Figure 3).** However, the other serum factors indicating pancreas function or clinical factors, such as age or sex, were not related to the HML of fibrosis (data not shown). The HMLs of interlobular fibrosis were calculated as pathologically positive and negative invasion for 123.5±17.1 and 119.0±51.9 (p=0.9407) in lymphatic vessels, 128.1±17.5 and 97.7.0±45.5 XX in veins (p=0.5818), and 117.7±17.7 and 191.2±24.7 in nervous system (p=0.1803), respectively, and the differences were not significant. The HMLs of intralobular fibrosis were also 13.3±2.3 and 10.0±3.9 (p=0.6778), 13.7±2.4 and 8.1±3.5 (p=0.4414), and 12.3±2.3 and 21.0±4.2 (p=0.2341), with no significant differences present. HMLs of both intralobular and interlobular fibrosis did not correlate with cancer progression factors (data not shown).

The FG and PSC activity also did not correlate with pancreas function and clinical factors (data not shown). The patients with a FG or PSC activity score of 3 were assigned to the hard pancreas group, and the patients with a FG and PSC activity score of 2 or less were assigned to the soft pancreas group, based on a previous report [14]. Both a FG of 3 and PSC activity score of 3 were found to be significantly associated with a higher number of positive lymph node metastases (p=0.0476 and 0.0304, respectively). These factors were then compared in terms of patient prognosis **(Figures 4, 5).** There were no significant differences between the two groups of whole pancreas cancer cases, but for T3 patients with a FG of 3 or lower in the hard and soft pancreas groups, 2-year

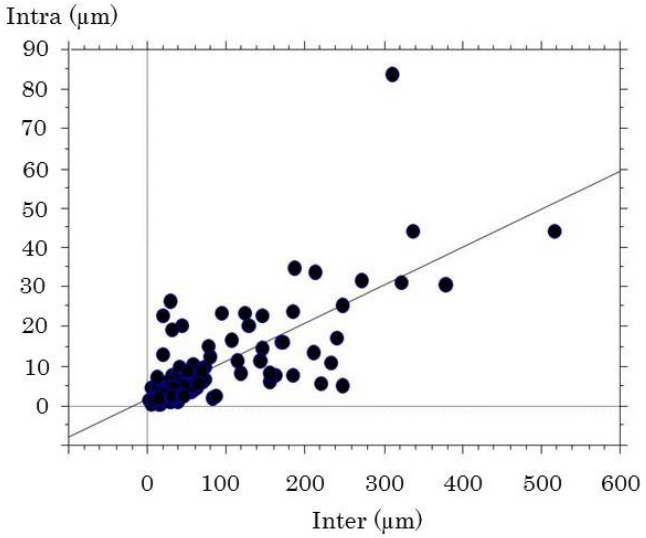

Figure 2: Correlation of HML between inter- and intra- lobular fibrosis. There was significant correlation of HML between intra-and inter-lobular fibrosis (p<0.0001).

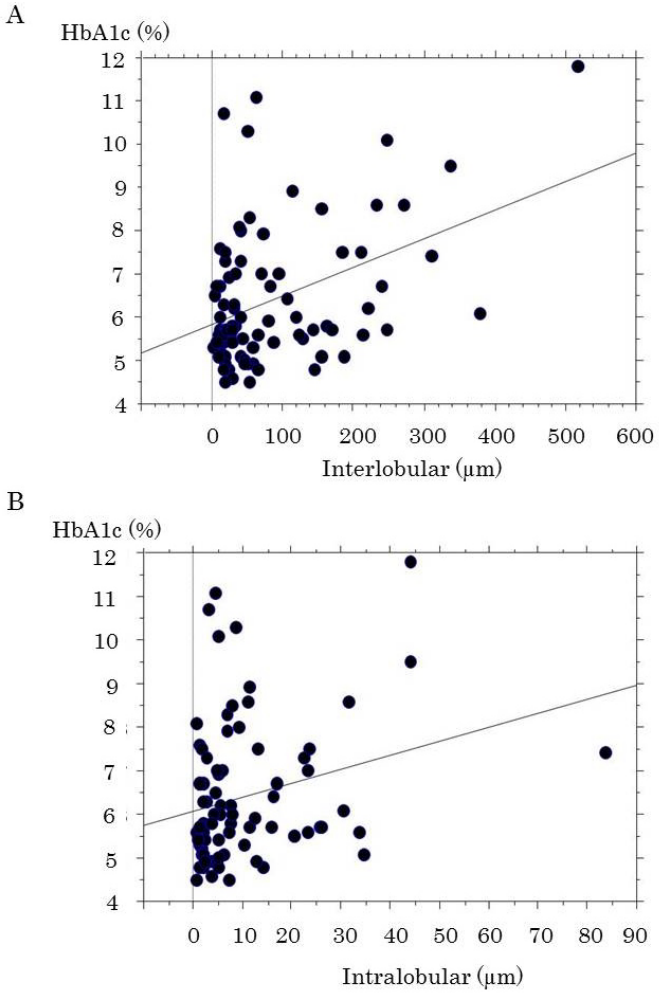

Figure 3: Correlation between preoperative HbA1C level and HML of inter- or intra- lobular fibrosis.
There was significant correlation between inter- **(a.).** or intra- **(b.).** lobular fibrosis and preoperative HbA1C (p=0.0001 or p=0.0158, respectively).

survival rate and median survival term (MST; days) were calculated as 45% and 71% (p=0.0464) and 505.3±80.6 and 1073.1±200.0 days (p=0.0007), respectively. For T3 patients with a PSC activity score of 3 or lower, 2-year survival rate and MST were 28% and 69% (p=0.0236) and 421.1±61.5 and 941.1±168.7 days (p=0.0002), respectively, for the two groups. Specifically for stage II/III patients with an FG of 3 or lower, survival rates were not significantly different, at 54% and 62% (p=0.2202), but the survival rate in patients with a PSC activity score of 3 was 38%, significantly lower than that of the other patients, at 89% (p=0.0494). Furthermore, when the site of cancer was limited to the pancreas head **(Figure 5),** 2-year survival rate and MST of T3 patients in the two groups were 40% *vs.* 67% (p=0.0498) and 430.1±78.1 *vs.* 718.9±99.1 days (p=0.0327) respectively and those of patients with stage II/III cancer were 38% *vs.* 68% (p=0.0397) and 703.1±224.5 *vs.* 1024.1±321.3 days (p=0.0467), respectively, for PSC activity. There were no significant differences for FG.

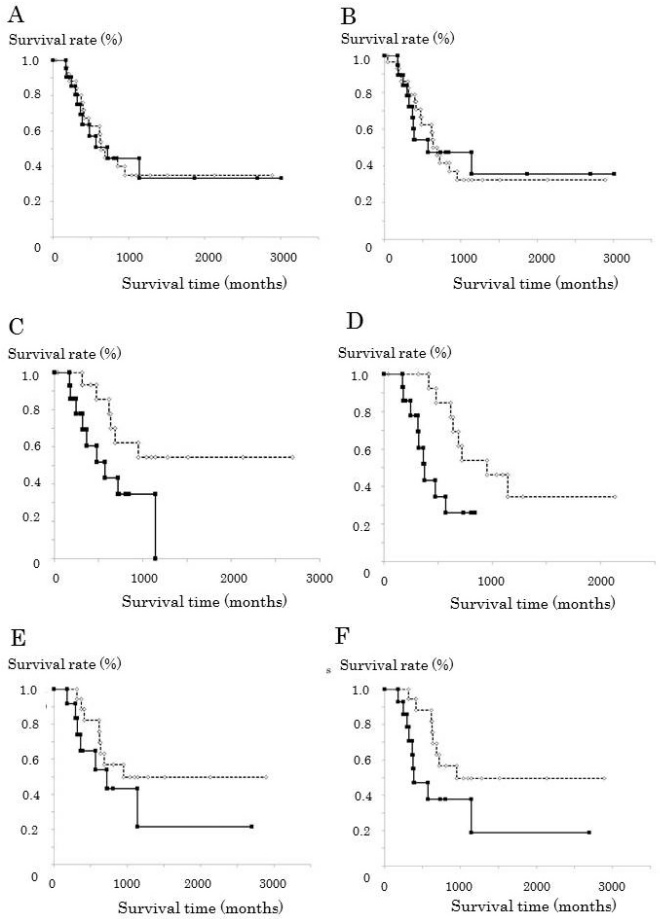

Figure 4: Patient prognosis in all cases.
Patient prognosis with hard (continuous line) and soft (dotted line) pancreas were described. Pancreas hardness was evaluated from fibrosis grade **(a.).** or PSC activity **(b.).** in all cases. T3 patients with a FG of 3 or lower in the hard and soft pancreas groups, 2-year survival rate and median survival term were calculated as 45% and 71% (p=0.0464) and 505.3±80.6 and 1073.1±200.0 days (p=0.0007), respectively **(c.).** For T3 patients with a PSC activity score of 3 or lower, 2-year survival rate and median survival term were 28% and 69% (p=0.0236) and 421.1±61.5 and 941.1±168.7 days (p=0.0002), respectively **(d.).** In stage II/III patients with an FG of 3 or lower, survival rates were not significantly different **(e.).**, at 54% and 62% (p=0.2202), but the survival rate in patients with a PSC activity score of 3 was 38%, significantly lower than that of the other patients **(f.).**, at 89% (p=0.0494).

DISCUSSION

Pancreatic fibrosis is generally defined as the accumulation of excessive amounts of ECM proteins in the

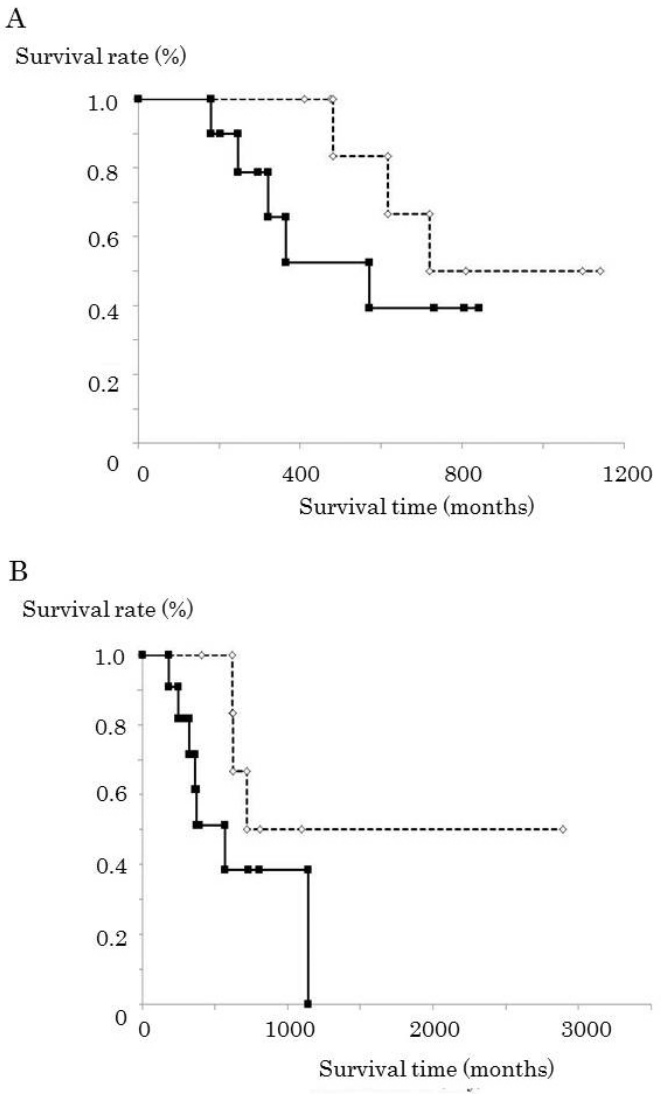

Figure 5: Prognosis of patients with pancreas head cancer
Patient prognosis with hard (continuous line) and soft (dotted line) pancreas were described. **(a.).** 2-year survival rate and median survival term of T3 patients in the two groups were 40% *vs.* 67% (p=0.0498) and 430.1±78.1 *vs.* 718.9±99.1 days (p=0.0327) respectively and **(b.).** those of patients with stage II/III cancer were 38% *vs.* 68% (p=0.0397) and 703.1±224.5 *vs.* 1024.1±321.3 days (p=0.0467), respectively, for PSC activity.

tissue. It is now generally accepted that fibrosis is not a mere end product of chronic injury but an active dynamic process that may be reversible in its early stages. In the past, pancreatic fibrosis was basically evaluated with several methods, of which one was the fibrosis grading system. In this method, fibrosis was graded according to the degree of interlobular/intralobular fibrosis and the presence of architectural destruction or acinar cell atrophy [21, 22]. As a novel concept to evaluate pancreatic fibrosis, the present study proposed the HML of fibrosis to measure the collagen width of interlobular and intralobular fibrosis. The HML of fibrosis was found to relate to the already estimated "fibrosis grade" [20], which evaluated the advancement of fibrosis. Chronic pancreatitis is a progressive fibro-inflammatory disease characterized by the destruction of pancreatic secretory parenchyma and replacement by fibrous tissue, eventually leading to malnutrition or diabetes. In fact, one of the features of chronic pancreatitis is the development of secondary diabetes due to the associated β-cell dysfunction. Actually, immunochemical and quantitative morphometric examination of pancreatic tissues revealed a significant 29% reduction in β-cell area in chronic pancreatitis, despite there being no change in the α-cell area [23]. In the present study also, a larger area of replacement of β-cells by fibrosis in pancreas tissue as measured by HML correlated with a higher serum level of HbA1c, indicating the loss of dysfunction.

Inflammatory cells also can influence fibrogenesis by supporting the activation of human PSCs, which consequently release ECM proteins that lead to fibrosis [24]. This activation of PSCs is driven by the release of cytokines from mononuclear cells, and following activation, they secrete autocrine factors that perpetuate their activation and contribute to the vicious cycle of inflammation and fibrosis [25]. From these results, fibrosis was indicated to occur unequally at different sites in the pancreas tissue, compared to that in the liver, which is controlled by serum factors. In the present study, the tissue near solid cancer areas was evaluated for PSC activation to observe the effect on the cancer progression. Although PSCs reduced the expression of insulin and induced apoptosis in pancreatic β-cells [26], the PSC activation score correlated well with FG from HML but not with pancreas function. In the early stages of chronic pancreatitis, patients usually do not have diabetes, and islet cells are reported to remain intact morphologically and functionally. By contrast, in advanced stages [23], but not yet at the onset of diabetes, the number of islet cells was shown to be reduced in accord with the stage of the pancreatitis [27]. Namely, patients with diabetes mellitus might have variable intra- and peri-islet fibrosis [28]. In addition, the PSCs can be activated by cancer cells, and the cross-talk between tumor cells and the surrounding stroma is a key modulator of carcinogenesis [29]. In the present study, to evaluate pancreas function, we included both patients with and without chronic pancreatitis. Therefore, inflammatory effect or cross-talk action due to causes such as cancer of the bile duct in normal pancreas tissue might increase the activity of PSCs.

Despite the strong link between fibrosis and carcinogenesis in the pancreas, less than 5% of patients with chronic pancreatitis go on to develop pancreatic cancer [30], indicating that fibrosis itself might have a weak effect on cancer progression. In support of this hypothesis, in the present study, although the level of fibrosis as evaluated by measuring HML was associated with pancreatic function, it was not associated with cancer progression. In contrast, PSCs are usually quiescent, with a low proliferation rate; however, upon activation, they not only synthesize ECM but also promote cancer progression [31]. PSCs have also been shown to promote cancer cell migration, during which cancer cells exhibit features of epithelial-mesenchymal transition, namely, decreased levels of epithelial markers such as E-cadherin concurrent with increased expression of mesenchymal markers [11]. It is possible that epithelial-

mesenchymal transition is responsible for the PSC-induced increased migration of cancer cells, supporting the present results for lymph node metastases. Further, one of the well-documented features of human pancreatic cancer is its resistance to both chemotherapeutic agents and radiotherapy. It is possible that this resistance may be mediated, at least in part, by the dense stroma produced by PSCs [32]. In support of this notion, it has been shown that sequestration of chemotherapeutic agents such as gemcitabine can occur before the drug can reach the cancer cells [33]. In fact, secretions of PSCs have been shown to confer a chemoresistant cancer cell phenotype by suppressing oxidative stress-induced apoptosis [34] and decrease sensitivity to chemotherapeutic agents or radiation [35]. Extensive neural remodeling is also known to occur in pancreatic cancer with the cancer stroma revealing neural hypertrophy and increased neural density [36]. It noteworthy that PSCs themselves express neural markers and also produce neurotrophic factors [37]. Thus, it would be reasonable to postulate that PSCs may act as neural elements in the tumor stroma, affecting the growth of nerves and survival of cancer cells that express receptors for neurotrophic factors. This hypothesis is supported by a report [38] demonstrating a positive correlation between the extent of desmoplasia and the degree of neural invasion in human pancreas cancer. Depending upon the location of the tumor site, i.e., the head or body/tail of the pancreas, or the tumor size, the situation of neural invasion is different. Therefore, with stricter management of tumor condition, prognosis in the present study was shown to be clearly different only in patients with T3 or Stage II/III cancer, despite the lack of significant differences among all of the patients combined. The activation of PSCs might be important in the prediction of patient prognosis clinically.

CONCLUSION

Inflammatory change evaluated by fibrosis was critical to understanding the mechanism of pancreatic dysfunction or cancer progression, but the situations for both occurrences need to be considered individually. Fibrotic change as measured by the amount of fibrotic tissue present favors the prediction of pancreatic function, whereas PSC activity favors the prediction of cancer progression.

Conflict of interest

All the authors have no conflicts of interest

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