Table 4: The rate and site of recurrence after resection of pancreatic cancer in relation to GPR54 expression.

	GPR54 expression Positive (n = 30)	GPR54 expression Negative (n = 23)	P value
Recurrence, n (%)	17 (56.7%)	16 (69.6%)	0.34
Site of recurrence			
Liver, n (%)	8 (26.7%)	7 (30.4%)	0.76
Local, n (%)	6 (20.0%)	3 (13.0%)	0.50
Peritoneum, n (%)	5 (16.7%)	4 (17.4%)	0.95
Lymph nodes, n (%)	2 (6.7%)	3 (13.0%)	0.43
Lungs, n (%)	1 (3.3%)	0	0.38
Bone, n (%)	0	1 (4.3%)	0.25
Unknown*, n (%)	0	1 (4.3%)	0.25

* Confirmed by elevated tumor marker during follow-up

both in esophageal squamous cell carcinoma was a significant predictor of lymph node metastasis. Finally, the survival of ovarian cancer patients with low *GPR54* mRNA expression is significantly worse than that of those with high expression[20].

On the other hand, studies in patients with breast cancer[19] and hepatocellular carcinoma (HCC) [15,21] have yielded opposite results, with a positive association between increased KiSS-1 levels and disease progression. Martin et al. [19] found that *KiSS-1* mRNA expression was increased in aggressive breast cancer. Ikeguchi et al. [15] reported that overexpression of *KiSS-1* and *GPR54* was correlated with the progression of HCC. Schmid et al. [21] performed an immunohistochemical study and concluded that high KiSS-1 expression was an independent prognostic factor for shorter survival of patients with HCC.

The mechanism by which the *KiSS-1/GPR54* system regulates tumor progression still remains unclear, although various studies have revealed the downstream signaling pathways activated by *KiSS-1* gene product. This might indicate that a complex signaling network exists with diverse physiological responses [23,28].

Stafford et al. [29] found that binding of *KiSS-1* peptide to the receptor leads to activation of G-protein-activated phospholipase C, which suggested a direct relation of *KiSS-1* to the Gαq-mediated phospholipase C-Ca^{2+} signaling pathway. In addition, activation of GPR54 has been shown to cause an increase of intracellular calcium [9-11],

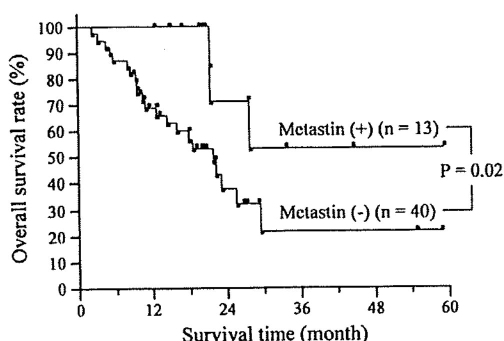

Figure 4
Impact of metastin expression on survival time of pancreatic cancer patients. Overall survival of patients whose tumors were positive (n = 13) or negative (n = 40) for metastin immunostaining. The survival of patients with positive tumors was significantly longer than that of patients with negative tumors (p = 0.02).

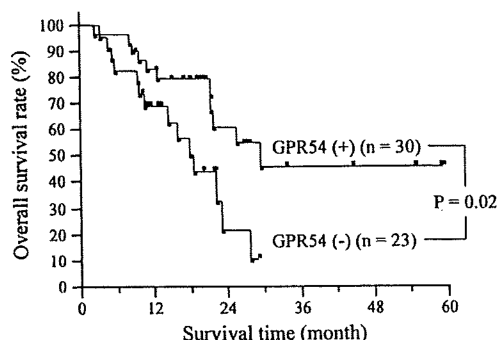

Figure 5
Impact of GPR54 expression on survival time of pancreatic cancer patients. Overall survival of patients whose tumors were positive (n = 30) or negative (n = 23) for GPR54 immunostaining. The survival of patients with tumors positive for GPR54 was significantly longer than that of those with negative tumors (p = 0.02).

Table 5: Univariate and Multivariate analyses of factors associated with survival after resection in patients with pancreatic cancer.

	Univariate analysis		Multivariate analysis	
Characteristics	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
Age (continuous variables)	1.01 (0.97–1.1)	0.50	1.03 (0.97–1.1)	0.29
Gender (male versus female)	1.09 (0.73–1.6)	0.66	1.16 (0.73–1.9)	0.52
Location of tumor (head versus body-tail)	1.08 (0.72–1.7)	0.72	0.71 (0.40–1.3)	0.25
Size of tumor (continuous variables)	1.01 (0.97–1.0)	0.63	1.01 (0.96–1.1)	0.69
Histopathological grading (G1 versus G2-4)	1.05 (0.70–1.7)	0.80	0.92 (0.49–1.8)	0.79
pT (pT1, pT2 versus pT3)	1.62 (0.88–4.0)	0.14	2.07 (0.86–6.7)	0.11
pN (pN0 versus pN1)	1.27 (0.85–2.0)	0.25	1.01 (0.58–1.8)	0.97
Lymphatic invasion (positive versus negative)	1.20 (0.80–1.8)	0.33	0.97 (0.54–1.7)	0.92
Venous invasion (positive versus negative)	1.01 (0.68–1.5)	0.95	0.91 (0.52–1.6)	0.73
Perineural invasion (positive versus negative)	1.57 (1.1–2.4)	0.03	1.47 (0.85–2.7)	0.17
pStage (I, II versus IV)	3.16 (1.6–5.8)	0.002	2.70 (1.1–6.8)	0.03
Residual tumor (R0 versus R1)	1.61 (1.0–2.5)	0.03	1.60 (0.91–2.9)	0.10
Metastin expression (positive versus negative)	1.93 (1.1–4.0)	0.01	2.08 (1.1–4.7)	0.03
GPR54 expression (positive versus negative)	1.62 (1.1–2.5)	0.02	1.22 (0.74–2.0)	0.43

arachidonic acid release [9], activation of mitogen-activated protein kinases (MAPKs), and activation of extracellular signal-regulated kinase (ERK) 1/2[9,14]. We have observed that exogenous metastin reduces migration of pancreatic cancer cells, while it induces the activation of ERK1 and p38[24]. Furthermore, the *KiSS-1* product was shown to repress 92-kDa type 4 collagenase and matrix metalloproteinase (MMP)-9 expression by decreasing the binding of NF-κB to the promoter [30]. Bilban et al. [31] also found downregulation of MMP-2 activity by the *KiSS-1* gene product in human trophoblasts, which implies an association between the tumor suppressor role of *KiSS-1* suggested in this study and our previous report that activation of MMP-2 has a significant role in invasion and metastasis of pancreatic cancer[32].

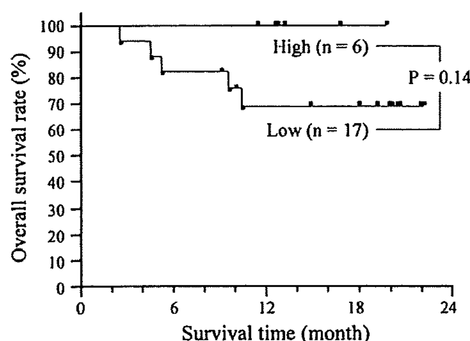

Figure 6
Impact of plasma metastin levels on survival time of pancreatic cancer patients. Overall survival of patients with high (n = 6) and low (n = 17) plasma metastin levels. There was no significant difference between the two groups (p = 0.14), but no patient with a high plasma metastin level died after surgery.

KiSS-1 has also been shown to influence cell adhesion by forming focal adhesions through phosphorylation of focal adhesion kinase and paxillin [11], and an association between loss of KiSS-1 expression and E-cadherin expression was reported in bladder cancer [16].

In our series, there were no significant differences of clinicopathological characteristics between the patients whose tumors showed positive and negative metastin immunostaining, and the result was similar for GPR54. On the other hand, patients whose tumors showed negative immunoreactivity for both metastin and GPR54 had significantly larger tumors than those with lesions positive for either molecule. In addition, recurrence was more frequent in the patients with metastin-negative tumors than in those with metastin-positive tumors. These results suggest that pancreatic cancer loses metastin and GPR54 expression along with its progression. The *KiSS-1* gene is mapped to chromosome 1q32-q41 [33] and *KiSS-1* expression is regulated by genes located on chromosome 6 within the region 6q16.3-q23 [13,28]. These findings are consistent with the fact that loss of 6q, 8p, 9p, 12q, 17p, and 18q is frequently observed in pancreatic cancer[34,35].

Finally, we measured the plasma metastin level in 23 of our patients with pancreatic cancer. We previously found

that the plasma metastin level of patients with pancreatic cancer is significantly higher than that of age- and gender-matched healthy volunteers (unpublished data), so we considered that there was potential to use plasma metastin as a novel tumor marker. In the present series, there was no significant difference of survival between the patients with high and low plasma metastin levels, but no patient with a high plasma metastin level died after surgery. Since the number of patients and the follow-up period are insufficient, more data and further investigation will be needed to clarify the value of measuring plasma metastin.

In this study, the plasma metastin level and metastin immunoreactivity in resected tumor tissues showed a weak correlation. It would be clinically useful if plasma metastin levels had prognostic significance because metastin expression in resected tumor tissues was shown to be a prognostic factor in this study.

Conclusion

In conclusion, expression of metastin and GPR54 was associated with better survival of patients with pancreatic cancer. Metastin expression by cancer tissue was an independent prognostic factor for better survival. Furthermore, the serum metastin level could become a non-invasive prognostic tool for patients with pancreatic cancer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KN conceived of the study and performed immunohistochemical studies and measurements of serum metastin. RD conceived of the study, and participated in its design and coordination and helped to draft the manuscript. FK and TI conceived of the study and performed immunohistochemical studies. AK and MK conceived of the study and performed measurements of serum meatstin. TM, YK, KT, SO and NF conceived of the study and performed experiments on pancreatic cancer tissues. SU conceived of the study, and participated in its design.

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ORIGINAL ARTICLE

Single-institution validation of the international consensus guidelines for treatment of branch duct intraductal papillary mucinous neoplasms of the pancreas

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Abstract
Background The international consensus guidelines (the guidelines) for management of intraductal papillary mucinous neoplasms (IPMNs) of the pancreas recommend surgical resection of branch duct IPMNs with any of the following features: cyst size >30 mm, mural nodules, main pancreatic duct diameter >6 mm, positive cytology, and symptoms. The aim of this study was to evaluate the usefulness of these guidelines for resection of branch duct IPMNs.
Methods We reviewed 84 consecutive patients with branch duct IPMNs who underwent surgical resection at our hospital between January 1984 and December 2007.
Results Sixty-nine patients had indications for resection according to the guidelines. Malignant IPMNs had significantly larger cysts than benign tumors ($P = 0.026$). Patients with malignant IPMNs had significantly more indications for resection than those with benign IPMNs (2.6 ± 1.0 vs. 1.7 ± 0.9, $P < 0.001$), and 36 of the 37 patients with malignant IPMNs had indications. The sensitivity of the guidelines for predicting malignancy was 97.3%. One of 15 patients without indications had malignancy, and the specificity was low (29.8%).
Conclusions The guidelines show a high sensitivity for predicting malignancy of branch duct IPMNs, but the specificity is low. The cyst size and the total number of indications in each patient should be taken into account when predicting the risk of malignancy for branch duct IPMNs.

Keywords Branch duct · Guidelines · Indications · Intraductal papillary mucinous neoplasm

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Introduction

Intraductal papillary mucinous neoplasms (IPMNs) are intraductal mucin-producing neoplasms of the pancreas that cause cystic dilation of the main pancreatic duct, branch ducts, or both [1–6]. This type of neoplasm was first described in 1982 by Ohashi et al. [7] as a mucin-producing tumor of the pancreas. It was termed IPMN by the World Health Organization (WHO) [8, 9] more than a decade later. Recently, IPMNs have been increasingly detected.

Intraductal papillary mucinous neoplasms have malignant potential and undergo transformation from adenoma to borderline neoplasms, followed by the development of carcinoma, including carcinoma in situ (CIS), and then the most advanced stage of invasive carcinoma [10]. Complete resection of noninvasive IPMNs (CIS or earlier disease) has been reported to achieve an excellent survival outcome [6, 11–14]. On the other hand, survival after resection of invasive IPMNs is far worse than after removal of noninvasive tumors [6, 11–14]. There is a general consensus that CIS or invasive IPMNs are potentially fatal unless complete surgical resection can be achieved [10].

Intraductal papillary mucinous neoplasms can be divided into the two major types based on imaging findings and histology: main duct tumors and branch duct tumors [10, 15]. Although a third category of mixed tumors with involvement of both the main and branch ducts has been suggested, it has not yet been clearly defined [10, 15]. The

frequency of malignancy and the survival rate after resection show marked differences between the two types of IPMNs. The international consensus guidelines [15] were published in 2006, and these recommend resection of main duct IPMNs, due to the high risk of malignancy, ranging from 60 to 100% [11, 14, 16–19], if the patient is a good surgical candidate with a reasonable life expectancy. On the other hand, branch duct IPMNs have a lower rate of malignancy (6–51%) [11, 14, 16–18], so the optimal management remains unclear, i.e., observation or resection (and when to resect if this is considered necessary) [15]. We recently reported our 22-year experience with 72 consecutive patients with IPMNs who underwent resection [14]. In that study, cyst size was identified as an independent predictor of malignancy for branch duct and mixed IPMNs, but we concluded that precise preoperative identification of malignancy was difficult. The guidelines include a flowchart covering the suggested procedures for surgical resection and follow-up of branch duct IPMNs. It is recommended that resection should be done if any of the following five factors are present: a cyst >3 cm in diameter, mural nodules, dilation of the main pancreatic duct to more than 6 mm in diameter, positive cytology, and symptoms attributable to the tumor [15].

Although the natural history and characteristics of IPMNs (especially branch duct tumors) are not fully understood yet, an increasing number of surgeons are following the guidelines for their management. The aim of this study was to assess the usefulness of the guidelines in terms of deciding the indications for surgical resection of branch duct IPMNs.

Methods

We retrospectively reviewed a total of 105 consecutive patients with IPMNs who underwent surgical resection between January 1984 and December 2007 at Kyoto University Hospital. The diagnosis of IPMN was histologically confirmed by at least two pathologists who examined the resected specimens.

As preoperative evaluation, computed tomography (CT) was performed in all patients. The patients also underwent endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance imaging (MRI), or both. Pancreatic juice was obtained for cytology during ERCP if possible. Endoscopic ultrasonography was performed in some patients to obtain more detailed information.

Based on the preoperative imaging findings, the tumors were classified as main duct or branch duct IPMNs. Lesions that predominantly involved the main pancreatic duct with dilation to more than 10 mm were classified as main duct IPMNs, while lesions that mainly involved a branch duct without nodules in the main duct were classified as branch duct IPMNs.

We investigated the usefulness of the guidelines for deciding the surgical indications for branch duct IPMNs. Each lesion was histologically graded as an adenoma, borderline neoplasm, CIS, or invasive carcinoma according to the WHO classification of IPMNs [9]. The tumors were also classified into a benign group (adenomas and borderline neoplasms) and a malignant group (CIS and invasive carcinomas).

We evaluated the clinical characteristics and morphological features of branch duct IPMNs based on preoperative imaging findings with respect to cyst size, main pancreatic duct diameter, and the presence of mural nodules. Information on clinical characteristics, including demographic data and presenting symptoms, was obtained from the medical records. Patients who had at least one of the following indications for resection suggested by the consensus guidelines were defined as patients for whom surgery was indicated; cyst size >3 cm, mural nodules, main pancreatic duct diameter >6 mm, positive cytology, and symptoms related to the neoplasm. We regarded Class 4 or 5 pancreatic juice cytology by the Papanicolaou method as positive.

To evaluate the usefulness of the surgical indications listed for branch duct IPMNs, we compared clinical characteristics between the patients with and without indications according to the guidelines, as well as between patients with benign and malignant tumors who had indications for surgery. We also analyzed the accuracy of the guidelines for preoperative diagnosis of malignant IPMNs (CIS and invasive carcinoma).

Continuous variables are expressed as the mean ± standard deviation. Comparison between two groups was performed with the Mann–Whitney U test, while categorical variables were compared by the χ^2 test. The sensitivity, specificity, positive predictive value, and negative predictive value of the guidelines for preoperative prediction of malignancy were calculated. For all analyses, a P value of <0.05 was considered to be statistically significant.

Results

Comparison of branch duct IPMNs with and without indications for resection

Among a total of 105 patients with IPMNs, 84 patients had branch duct IPMNs; their clinicopathological characteristics are shown in Table 1. There were 48 men (57.1%) and 36 women (42.9%) with a mean age at diagnosis of 62.9 years (median age 63 years; range 41–85 years). Pancreatoduodenectomy was performed in 43 patients

Table 1 Comparison of the branch duct intraductal papillary mucinous neoplasms with and without guidelines indications for resection

Clinicopathological characteristics	All patients (n = 84)	With indications (n = 69)	Without indications (n = 15)	P value
Age (years)	63 ± 9	63 ± 9	65 ± 8	0.49
Gender (male:female)	48:36	43:26	5:10	0.04
Tumor location (head:body-tail)	50:34	44:25	6:9	0.09
Cysts size (mm)	34 ± 18	37 ± 18	19 ± 6	<0.001
Presence of mural nodules, n (%)	33 (39.3%)	33 (47.8%)	0	<0.001
Diameter of MPD (mm)	6.2 ± 9.5	7.0 ± 10	2.1 ± 1.4	<0.001
Cytology (positive:negative)	12:23	12:19	0:4	0.12
Symptomatic, n (%)	32 (38.1%)	32 (46.4%)	0	<0.001
Pathology, n (%)				
Adenoma	26 (31.0%)	19 (27.5%)	7 (46.7%)	0.15
Borderline neoplasms	21 (25.0%)	14 (20.3%)	7 (46.7%)	0.03
CIS	17 (20.2%)	16 (23.2%)	1 (6.7%)	0.15
Invasive carcinoma	20 (23.8%)	20 (29.0%)	0	0.02
Malignancy (CIS + invasive carcinoma), n (%)	37 (44.0%)	36 (52.2%)	1 (6.7%)	0.001

MPD main pancreatic duct, *CIS* carcinoma in situ

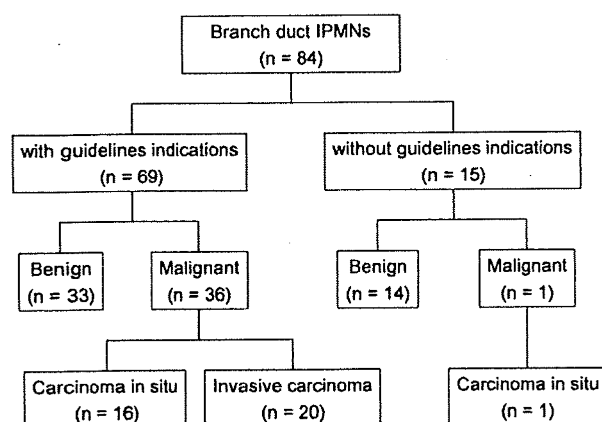

Fig. 1 Classification of 84 resected branch duct intraductal papillary mucinous neoplasms (*IPMNs*) based on the presence or absence of consensus indications for resection and the histological diagnosis. Thirty-six (52.2%) out of 69 patients with indications for resection had malignant tumors (16 carcinomas in situ and 20 invasive carcinomas). Only one (6.7%) of 15 patients without any indications for resection had a malignant tumor (carcinoma in situ)

(51.2%), distal pancreatectomy in 26 (31.0%), total pancreatectomy in ten (11.9%), and partial resection in five (6.0%).

Among the 84 patients with branch duct IPMNs, 37 patients (44.0%) had malignant tumors (CIS in 17 patients and invasive carcinoma in 20). Sixty-nine patients (82.1%) had at least one indication for resection according to the guidelines, whereas 15 patients (17.9%) did not have any indications (Fig. 1). There was no significant difference of age between the patients with and without indications ($P = 0.49$), but the percentage of men was significantly higher among patients with indications than among those without indications ($P = 0.04$).

Of the 69 patients with indications, 36 patients (52.2%) had malignant IPMNs, including 16 patients with CIS and 20 with invasive carcinoma.

Among the 15 patients without indications, only one patient (6.7%) had malignancy (CIS). This patient was a 69-year-old man who had been followed up for chronic hepatitis C. A cystic lesion in the pancreatic body was incidentally found on the CT scan. Both the CT and ERCP scans showed a cystic lesion measuring about 25 mm in diameter without intramural nodules, which communicated with the main pancreatic duct (dilated to 5 mm in diameter). Distal pancreatectomy was performed, and pathological examination of the resected specimen revealed a branch duct IPMN with CIS. At 63 months after surgery, the patient is alive without any evidence of recurrence.

Comparison of benign and malignant branch duct IPMNs with indications for resection according to the guidelines

A comparison of benign and malignant branch duct IPMNs with indications for resection according to the guidelines in terms of patient characteristics is shown in Table 2. Age and gender were similar for the patients with indications whether they had benign or malignant tumors. Malignant IPMNs had significantly larger cysts than benign tumors ($P = 0.026$). In contrast, the main pancreatic duct diameter was significantly greater in benign IPMNs than in malignant tumors. Patients with malignant IPMNs had significantly more indications for resection than those with benign IPMNs (2.6 ± 1.0 vs. 1.7 ± 0.9, $P < 0.001$).

Table 2 Comparison of benign and malignant branch duct IPMNs with indications for resection according to the guidelines

Clinicopathological characteristics	Benign ($n = 33$)	Malignant ($n = 36$)	*P* value
Age (years)	62 ± 8	63 ± 10	0.81
Gender (male:female)	20:13	23:13	0.78
Tumor location (head:body-tail)	20:13	24:12	0.60
Cysts size (mm)	31 ± 13	42 ± 20	0.026
Cyst size >3 cm, *n* (%)	19 (57.6%)	27 (75.0%)	0.13
Presence of mural nodules, *n* (%)	12 (36.4%)	21 (58.3%)	0.07
Diameter of MPD (mm)	7.2 ± 14	6.8 ± 5.1	0.049
Diameter of MPD >6 mm, *n* (%)	11 (33.3%)	16 (44.4%)	0.34
Cytology (positive:negative)	4:9	8:10	0.44
Symptomatic, *n* (%)	10 (30.3%)	22 (61.1%)	0.18
Number of indication factors	1.7 ± 0.9	2.6 ± 1.0	<0.001

Table 3 Assessment of the predictive accuracy in relation to the number of indication factors for resection

	Benign ($n = 47$)	Malignant ($n = 37$)	Total ($n = 84$)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
NIR ≥ 1	33	36	69	97.3	29.8	52.2	93.3
NIR = 0	14	1	15				
NIR ≥ 2	15	30	45	81.1	68.1	66.7	82.1
NIR < 2	32	7	39				
NIR ≥ 3	7	20	27	54.1	85.1	74.1	70.2
NIR < 3	40	17	57				
NIR ≥ 4	1	8	9	21.6	97.9	88.9	61.3
NIR < 4	46	29	75				

NIR number of indication factors for resection, *PPV* positive predictive value, *NPV* negative predictive value

Accuracy of the guidelines for preoperative diagnosis of malignancy in branch duct IPMNs

The predictive accuracy of the guidelines for preoperative diagnosis of malignancy was assessed in relation to the number of indications per patient (Table 3). Among 37 patients with malignant IPMNs, 36 patients (97.3%) had indications for surgery according to the guidelines. The sensitivity of the guidelines for preoperative diagnosis of malignancy was 97.3%. In contrast, one of the 15 patients without any indications had a malignant tumor, so the negative predictive value was 93.3%. However, the specificity (29.8%) and positive predictive values (52.2%) were both relatively low.

As shown in Table 3, we also assessed the diagnostic accuracy in relation to the number of indications for each patient. As patients had more indications, the specificity and positive predictive value of the guidelines for making a preoperative diagnosis of malignancy showed an increase, but the sensitivity and negative predictive value decreased.

Discussion

While there is a general consensus that all main duct IPMNs should be resected [15], the indications for resection of branch duct IPMNs remain controversial because of their lower frequency of malignancy. Studies on the surgical indications for branch duct IPMNs have been conducted to identify preoperative predictors of malignancy, with most of these focusing on an evaluation of the relationship between the morphological features of the neoplasm and the pathological diagnosis of the resected specimen (i.e., pathologically benign or malignant). Based on such studies, indications for resection of branch duct IPMNs were developed for the consensus guidelines [15]. Here, we have evaluated the usefulness of the surgical indications suggested for branch duct IPMNs by the guidelines.

Within the accepted framework that IPMNs with CIS or invasive carcinoma are potentially fatal unless resected [10], while complete resection of noninvasive IPMNs (CIS or earlier disease) achieves excellent survival [6, 11–14], the aim of studies on the indications for resection should be to establish criteria with a high sensitivity for detecting malignant IPMNs. From this perspective, the guidelines were found to be valid for determining surgical indications. In our series, 36 of 37 patients with malignant IPMNs (97.3%) had indications listed in the guidelines. On the other hand, there is the problem that many patients with benign disease might also undergo surgery based on the guidelines, since 33 out of 69 patients (47.8%) with indications actually had benign tumors. Pelaez-Luna et al. [20]

recently reported similar results: the guidelines had a sensitivity of 100% but a low specificity of 23% for the prediction of malignancy in branch duct IPMNs.

In this study, we compared patients with benign and malignant branch duct IPMNs who had indications for resection. We found that the malignant IPMNs had significantly larger cysts than the benign tumors ($P = 0.026$). In contrast, the main pancreatic duct diameter showed the reverse correlation between the two groups ($P = 0.049$), but the actual difference was relatively small and the standard deviation of the diameter was large. These results imply that the cyst size is the most important indication for resection in the guidelines. In addition, the total number of indications was significantly larger in patients with malignant IPMNs (2.6 ± 1.0) than in those with benign tumors (1.7 ± 0.9, $P < 0.001$). We also assessed the diagnostic accuracy in relation to the number of indications for each patient. As patients had more indications, the guidelines showed a higher specificity but a lower sensitivity for predicting malignancy. Because the indications listed in the guidelines have a high sensitivity for predicting malignancy, we are basically following the guidelines in considering surgical resection of branch duct IPMNs in order to avoid losing the chance to cure the disease. In addition, our results suggest that we should take the cyst size and the number of indications for each patient into account when predicting malignancy using these guidelines. Needless to say, the decision about management of the disease must be individualized with due consideration for each patient's age, co-morbidities, and willingness to undergo surgery or to be followed up with surveillance.

In addition, those patients with indications included a higher percentage of men than those without indications, but among the patients with indications, the gender balance did not differ between benign and malignant tumors. Men tend to fit into the group with indications more often, but the reason for this tendency is unclear.

There is increasing evidence that most branch duct IPMNs can be followed up carefully [20–28]. Salvia et al. [25] prospectively evaluated the effectiveness of follow up for a median of 32 months in 89 asymptomatic patients who had branch duct IPMNs (<3.5 cm) without mural nodules. Only five (5.6%) patients showed an increase of lesion size and underwent surgery, and none of them had cancer on examination of the resected specimen. Similarly, Tanno et al. [28] recently reported a prospective study. They found that 69 of 82 patients (84.1%) who had branch duct IPMNs without mural nodules showed no changes of their lesions during a median follow-up period of 61 months. Thus, there are no discrepancies between the evidence obtained by follow-up studies and the surgical indications suggested by the guidelines. In addition to the factors included in the consensus guidelines, accumulating more data on the natural history of branch duct IPMNs should increasingly support the concept of observation and provide more detailed information for selecting patients who can be followed up without surgery. Moreover, the most appropriate surveillance program for improving the survival of patients will need to be established.

In conclusion, based on the current indications for resection, the sensitivity of the guidelines for predicting malignancy of branch duct IPMNs (CIS and invasive carcinoma) is high, but the specificity is low. The cyst size and the number of indications per patient should be taken into account when predicting malignancy for the management of branch duct IPMNs.

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Original Paper

Usefulness of Gemcitabine Combined With 5-Fluorouracil and Cisplatin (GFP) in Patients for Unresectable Biliary Carcinoma

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ABSTRACT:
Background/Aims: Advanced biliary carcinoma have poor prognosis and chemotherapy has been shown to have little impact. The aim of the present study is to clarify the effectiveness of GEM combined with CDDP and 5FU (GFP) therapy for unresectable biliary carcinoma.
Methodology: Fourteen patients with biliary carcinoma (4 patients; gallbladder cancer, 10 patients; biliary tract) who had no prior chemotherapy were enrolled. A triple combination of agents was administered with a 4-week cycle GFP chemotherapy consisting of GEM at 1000mg/m^2 on days 1 and of 5-FU at 250mg/m^2 and CDDP at 3mg/m^2 on days 1 to 5.
Results: No patient achieved CR, while five patients achieved PR as assessed by RECIST. The overall response rate from the intent-to-treat analysis was 21.4%. Stable disease was observed in 9 (64.3%) patients. Clinical benefit rate was observed in 14 (85.7%) patients. According to the tumor site, overall response rate was 20.0% in biliary tract carcinoma, on the other hand, 25.0% in gallbladder carcinoma.
Conclusions: The significant antitumor activity of GFP chemotherapy has been seen in patients with advanced biliary carcinoma. However, further evaluation in large numbers of patients is needed to determine the difference in chemosensitivity according to the tumor site.

KEY WORDS:
Biliary carcinoma; Gemcitabine; Combined chemotherapy

ABBREVIATIONS:
Gemcitabine (GEM); Cisplatin (CDDP); 5-Fluorouracil (5-FU); Complete Response (CR); Partial Response (PR); Progressive Disease (PD); Stable Disease (SD); Time to Disease Progression (TTP)

INTRODUCTION:

Biliary carcinoma is a relatively uncommon malignancy worldwide. However, the incidence of this disease has increased markedly in Japan over the past several decades. Vital statistics in 2004 in Japan showed biliary carcinoma was the sixth leading cause of carcinoma death with approximately over 16,000 deaths and a mortality rate of 13.0 per 100,000 (1). Clinically, biliary carcinoma is one of the most aggressive tumors and has a poorer prognosis. Complete surgical resection of this disease is the only therapy to have shown a survival benefit, however, only a small minority (<25%) of patients with this disease are eligible for surgery because of metastasis or invasion of the tumor directly into adjacent organs at diagnosis. Moreover, even patients who have undergone surgical resection eventually have a recurrence of disease (2-7). However, to date, chemotherapy has been shown to have little impact on this disease and effective chemotherapeutic agents and regimens for this carcinoma have not been established as yet. Therefore, it is essential to develop new therapeutic strategies to affect clinical outcomes in this patient population.

Gemcitabine (GEM) is a novel nucleoside analog demonstrating biological activities in a broad spectrum of solid tumors including pancreas cancers (8, 9). Because the biliary apparatus (gallbladder and bile ducts) shares a common embryological origin with the exocrine pancreas), the possibility that GEM may be an active agent for this disease has recently been investigated. However, clinical efficacy with GEM as a single agent remains poor in some clinical studies (10), and GEM-based combinations are needed to improve outcomes. Over the past few years, there has been a development with the use of GEM plus cisplatin (CDDP) in the treatment of patients with several types of malignant disease (11, 12). The choice of such a combination of drug therapy was based on theoretical considerations and results of laboratory experiments (13). Moreover, GEM and 5-fluorouracil (5-FU) in combination appear to have synergy in preclinical studies (14). A phase II trial, evaluating in a protracted intravenous infusion plus weekly GEM in patients with pancreatic carcinoma, showed promising activity (15). So, the results regarding the effect of GEM combined with 5-FU and CDDP (GFP) chemotherapy for advanced biliary tree

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cancers have been reported in a pilot study (16). However, the number of patients participating in this pilot study was very small and the most effective combination of chemotherapy for advanced biliary carcinomas remains unclear.

In regard to these considerations, we applied GEM combined with 5-FU and CDDP on the unresectable biliary carcinoma from 2004 in our department. In this study, we investigated the effectiveness of GFP chemotherapy for unresectable biliary carcinoma. In addition, the prognosis of patients who received GFP chemotherapy is compared to our previous patients before the application of GFP chemotherapy.

METHODOLOGY

Eligibility criteria

Enrolled patients had histologically confirmed advanced biliary tract carcinoma (intrahepatic and extrahepatic) or adenocarcinoma of the gallbladder. The eligibility criteria for this study were as follows; 1) age between 18 and 85 years; 2) at least 1 measurable lesion according to the Response Evaluation Criteria in Solid Tumors17) (RECIST, National Cancer Institute, Cancer Therapy Evaluation Program, available from URL: http://ctep.cancer.gov/guidelines/recist.html); 3) Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; 4) a life expectancy of at least 2 months; 5) adequate bone marrow function white blood cell count (WBC) of at least 1500/mm^3, platelet count of at least 100,000/mm^3, aspartate aminotransferase/alanine aminotransferase (AST/ALT) of no more than 5.0 times the upper limit of normal, total bilirubin of 5.0mg/dl or less, serum creatinine of 1.5mg/dl or less; and 6) written informed consent.

Patients who had undergone prior chemotherapy were excluded. Patients were excluded from the study if they had pulmonary fibrosis, interstitial pneumonia, New York Heart Association class III or IV congestive heart failure, myocardial infarction within the preceding 6 months, diabetes mellitus with severe complications, marked pleural or pericardial effusion, marked peripheral edema, or active infection. The study was conducted in accordance with the ethical principles stated in the most recent version of the Declaration of Helsinki or the applicable guidelines on good clinical practice, whichever represented the greater protection of the individual.

Treatment Plan

One 4-week course of treatment included a triple combination of agents that were administered. GEM (1000 mg/m^2) diluted with 100 mL solution of normal saline was administered intravenously (i.v.) over 30 minutes on days 1, 8, 15 and 22. CDDP at 3mg/m^2/day and 5-FU at 250 mg/m^2/day were given via a peripherally on days 1 to 5, 8 to 12, 15 to 19 and 22 to 26. Furthermore, after 2 cycles, for outpatients, Gemcitabine (1000 mg/m^2) and CDDP (3mg/m^2/day) diluted with 100 mL solution of normal saline respectively were administered intravenously (i.v.) over 30 minutes on days 1 and 15. UFT at 300 mg/body were orally given on days 1 to 5, 8 to 12, 15 to 19 and 22 to 26.

Patient evaluation

The antitumor response was evaluated by CT or MRI every 2 cycles of chemotherapy. Responses to chemotherapy were assessed according to RECIST criteria. A complete response (CR) was defined as the complete disappearance of all evidence of the tumor. A partial response (PR) was defined as a decrease of at least 30 % in the sum of the longest diameters of target lesions without appearance of new lesions or progression of non-target lesions. To be assigned a status of response, changes in tumor measurement were confirmed by repeat assessment that was performed no less than 4 weeks after the criteria for response were first met. A progressive disease (PD) was defined as a 20% increase in the sum of the largest diameters of target lesions or as appearance of new lesions or as progression of non-target lesions. A stable disease (SD) was defined as no sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD in the sum of the largest diameters of target lesions without appearance of new lesions or progression of non-target lesions. Disease control was defined as the absence of tumor progression for at least two months. Time to disease progression (TTP) was calculated from the date of patient enrollment to the date of the following events of disease progression. Adverse events were evaluated using the NCI's Common Toxicity Criteria, Version 2.0 (NCI CTC V2.0; available from URL: http://ctep.cancer.gov/reporting/ctc.html).

Dose modification

In cases of grade 3–4 leukocytopenia or thrombocytopenia, the next chemotherapy schedule would be delayed until there was sufficient recovery of WBC or platelet count (WBC 3000/mm^3, platelet count 75 000/mm^3). The dose of gemcitabine would not be reduced in the next cycles. Patients with PD were withdrawn from the study. If the patients' medical conditions could tolerate more chemotherapy, they could receive second-line chemotherapy with TS-1 based chemotherapy at the physician's discretion. In patients with PR or SD, treatment was continued until unacceptable toxicities occurred or there was evidence of disease progression. All measurable lesions would be re-evaluated by imaging studies after every two cycles of chemotherapy to re-assess the response.

Statistical analysis

Both overall survival and progression free survival were calculated from the date of patient enrollment to the date of death or the date of the following events of disease progression using the Kaplan-Meier method. The difference in clinical parameters between responders and non-responders to protocol

treatment was evaluated by χ^2 test or Wilcoxon rank-sum test. A p value < 0.05 was considered statistically significant.

RESULTS

Patients' characteristics

From April 2004 to January 2007, 14 chemotherapy-naive patients with locally advanced or metastatic biliary carcinomas who met the inclusion criteria were enrolled. The clinical characteristics of these patients are summarized in **Table 1**. The median age was 65 years (range 39–85). There were 12 men (86.0%) and 2 women (14.0%). Nine patients (64.3%) had a performance status of 0 or 1, and 5 patients (35.7%) had a performance status of 2. Four (28.6%) had gallbladder adenocarcinoma, seven patients (50.0%) had intrahepatic biliary tract carcinoma and three (21.4%) had extrahepatic biliary tract carcinoma. Evidence of metastatic disease was present in all 14 patients at the time of study enrollment. The most common metastatic site was lymph node. A total of 3 patients had obstructive jaundice that required biliary drainage (2 percutaneus transhepatic biliary drainage; one internal biliary stent) before enrollment. All patients were assessable for efficacy and toxicity analysis.

Delivery of drugs (treatment)

In this study, a total of 71 cycles of the regimen were administered with a median of 5 cycles per patient (range, 2-16 cycles). Seven patients (50%) completed at least 5 cycles of therapy. Dose or schedule modification was necessary in all patients. The most common causes of dose modification were leucopenia (71.4%) and thrombocytopenia (28.6%).

Efficacy (Response and survival)

All patients were evaluated for response. No patient achieved CR, while five patients (one woman; two men) achieved PR. The overall response rate from the intent-to-treat analysis was 21.4%. An additional 9 (64.3%) patients were observed and SD as well as PR was experienced in 2 (14.3%) patients. The disease control rate was observed in 12 (85.7%) patients. The median progression-free and overall survival time of all 14 patients were 6.0 months and 8.7 months, respectively **(Fig. 1)**. This result was superior to historical data (n=37) before application of GFP chemotherapy. At the end of periodic monitoring for this study, the overall survival for the five responders was 7.6, 8.3 and 18.3 months respectively.

According to tumor site, overall response rate was 20.0% (2 patients) in biliary tract carcinoma, on the other hand, 25.0% (1 patient) in gallbladder carcinoma **(Table 2)**. However, overall survival time and median time to progression in biliary tract carcinoma was superior to that in gallbladder **(Figure 2)**. Furthermore, in gallbladder carcinoma, three patients who met the criteria for response only did so up to 2.2 months after treatment and TTP of PR was shorter than that of SD (data not shown).

There was no significant difference in the clinicopathological features between the responders and non-responders in terms of age, sex, performance status, number or site of involved organs, or site of primary tumor except gender. All responders had improvement in tumor-related symptoms and performance status.

Table 1 Patients' Demographics

Characteristics		No. of patients	%
Age	Median (Range)	65 (39-85)	
Gender	Male	12	86
	Female	2	14
ECOG performance	0-1	9	64
Status	2	5	36
Primary site	Gallbladder carcinoma	4	29
of tumor	Biliary		
	intrahepatic	7	50
	extrahepatic	3	21
Site of metastatic	Liver	8	57
disease	Lymph nodes	9	64
	Peritoneal implants	2	14
	Lung	4	29
	bone	2	14
Obstructive jaundice		3	21

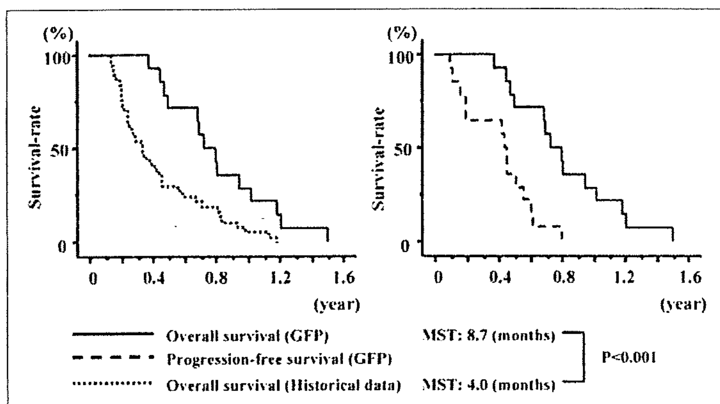

FIGURE 1 Overall and progression free survival curve; GFP chemotherapy improved overall survival curve of patients with biliary carcinoma.

Table 2 Tumor Response of GFP Chemotherapy

		Biliary (n=10)	Gallbladder (n=4)	Overall (n=14)
Response	Complete response	0 (0%)	0 (0%)	0 (0%)
	Partial responce	2(20.0%)	1 (25.0%)	3 (21.4%)
	Stable disease	7 (70.0%)	2 (50.0%)	9 (64.3%)
	Progression of disease	1 (10.0%)	1 (25.0%)	2 (14.3%)
Overall	Median (months)	9.6	6.0	8.7
survival	Range (months)	5.4-18.0	4.5-11.3	4.5-18.0
Time to	Median (months)	6.0	2.2	6.0
progression	Range (months)	1.3-11.0	1.6-7.0	1.3-11.0

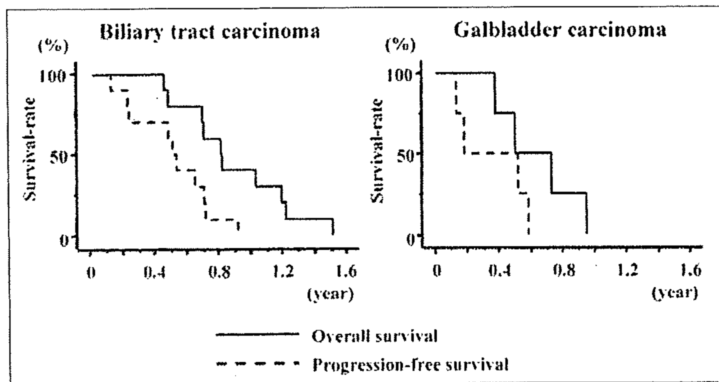

FIGURE 2 Overall and progression free survival curve according to the tumor site.

Table 3 Toxicity Profile of All 14 Patients Enrolled

NCI-CTC Grade		Number of patients (%) 1	2	3	4
Hematological	Anemia	3 (21.4)	6 (42.9)	4 (28.6)	0 (0.0)
	Leukemia	0 (0.0)	4 (28.6)	9 (64.3)	1 (7.1)
	Thrombocytopenia	5 (35.7)	5 (35.7)	4 (28.6)	0 (0.0)
Non-hematological	Anorexia	3 (21.4)	1 (7.1)	0 (0.0)	0 (0.0)
	Nausea	1 (7.1)	2 (14.2)	0 (0.0)	0 (0.0)
	Diarrhea	0 (0.0)	1 (7.1)	1 (7.1)	0 (0.0)
	Fatigue	0 (0.0)	1 (7.1)	0 (0.0)	0 (0.0)

Toxicities

All patients were evaluated for toxicity, and toxicities observed during treatment are listed in **table 3**. This GFP chemotherapy was generally well tolerated. There were no treatment-related deaths. No patients discontinued treatment because of toxicity.

NCI-CTC Grade 3 or 4 hematologic toxicities included leucopenia in 10 (71.4%) patients , thrombocytopenia 4 patients (28.6%) and anemia in 4 (28.5%) patient. One of 10 patients with Grade 3 or 4 neutropenia had febrile episodes (7.1%). NCI-CTC Grade 3 or 4 non hematologic toxicities included diarrhea in 1 (7.1%) patient.

Presentation of a case with partial response

A 45-years old male patient with intrahepatic biliary tract carcinoma was examined for treatment. Most of the liver was occupied by a massive tumor and swelling, and paraaortic lymph nodes metastasis was suspected. Pathological diagnosis using a needle biopsy of the liver was moderately differentiated adenocarcinoma. A partial response was reached after 2cycles of GFP chemotherapy, and the liver tumor obviously reduced **(Figure 3B)**. Furthermore, after 4 cycles, the liver tumor almost disappeared **(Figure 3C)**. This patient received 6 cycles of GFP chemotherapy. However, 9 months after induction of GFP chemotherapy, the main liver tumor became enlarged and lung metastasis was observed. Unfortunately, this patient died 18.3 months after induction of GFP chemotherapy. Patient's quality of life had been kept constant for the treatment period.

DISCUSSION

To date, the optimal role of chemotherapy has not yet been established for advanced biliary carcinoma, and there is no standard first line chemotherapy. In our trial, with an overall response rate of 21.4%, an additional 64.3% with durable stable disease and modest hematological and non-hematological toxicity, our results compare very favorably with other regimens evaluated tumor site. The TTP and OS were 6.0 and 8.7 months, respectively.

In preclinical studies, it has been shown that gemcitabine may increase the formation of DNA-platinum adducts, and cisplatin may increase the incorporation of gemcitabine into DNA (17). It has been shown that this synergistic interaction is related to a decrease in repair of DNA-platinum adducts (18, 19). The combination of gemcitabine and cisplatin has been explored in several studies (11-12, 20-21). Furthermore, gemcitabine and 5-FU in combination appear to have synergy in the laboratory data (14). On the basis of pre-clinical studies, gemcitabine potentially augments the activity of 5-FU by its direct inhibition of ribonucleotide reductase. Furthermore, a phase II trial, evaluating in a protracted 5-FU intravenous infusion plus weekly gemcitabine in patients with pancreatic carcinoma, showed promising activity. Recently, several Phase II trials of gemcitabine and various other agents with various schedules have been evaluated for the treatment of advanced biliary tract carcinoma. These trials showed various objective response rates ranging from 20.0% to 45.0%, and median survival time ranging

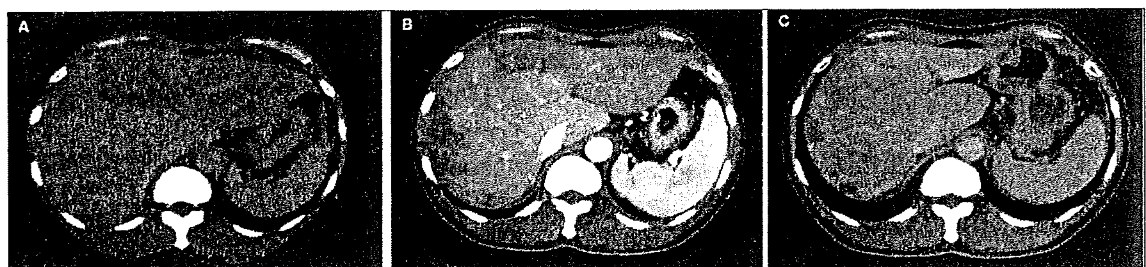

FIGURE 3 Effective case with intrahepatic biliary tract carcinoma. **A**; Most of the liver was occupied by a massive tumor and swelling, **B**; The liver tumor obviously reduced after 2cycles of GFP chemotherapy, **C**; The liver tumor almost disappeared after 4cycles of GFP chemotherapy.

from 5.0 to 14.0 months, which seem to be much higher than in previous trials using gemcitabine alone (22-25). Gemcitabine combined with other agents may provide other potential options of building on the activity of gemcitabine. Therefore, we thought that a further synergy effect was obtained by using triple agent chemotherapy for advanced biliary carcinoma.

In this trial, we did not experience an impressive response such as that stated in the previous report. It is difficult to compare directly the response rate in different trials because of the relatively small sample size and the heterogeneous patient populations of their series. However, in this trial, both response rates and median survival was relatively favorable compared with other reports. Furthermore, a relatively high disease-control rate was observed. This combination of triple agents was relatively well tolerated. While non-hematological toxicity of grade 3/4 was not observed through this trial, the relatively high incidence of leucocytopenia in our patients compared to the previous report is an important concern. Therefore, dose modification was needed in particular this morbid patients. However, there were no serious outcomes of adverse events in this trial.

Interestingly, according to the tumor site, the difference of tumor response was experienced in this trial. While similar response rates were observed in gallbladder (25.0%) carcinoma than that in biliary tract carcinoma (20.0%), over all survival time (median: 6.0 months *v.s.* 9.6 months) and TTP (median: 2.2 months *v.s.* 6.0 months) was better in biliary tract carcinoma than in gallbladder. Moreover, the disease control period in gallbladder carcinoma was within about 2 months, and this period was very much shorter than that of the biliary tract carcinoma. A reason for this is that carcinomas of biliary and gallbladder may be biologically sufficiently different as to lead to different sensitivities to chemotherapy. In other several reports, response rates of patients with gallbladder carcinoma were higher compared with biliary tract carcinoma, though there was no significant difference in overall survival rates between gallbladder carcinoma and biliary tract carcinoma (26). However, the relationship between chemosensitivity and cellular origin of biliary carcinoma, as well as the differential activity of gemcitabinem, cisplatin and 5-FU on advanced biliary carcinoma, remains to be clarified.

In conclusion, our trial indicates that a regimen of gemcitabine combined with 5-fluorouracil and cisplatin is an active and relatively well tolerated new therapeutic option as first line chemotherapy for patients with advanced biliary tract carcinoma. Furthermore, this regimen warrants further evaluation in detail to determine whether biological activity according to the tumor site of the biliary carcinoma reflects chemosensitivity in a stratification strategy.

FOOTNOTES:

The authors have no conflict of interest to declare regarding the contents of this manuscript.

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Tumor–stromal interactions with direct cell contacts enhance proliferation of human pancreatic carcinoma cells

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Pancreatic ductal adenocarcinoma is often characterized by an abundant desmoplastic stroma that is partially induced by activated pancreatic stellate cells (PSCs). Indirect co-culture has often been used to investigate the effects of cancer–stromal interactions on the proliferation of cancer cells, but the effects of cell–cell adhesion and juxtacrine signaling between cancer and stromal cells cannot be evaluated using this method. This study aimed to establish a simplified direct co-culture system that could be used to quantify populations of cancer cells in co-culture with PSCs, and to evaluate the effects of direct cell contact on the proliferation of cancer cells. We established three green fluorescent protein (GFP)-expressing pancreatic cancer cell lines and were able to quantify them with high reliability and reproducibility, even when co-cultured directly with PSCs, using a color plate reader. We assessed the differential effects of direct and indirect co-culture with PSCs on the proliferation of cancer cells, and found that the proliferation of GFP-expressing pancreatic cancer cell lines was dramatically enhanced by direct co-culture with PSCs, compared with the indirect co-culture system. We also found that direct co-culture of cancer cells and PSCs activated the Notch signaling pathway in both cell types. Direct cell contact between cancer cells and PSCs plays an important role in the control of cancer cell proliferation, and is essential to the understanding of tumor–stromal interactions. (*Cancer Sci* 2009; 100: 2309–2317)

Pancreatic ductal adenocarcinoma (PDAC) is often characterized by an abundant desmoplastic stroma,[1–3] which is defined as a proliferation of fibrotic tissue with an altered extracellular matrix (ECM) that is conductive to tumor growth and metastasis.[4–7] The host's desmoplastic reaction is characterized by complex interactions between normal host epithelial cells, invading tumor cells, stromal fibroblasts, inflammatory cells, proliferating endothelial cells, the altered ECM, and growth factors, which activate oncogenic signaling pathways by autocrine and paracrine mechanisms.[7–10] Recently, a pronounced increase in the number of α-smooth muscle actin (α-SMA)-positive myofibroblasts was reported in PDAC.[3] In addition, other studies have demonstrated that pancreatic stellate cells (PSCs) are associated with tumor desmoplasia.[1,8,11] Although the desmoplastic reaction was initially regarded as a host barrier against tumor invasion, it has become evident that pancreatic cancer cells induce fibrosis by activating PSCs to synthesize excessive ECM.[1,3,12] The ECM influences the growth, differentiation, survival, and motility of cells by both providing a physical scaffold and acting as a reservoir for soluble mitogens.[5,6,9,10] PSCs have also been reported to inhibit apoptosis[13,14] and enhance the migration and invasion of pancreatic cancer cells.[14,15] The tumor-supportive microenvironment is thus a dynamic environment that promotes tumor growth and invasion.

Several models have been established to investigate tumor–stromal interactions, including *in vivo* xenograft models,[16–20] *in vitro* three-dimensional co-culture models,[21–24] *in vitro* two-chamber co-culture models using culture inserts,[10,18,25–29] and *in vitro* direct co-culture models.[30–32] The two-chamber co-culture models, which are often used for *in vitro* experiments, are not suitable for investigating the effects of direct cell contacts between stromal cells and cancer cells on tumor biology. In contrast, the *in vivo* xenograft and *in vitro* direct co-culture models can be used to evaluate the effects of cell–cell adhesion and juxtacrine signaling, but simple and reproducible quantitative assessment of cell populations using these methods remains problematic. Krtolica *et al.*[33,34] established a method for quantifying a population of epithelial cells directly co-cultured with fibroblasts using fluorescence imaging of 4,6-diamidino-2-phenylindole (DAPI) and green fluorescent protein (GFP). In the present study, we modified and simplified this method to investigate the parameters affecting cell growth with high sensitivity, high reproducibility, and ease of handling, which are difficult to achieve with other available methods. We quantified the population of GFP-expressing cells using a color plate reader,[35–38] and were able to quantitatively detect GFP-expressing cancer cells, even in direct co-culture with PSCs. We compared the use of direct and indirect co-culture systems for investigating the effects of cell interactions with PSCs on the proliferation of GFP-expressing pancreatic cancer cell lines. Furthermore, to investigate the effects of the juxtacrine mechanism, we assessed the associations of the Notch signaling pathway with these two co-culture systems.

Materials and Methods

Establishment of cell lines constitutively expressing GFP. We used three pancreatic cancer cell lines in our study (Table 1). SUIT-2 and Panc-1 were generously provided by Dr H. Iguchi, (National Shikoku Cancer Center, Matsuyama, Japan), and MIA PaCa-2 was obtained from the Japanese Cancer Resource Bank (Tokyo, Japan). Cells were maintained as previously described.[18] A pAcGFP1-N1 vector (Clontech, Palo Alto, CA, USA) encoding GFP was used to create stable GFP-expressing cell lines (GFP-SUIT-2, GFP-Panc-1, and GFP-MIA PaCa2). The pAcGFP1-N1 vector was electroporated into SUIT-2, Panc-1, and MIA PaCa-2 cell lines using Nucleofector (Amaxa Biosystems, Koln, Germany), according to the manufacturer's instructions. SUIT-2 cells electroporated with pAcGFP1-N1 were selected for neomycin resistance (G418, 800 μg/mL) in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical

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Table 1. Cell lines

Cell line	Tissue origin	Diagnosis	Doubling time (h)
Pancreatic cancer cell lines constitutively expressing GFP			
GFP-SUIT-2	Liver metastasis	PDAC	19.56 ± 0.73
GFP-Panc-1	Pancreas	PDAC	22.24 ± 1.10
GFP-MIA PaCa-2	Pancreas	PDAC	16.81 ± 0.15
Fibroblast cell line			
MRC5	Human embryonic lung		27.75 ± 1.37
Primary cultured myofibroblasts			
NPF-1	Normal pancreas	Benign endocrine tumor	24.26 ± 2.48
NPF-2	Normal pancreas	Bile duct carcinoma	53.28 ± 2.47
PCF-1	Pancreatic cancer	PDAC	40.60 ± 0.75
PCF-2	Pancreatic cancer	PDAC	21.74 ± 2.18
MCF-1	Metastatic tumor of abdominal wall	PDAC	30.84 ± 3.15

GFP, green fluorescence protein; PDAC, pancreatic ductal adenocarcinoma.

Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and penicillin (100 U/mL). Green colonies were isolated and grown in the absence of selective pressure for several months. Panc-1 and MIA PaCa-2 cells electroporated with pAcGFP1-N1 were sorted using a cell sorter (Epics Altra; Beckman Coulter, Fullerton, CA, USA), according to the manufacturer's instructions.

Fibroblasts and myofibroblasts including PSCs. We used a human fibroblast cell line MRC5 (Riken, Tokyo, Japan). Two cultures of PSCs derived from normal pancreases without pancreatitis (NPF-1, from a patient with a benign endocrine tumor of the pancreas, and NPF-2, from a patient with bile duct cancer), two cultures of PSCs derived from pancreatic cancer tissues of patients with PDAC (PCF-1 and PCF-2), and a culture of myofibroblasts derived from a metastatic tumor of the abdominal wall in a patient with PDAC (MCF-1) were also used in this study (Table 1). All primary cultures of myofibroblasts were isolated using the outgrowth method, as described previously.[39] Cells were maintained as described previously.[18]

Propidium iodide (PI) assay. To calculate the doubling time of each cell line, cells were seeded in 24-well plates (Becton Dickinson Labware, Bedford, MA, USA) at a density of 1×10^4 cells/well, using cell numbers previously counted using a particle distribution analyzer (CDA 500; Sysmex, Kobe, Japan). Cell populations were evaluated by measuring the fluorescence intensity of PI at specified times, as described previously.[40]

GFP fluorescence measurements. The fluorescence of cells in multiwell plates was quantified in triplicate using a Cytofluor II (Perseptive Biosystems, Framingham, MA, USA) at gain 80, with filter settings of excitation at 485 nm with a bandwidth of 20 nm and emission at 530 nm with a bandwidth of 25 nm, as described previously.[35–38] Fluorescence intensity was calculated in relative fluorescence units (RFU). The nonspecific signal of wells containing cell-free medium or PSCs alone (blank value) was subtracted from the results to give the fluorescence signal of the GFP-expressing cells. To obtain sensitive and reproducible measurements, we used DMEM without phenol red.

***In vitro* direct co-culture system.** For proliferation assays, 1×10^4 GFP-expressing cancer cells were mixed with 1×10^4 stromal cells. Each cell mixture was seeded in a 24-well plate (1×10^4 cancer cells/well) in triplicate, and cultured in DMEM supplemented with 1% or 10% FBS. The fluorescence signals of each well were detected at specified times. To analyze GFP expression, 5×10^4 GFP-SUIT-2 cells were mixed with 5×10^4 PSCs, seeded in a six-well plate in triplicate, and cultured in DMEM supplemented with 10% FBS for 3 days. After harvesting the cells, the total cell number was determined using the CDA 500. The PSC/GFP-SUIT-2 cell proportion was determined using a cell sorter (Epics Altra) based on the GFP fluorescence as described previously,[30,32] and we isolated GFP-SUIT-2 cells and GFP-negative PSCs according to the manufacturer's instructions.

***In vitro* indirect co-culture system.** For proliferation assays, 1×10^4 GFP-expressing cancer cells were seeded in triplicate into the lower wells of a transwell cell culture system (24-well type, fluoroblock membrane with 3-μm pores; Becton Dickinson, San Jose, CA, USA) and 1×10^4 PSCs were seeded into the upper chambers (cell culture inserts), and cultured in DMEM supplemented with 1% or 10% FBS. The fluorescence signals of each well were detected at specified times. To analyze GFP expression, 5×10^4 GFP-SUIT-2 cells were seeded in triplicate into the lower wells of a transwell cell culture system (six-well type, 3-μm pores; Becton Dickinson) and 5×10^4 PSCs were seeded into the upper chambers, and cultured in DMEM supplemented with 10% FBS for 3 days. After harvesting the cells, the total cell number was determined using the CDA 500.

Immunoblot analysis for α-smooth muscle actin (α-SMA). Immunoblot analysis for α-SMA was performed as described previously.[18] Briefly, whole-cell lysates were fractionated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was incubated with 1:500 dilutions of monoclonal mouse antihuman α-SMA antibody (Dako, Glostrup, Denmark) overnight at 4°C, and then probed with antimouse IgG conjugated with horseradish peroxidase (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. Immunoblots were detected using the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK) and visualized with a Molecular Imager (ChemiDoc XRS System; Bio-Rad Laboratories, Hercules, CA, USA). The membrane was stripped and probed with anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), as an internal control.

Immunofluorescence staining of α-SMA. PSCs (5×10^4) were seeded on six-well plates and cultured in DMEM supplemented with 10% FBS for 24 h. Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100, blocked with blocking solution (1% FBS and 1% BSA in PBS), and incubated with 1:500 dilutions of monoclonal mouse antihuman α-SMA antibody (Dako) for 2 h at room temperature. The cells were then incubated for 1 h with Alexa 546-conjugated antimouse IgG (Molecular Probes, Eugene, OR, USA) and 0.05 μg/mL DAPI. A TE-2000U inverted microscope (Nikon, Tokyo, Japan) was used for immunofluorescence microphotography and images were managed using VB-Viewer software (Keyence, Osaka, Japan).

Flow cytometry. Cellular expression of α-SMA was examined by flow cytometry (Epics Altra) using a phycoerythrin (PE)-conjugated monoclonal mouse antihuman α-SMA antibody (R&D

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Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Non-specific mouse IgG (Miltenyi Biotec, Auburn, CA, USA) was used as a negative control.

Quantitative real-time reverse transcription–polymerase chain reaction (qRT–PCR). Total RNA was extracted from cultured cells using a High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. qRT–PCR was performed using a QuantiTect SYBR Green RT–PCR Kit (Qiagen, Tokyo, Japan) with Opticon4 (Bio-Rad Laboratories), as described previously.[41] Briefly, the reaction mixture was first incubated at 50°C for 15 min to allow reverse transcription. PCR was initiated with one cycle at 95°C for 10 min to activate modified Taq polymerase, followed by 45 cycles at 94°C for 15 s, 55°C for 20 s, and 72°C for 10 s, and one cycle at 95°C for 0 s, 65°C for 15 s, and +0.1°C/s to 95°C for melting analysis. Each sample was run in triplicate. The 10% deviation was calculated from the concentrations determined from the calibration curve. The level of mRNA expression was calculated from a standard curve constructed using total RNA from MRC5 cells. We designed specific primers (Table 2), and screened a database with BLASTN to confirm the specificity of these primers. Primers for *Snail* were designed by Takara Bio (primer set ID: HA075019; Ohtsu, Shiga, Japan). Expression of each mRNA was normalized to that of *18S rRNA*.

Statistical analysis. Statistical analyses and graph presentations were carried out using JMP 7 (SAS Institute, Cary, NC, USA). Values were expressed as the mean ± SD. Comparisons between two groups were performed using Student's *t*-test. The level of statistical significance was set at $P < 0.05$. Correlations between two groups were statistically evaluated by regression analysis and by calculating Spearman's rank-correlation coefficient.

Results

Correlation between the number of cells and fluorescence intensity of GFP. We established three pancreatic cancer cell lines constitutively expressing GFP, as described in the Materials and Methods (Fig. 1a). These clones were confirmed by flow cytometry to be >99% GFP-positive in comparison with the non-GFP-expressing parental cell lines (Fig. 1b). In the first series of experiments, we evaluated the efficiency of GFP fluorescence for the determination of cell numbers (Fig. 1c). Regression analysis confirmed that the fluorescence intensity of total GFP-SUIT-2 cells was correlated with the cell numbers counted within the range from 5×10^3–1×10^6 cells/well (Fig. 1d; Spearman's rank-correlation coefficient: 0.995, $P < 0.0001$). Similarly, there were significant correlations between fluorescence intensity and the numbers of GFP-Panc1 and GFP-MIA PaCa-2 cells (data not shown). In addition, we found a significant correlation between GFP fluorescence and PI fluorescence calculated by PI assay as another method for evaluating cell proliferation (Supporting Information Fig. S1a,b; Spearman's rank-correlation coefficient: 0.998, $P < 0.0001$). The lower limit of detection for GFP fluorescence was in the order of 1000 cells/well for these cell lines (data not shown). At $\geq 1 \times 10^6$ cells/well, cells became confluent in flat-bottomed 24-well plates; thus, 1×10^4–5×10^5 cells were used in subsequent experiments.

α-SMA expression in myofibroblasts. To elucidate tumor–stromal interactions between pancreatic cancer cells and PSCs, we isolated bulky lines of myofibroblasts from resected normal pancreas, pancreatic cancer tissue, and a metastatic tumor from a patient with PDAC using the outgrowth method, as described previously.[39] To confirm that PSCs expressed high levels of α-SMA and collagen type I (COL1),[1,12,39,42] we analyzed the expression levels of *α-SMA* and *COL1* mRNAs in these myofibroblast cultures. All myofibroblast cultures expressed higher levels of *α-SMA* and *COL1* mRNA than MRC5 and cancer cells (Fig. 2a,b). Interestingly, MCF-1 myofibroblasts derived from a metastatic tumor also expressed high levels of *α-SMA* and *COL1* mRNAs. Immunoblot analysis and immunofluorescence staining revealed that these myofibroblast cultures expressed α-SMA protein (Fig. 2c,d). We further found that >80% of PSCs and myofibroblasts expressed α-SMA by flow cytometry (Fig. 2e,f). We used these four PSC cultures and one myofibroblast culture to establish a simplified direct co-culture system using GFP-expressing cancer cells in the following experiments.

Effects of co-culture on cell morphology. Indirect co-culture has often been used to investigate the effects of cancer–stromal interactions on the proliferation of cancer cells, because of its easy evaluation (Fig. 3a). However, the effects of cell–cell adhesion and juxtacrine signaling between cancer and stromal cells cannot be evaluated by this method. To evaluate these effects, we established a direct co-culture system using GFP-expressing cells (Fig. 3a). Initially, we assessed the effects of direct co-culture with PSCs on the morphology of cancer cells. Monocultured GFP-SUIT-2 cells were almost round in shape (Fig. 3b), whereas indirectly and directly co-cultured cells exhibited a fibroblastoid morphology (Fig. 3b). These findings suggest that co-culture with PSCs promoted the epithelial–mesenchymal transition (EMT)[43] of GFP-SUIT-2 cells. We evaluated the effects of the morphological alterations on the GFP expression levels in GFP-SUIT-2 cells by flow cytometry and found that there was no significant difference between monocultured and co-cultured cells (Fig. 3c). To confirm the induction of the EMT in co-cultured GFP-SUIT-2 cells, we isolated them using a cell sorter (Fig. 3d), and quantified the mRNA levels of EMT markers, including *Snail*, *Vimentin*, and *N-cadherin* (Fig. 3e).

Table 2. Primer sequences and product size

Primer	Forward	Reverse	Product size
	Sequence 5′–3′	Sequence 5′–3′	
α-SMA	ccgggagaaaatgactcaaa	gcgtccagaggcatagagag	97
COL1	acgtgatctgtgacgagacc	agcaaagtttcctccgaggc	250
Snail	Takara Bio (primer set ID: HA075019)		
Vimentin	tgcccttaaaggaaccaatg	gcttcaacggcaaagttctc	72
N-cadherin	aggatcaaccccatacacca	tggtttgaccacggtgacta	125
Notch-1	tccaccagtttgaatggtca	cgcagagggttgtattggtt	80
Hes-1	ccaaagacagcatctgagca	tcagctggctcagactttca	91
Jagged-1	ctgcctctctgatccctgtc	tggggaacactcacactcaa	76
18S rRNA	gtaacccgttgaaccccatt	ccatccaatcggtagtagcg	151

α-SMA, α-smooth muscle actin; COL1, collagen type I; Hes-1, hairy and enhancer-of-split homolog-1.

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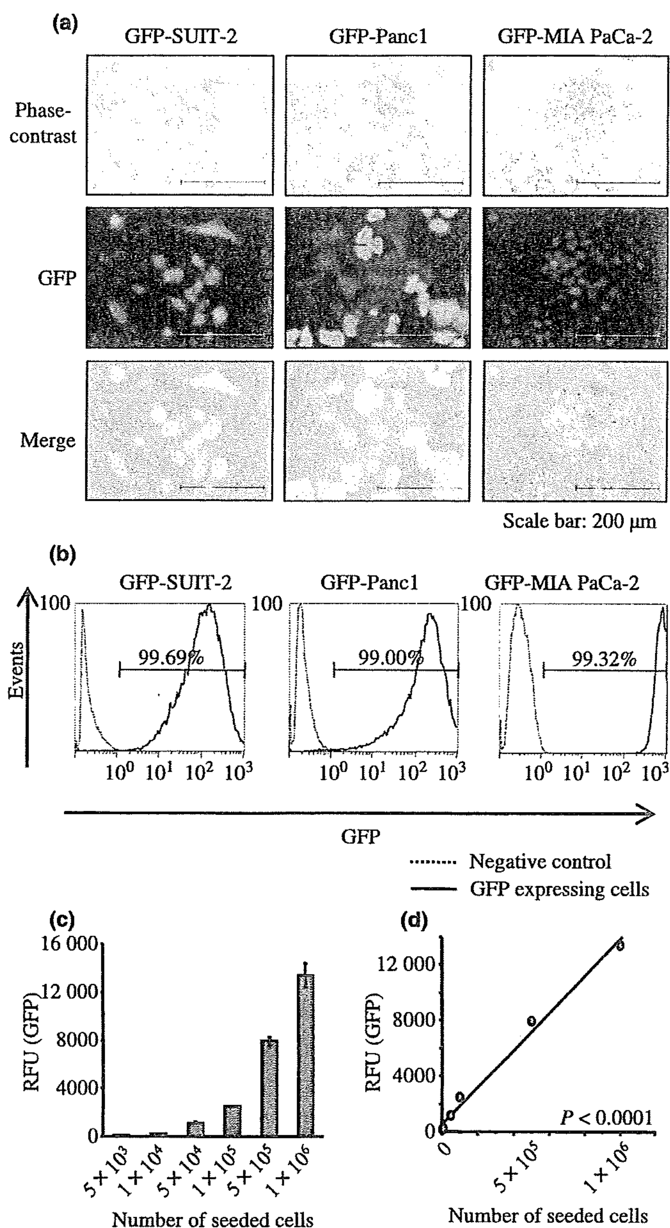

Fig. 1. (a) Microphotographs of three pancreatic cancer cell lines constitutively expressing green fluorescent protein (GFP). (b) The GFP-SUIT-2, GFP-Panc-1, and GFP-MIA PaCa-2 clones used for subsequent experiments were confirmed by flow cytometry to be >99% GFP-positive in comparison with the non-GFP-expressing parental cell lines. (c) GFP fluorescence intensity of GFP-SUIT-2 cells. (d) Regression analysis confirmed that the fluorescence intensity of GFP-SUIT-2 cells was correlated with the number of cells counted within the range from 5×10^3–1×10^6 cells/well (Spearman's rank-correlation coefficient: 0.994, $P < 0.0001$).

Directly co-cultured GFP-SUIT-2 cells expressed significantly higher levels of these mRNAs than monocultured cells.

Proliferation of cancer cells in direct and indirect co-culture systems. In our preliminary study, we evaluated the effects of co-culture on the fluorescence intensity of GFP-SUIT-2 cells. Both types of GFP-SUIT-2 (5×10^4) co-cultures expressed similar levels of fluorescence intensity to monocultured cells, regardless of the number of co-cultured PSCs (Supporting Information Fig. S1c), and the fluorescence intensity of GFP-SUIT-2 cells (1×10^4–2×10^5) was correlated with the number of cells, despite the coexistence of PSCs (4×10^4) and their EMT-like morphological changes (Supporting Information Fig. S1d,e; Spearman's rank-correlation coefficient: 0.993, $P < 0.0001$).

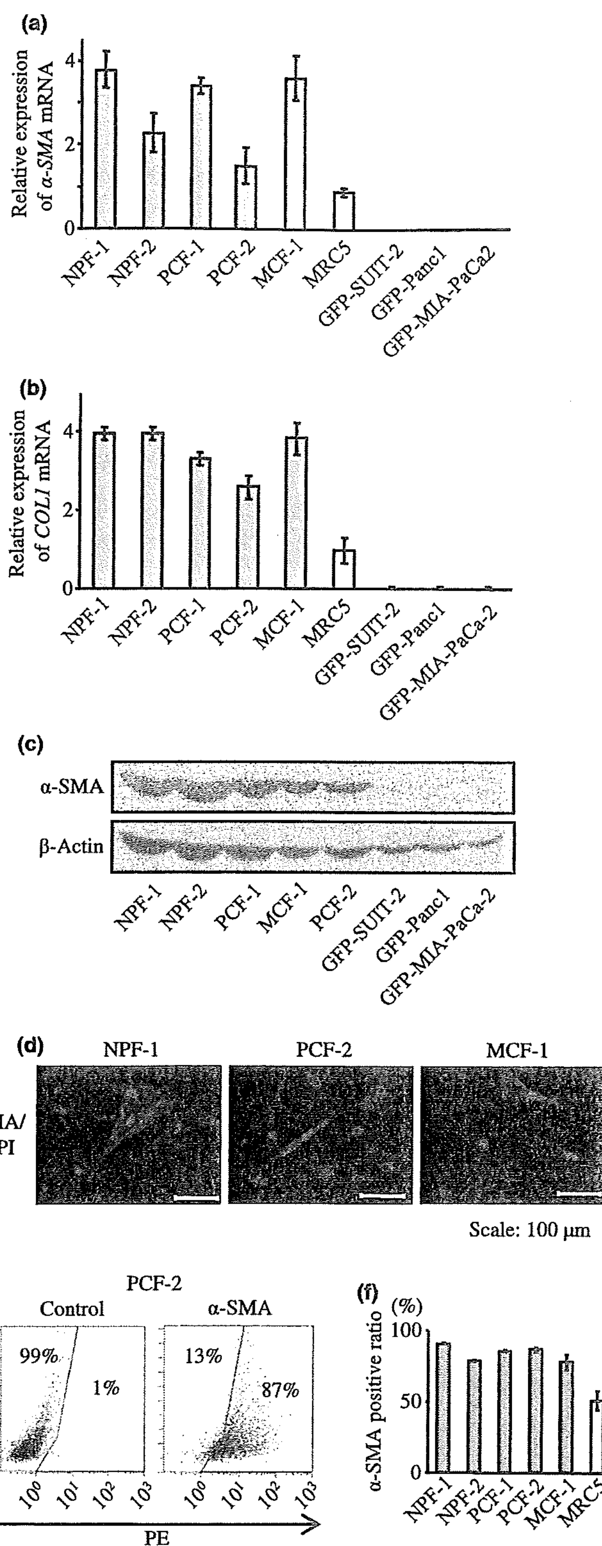

Fig. 2. (a,b) Pancreatic stellate cells (PSCs) expressed higher levels of *α-smooth muscle actin* (*α-SMA*) and *collagen type I* (*COL1*) mRNAs than MRC5 and cancer cells. (c,d) Immunoblot analysis and immunofluorescence staining revealed that these myofibroblasts expressed α-SMA protein. (e,f), Flow cytometry demonstrates that >80% of the PSCs and myofibroblasts expressed α-SMA.

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