Background

Pancreatic cancer remains a lethal disease and is the fourth to fifth leading cause of cancer-related death in the Western world, despite a significant reduction of the postoperative morbidity and mortality associated with pancreatectomy[1,2]. While surgical resection represents the only definitive option for cure of this disease and complete tumor resection is associated with longer survival, only 10% to 15% of patients have resectable disease[3,4]. Most patients with pancreatic cancer have locally advanced tumors, metastases, or both at the time of diagnosis. In addition, tumors frequently recur, even after margin-free curative resection, and most patients with recurrence have metastasis, which is often fatal. To improve the survival of patients with pancreatic cancer, we need a new strategy for the treatment of advanced disease that is unsuitable for surgical resection.

Metastasis is a multistep process in which tumor cells migrate through the stroma and invade a vessel, after which the cells are transported through the circulation to re-invade and proliferate at a distant site. Dozens of regulators influence each step of the metastatic cascade[5,6]. In 1996, *KiSS-1* was identified as a human metastasis-suppressing gene in melanoma cells[7] and breast cancer cells[8]. Then, the *KiSS-1* gene product was isolated from human placenta as the endogenous ligand of an orphan G-protein-coupled receptor known as GPR54[9], AXOR12[10], or hOT7T175[11]. *KiSS-1* encodes a 145-amino acid peptide which is further processed to a C-terminally amidated peptide with 54 amino acids called metastin[11] or kisspeptin-54, as well as to peptides with 14 amino acids (kisspeptin-14) and 13 amino acids (kisspeptin-13)[9].

The bioactive sequence of the *KiSS-1* gene product is the C-terminal 10 amino acids, metastin (45–54) (metastin-10 or kisspeptin-10)[12]. Metastin was shown to inhibit the chemotaxis and invasion of *GPR54*-transfected Chinese hamster ovary cells *in vitro*, while it inhibited the pulmonary metastasis of *GPR54*-transfected melanoma cells *in vivo*[11]. The prognostic relevance of *KiSS-1* has been demonstrated for some solid tumors [13-21].

In addition to the inhibition of tumor metastasis, *KiSS-1* shows neuroendocrine activity and has a role in the gonadotropin-releasing hormone cascade, puberty, placentation, and reproduction, as shown by recent studies[22,23]. In normal tissues, the highest level of *KiSS-1* mRNA expression has been detected in the placenta, with moderate to weak expression in the central nervous system, testis, liver, pancreas, and intestine[7,10,11]. In the case of *GPR54* mRNA, high levels of expression are found in the placenta, pancreas, and central nervous system [9-11].

We previously found that expression of *KiSS-1* mRNA was lower and expression of *GPR54* mRNA was higher in pancreatic cancer tissue compared with normal pancreatic tissue[24]. However, the clinical significance of *KiSS-1* and *GPR54* expression by pancreatic cancer remains unclear. We hypothesized high levels of *KiSS-1* and *GPR54* expression could be associated with better survival of pancreatic cancer patients. Therefore, we investigated immunohistochemical expression of the *KiSS-1* gene product (metastin) and that of GPR54 in pancreatic cancer tissues obtained by surgical resection. We also measured plasma metastin levels in pancreatic cancer patients by using an enzyme immunoassay (EIA) that we previously established[25] and evaluated the clinical applicability of these two parameters.

Methods

Patients

A total of 53 consecutive patients with pancreatic cancer who underwent surgical resection between July 2003 and May 2007 at Kyoto University Hospital were studied. The diagnosis of ductal adenocarcinoma of the pancreas was confirmed histologically by at least two pathologists who examined the resected specimens. None of the patients received preoperative chemotherapy or radiation therapy, and all patients gave written informed consent to participation in the study. Follow-up information was obtained from the medical records or by direct contact with patients or their referring physicians.

We evaluated the following clinicopathological characteristics according to the sixth edition of the TNM classification of the international union against cancer (UICC)[26]: tumor location, tumor size, tumor extent (pT), lymph node metastasis (pN), pStage, histopathological grade (G), lymphatic invasion, venous invasion, perineural invasion, and residual tumor (R).

Immunohistochemical staining for metastin and GPR54

Immunohistochemical staining of resected pancreatic tissues was done in 53 patients with ductal adenocarcinoma of the pancreas. We chose sections that contained cancer tissue and adjacent non-cancerous tissue in the same section.

Paraffin-embedded tissue blocks were cut into 4 μm sections, dried overnight at 37°C, and then deparaffinized with xylene and rehydrated in a graded ethanol series. Sections were treated with Dako target retrieval solution (Dako, Carpinteria, CA, USA) before antigen retrieval was done by heating at 95°C for 40 min. Then the sections were cooled to room temperature, and were treated with dilute hydrogen peroxide to block endogenous peroxidase activity. Nonspecific binding was minimized by incubation with Dako protein block (Dako) for 30 min. Rabbit

anti-human polyclonal antibodies for metastin (1–54)-Amide (catalogue number: H-048-59, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) and GPR54 (375–398) (catalogue number: H-048-61, Phoenix Pharmaceuticals) were applied overnight at 4°C at a dilution of 1:400. On the next day, sections were incubated for 1 hr at room temperature with anti-rabbit IgG conjugated to a horseradish peroxidase (HRP) -labelled polymer (Dako Envision™ + System, Dako), treated with 3,3'-diaminobenzidine tetrahydrochloride (DAB), and counterstained with Mayer's hematoxylin. As a positive control, human placental tissue was stained with the anti-metastin and anti-GPR54 antibodies (Figure 1A, 1B). For negative control slides, the primary antibody was substituted with irrelevant rabbit serum.

Assessment of metastin and GPR54 expression

Five fields (at a × 400 magnification) were randomly chosen to evaluate staining. The intensity of staining in cancer tissues was graded according to a 3-point scale as follows: 0 was weak; 1 was mild (the same staining intensity as that of non-cancerous pancreatic ducts as an internal control on each slide); and 2 was strong. The percentage of tumor cells showing each staining intensity was estimated to calculate an intensity score ([0 × %weak] + [1 × %mild] + [2 × %strong]) that could range from 0 to 200. A score ≥ 100 was defined as positive staining and a score <100 was defined as negative staining.

Then we compared clinicopathological characteristics between patients with positive and negative staining for metastin and GPR54.

Blood sampling and EIA for plasma metastin

Plasma levels of metastin were measured by EIA, as described previously[25], in 23 consecutive patients who underwent resection between July 2006 and May 2007.

A blood sample was collected in the morning before surgery, placed in a chilled tube containing aprotinin (500 KIU/ml) and EDTA (1.2 mg/ml), and immediately centrifuged. The plasma thus obtained was diluted five-fold with 4% acetic acid (pH 4.0), and loaded onto a column with a C18 reversed-phase cartridge (Sep-Pak C18, Millipore, Milford, MA, USA). After washing with 4% acetic acid, peptides were eluted with 70% acetonitrile in 0.5% acetic acid (pH 4.0). The eluted samples were concentrated by spin-vacuum evaporation, lyophilized, and stored at -40°C until assay.

EIA was performed by the delayed-addition method with separation of bound and free antigens on anti-rabbit IgG-coated immunoplates. Human metastin (45–54) was conjugated with β-D-galactosidase using *N*-(ε-maleimidocaproyloxy)-succinimide, as reported previously[27]. The EIA was sensitive and specific for all bioactive *KiSS-1* gene products (metastin, kisspeptin-14, and kisspeptin-13)[25].

The third quartile value was set as a cut-off for the plasma metastin level. We evaluated the association between the plasma level of metastin and metastin immunoreactivity in resected pancreatic cancer tissues, and also the associations between plasma metastin and the clinicopathological characteristics of the patients.

Statistical analysis

Continuous variables are presented as the mean ± standard deviation or as the median and range. Comparison of the groups was done with the Mann-Whitney U test, while categorical variables were compared by the χ^2 test. Correlations between metastin and GPR54 immunoreactivity were investigated by calculation of Pearson's correlation coefficient (r) values and scatter plots with a linear regression line were drawn. An r value of 0–0.19 was defined as a very weak correlation, while 0.2–0.39 was weak, 0.40–0.59 was moderate, 0.6–0.79 was strong, and 0.8–1 was very strong. Overall survival curves were drawn by the Kaplan-Meier method, and were compared by the log-rank test. Prognostic factors for survival were examined by univariate and multivariate analyses using Cox's proportional hazards model. For all analyses, $p < 0.05$ was considered to be statistically significant.

Results

Demographic and clinicopathological characteristics

There were 25 men (47.2%) and 28 women (52.8%) with a mean age at diagnosis of 65.6 years (median age: 68 years; range: 32 – 86 years). The tumor was located in the head of the pancreas in 38 patients (71.7%), while it was found in the distal pancreas in 15 patients (28.3%). Pancreatoduodenectomy was performed in 36 patients (67.9%), while distal pancreatectomy was performed in 13 patients (24.5%), and total pancreatectomy in 4 patients (7.5%). On histopathological examination, one patient (1.9%) had pStage IA disease, three patients (5.7%) had pStage IB, 16 patients (30.2%) had pStage IIA, 29 patients (54.7%) had pStage IIB, and four patients (7.5%) had pStage IV.

Twenty-nine patients received adjuvant chemotherapy, which consisted of S-1 (n= 18), gemcitabine (n = 8), 5-fluorouracil (n = 2), and tegafur-uracil (n = 1). This was excluded from statistical analysis because of variations in the duration and type of chemotherapy.

Immunostaining for metastin and GPR54

Pancreatic cancer tissues showed heterogenous immunoreactivity for metastin and GPR54 (Figure 1). Acinar cells and islet cells did not exhibit any immunoreactivity, while

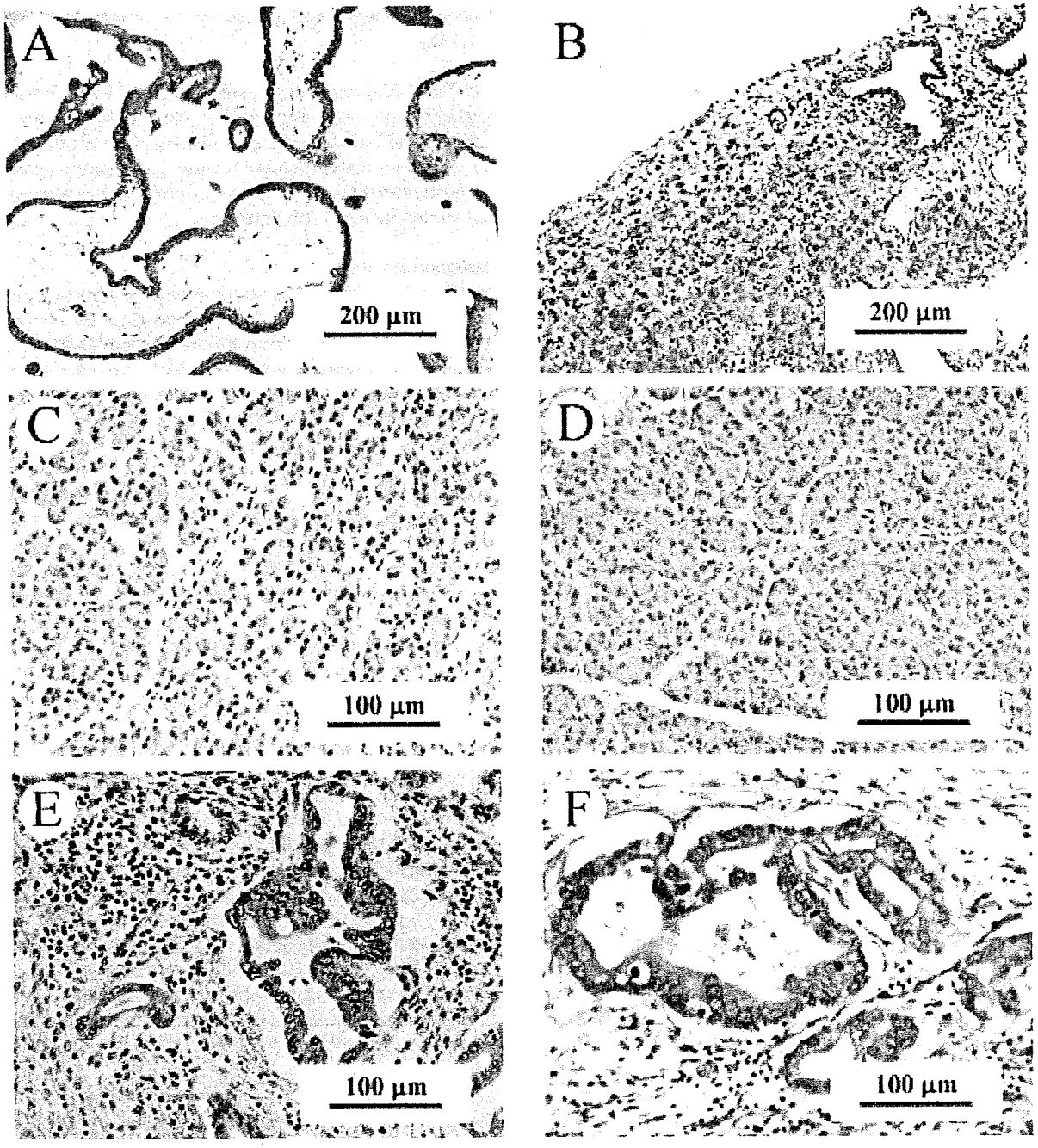

Figure 1
Immunohistochemical staining of non-cancerous pancreatic tissues and pancreatic cancer tissues. (A, B); Immunohistochemical staining of human placental tissues as a positive control. Tissues were stained with anti-metastin (A) and anti-GPR54 antibody (B). (Original magnification, × 200). (C, D); Non-cancerous and cancerous tissues were stained with anti-metastin and anti-GPR54 antibody. (Original magnification, × 400). Weak positivity of non-cancerous ductal cells for metastin (C) and GPR54 (D). (E, F); Pancreatic cancer tissues were stained with anti-metastin and anti-GPR54 antibody. Heterogeneous strong positive immunostaining of carcinoma cells for metastin (E) and GPR54 (F) are shown.

metastin and GPR54 were both weak or mildly positive in the cytoplasm of normal pancreatic ductal cells.

The mean intensity score for metastin was 72.1 ± 54.9 (n = 53) and that for GPR54 was 99.9 ± 55.1 (n = 53) (Figure 2).

Positive metastin staining was detected in 13 tumors (24.5%), while GPR54 was positive in 30 tumors (56.6%). Immunoreactivity for metastin and GPR54 showed a strong positive correlation ($r = 0.62$, $p < 0.001$; Fig. 3).

Demographic and clinicopathological characteristics showed no significant differences between patients whose tumors were positive or negative for metastin (Table 1), and the outcome was similar for GPR54 (Table 2). However, tumors that were negative for both metastin and GPR54 showed a significantly larger size than tumors positive for metastin and/or GPR54 (median of 2.5 cm and range of 0.8–5.0 cm versus median of 3.0 cm and range of 1.5–6.5 cm, $p = 0.047$).

Recurrence and survival

The median postoperative follow-up period was 18.5 months (range: 2.6–59.2 months). There were no operative deaths in this series. During the follow-up period, 33 patients (62.3%) showed recurrence and 25 patients (47.2%) died of their cancer. Recurrence was detected in the liver (n = 15), local region (n = 9), peritoneum (n = 9), lymph nodes (n = 5), lungs (n = 1), and bone (n = 1), while it was at an unknown location in 1 patient (elevated tumor marker). No patient died of any other disease or cause.

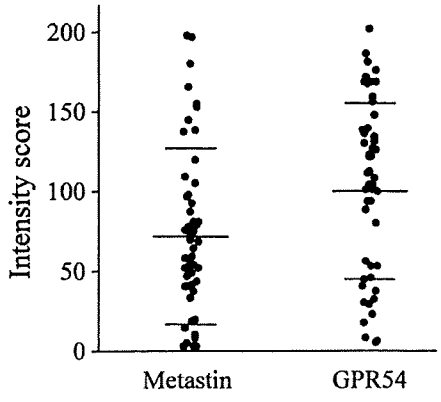

Figure 2
Expression of metastin and GPR54 in pancreatic cancer tissues. Immunoreactivity for metastin and GPR54 in resected pancreatic cancer tissues (n = 53) shown as the intensity score of each patient. The mean metastin intensity score was 72.1 ± 54.9 and that for GPR54 was 99.9 ± 55.1. The horizontal bar indicates the mean ± SD.

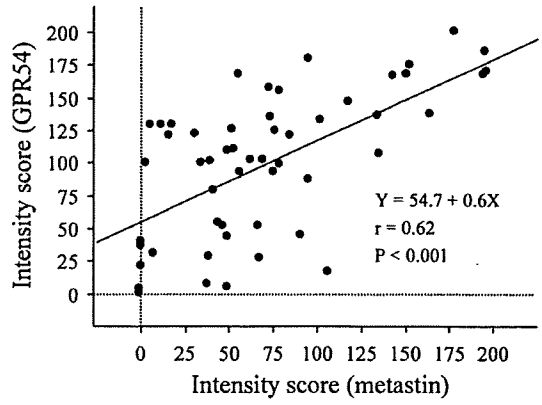

Figure 3
Correlation between metastin and GPR54 expression in pancreatic cancer tissues. Scatter plot showing the correlation between immunoreactivity for metastin and GPR54. A strong correlation was found ($r = 0.62$, $p < 0.001$).

The recurrence rate was significantly lower in the patients whose tumors were positive for metastin than in those with negative tumors (38.5% versus 70.0%, $p = 0.04$) (Table 3). There were no significant differences of the recurrence rate at each site between the patients with metastin-positive and -negative tumors (Table 3), and the same was found for GPR54 (Table 4).

The overall survival of patients whose tumors were positive for metastin was significantly longer than that of patients with negative tumors ($p = 0.02$) (Figure 4). Similarly, the overall survival of patients with tumors that were positive for GPR54 was significantly longer than that of patients with negative tumors ($p = 0.02$) (Figure 5).

Prognostic factors according to multivariate analysis

Univariate and multivariate analysis were performed to identify parameters associated with overall survival according to the Cox proportional hazards model. The univariate analysis revealed the following five factors to be associated with survival: perineural invasion, pStage, residual tumor, metastin expression, and GPR54 expression. In the multivariate analysis, as well as the UICC pStage (I + II versus IV), overexpression of metastin was an independent prognostic factor for better survival (hazard ratio, 2.08; 95% confidence interval, 1.05–4.71; $p = 0.03$) (Table 5).

Table 1: Comparison of the patients with pancreatic cancer who had positive immunostaining for metastin and those negative.

Characteristics	Positive for metastin (n = 13)	Negative for metastin (n = 40)	P value
Age	68.8 ± 7.2 (71, 56–78)	64.5 ± 10.5 (65.5, 32–86)	0.19
Gender			
Male	6	19	0.93
Female	7	21	
Location of tumor			
Pancreas head	8	30	0.35
Pancreas body-tail	5	10	
Size of tumor, cm	2.5 ± 0.9 (2.5, 1.2–4.5)	3.0 ± 1.2 (2.8, 0.8–6.5)	0.34
Histopathological grading			
G1	5	9	0.26
G2-4	8	31	
pT			
pT1, pT2	2	6	0.97
pT3	11	34	
pN			
pN0	6	15	0.58
pN1	7	25	
Lymphatic invasion			
Positive	7	24	0.70
Negative	6	16	
Venous invasion			
Positive	7	23	0.82
Negative	6	17	
Perineural invasion			
Positive	6	22	0.58
Negative	7	18	
pStage			
I, II	13	36	0.24
IV	0	4	
Residual tumor			
R0	11	28	0.30
R1	2	12	

Median and range are shown in parentheses.

Plasma metastin level

The mean plasma level of metastin before surgery was 22.7 ± 17.2 fmol/ml (median, 21.5 fmol/ml; range, 4.0–58.9 fmol/ml). Plasma metastin levels and the intensity score for metastin immunoreactivity in resected tissues showed a weak correlation (r = 0.23, p = 0.30). When we used the third quartile plasma metastin level (28.0 fmol/ml) as a cut-off value, there were no significant differences of demographics and clinicopathological characteristics between patients with a high (n = 6) or low (n = 17) plasma metastin level.

Overall survival curves of the patients with high and low plasma metastin levels are shown in Fig. 6. The median postoperative follow-up period was 14.8 months (range: 2.6–22.1 months, n = 23). While survival showed no significant difference between the two groups (p = 0.14), no patient with a high plasma metastin levels died after surgery (Figure 6).

Discussion

In this study, we investigated the clinical significance of immunohistochemical metastin and GPR54 expression in resected pancreatic cancer tissues. We found that strong expression of metastin or GPR54 was associated with better survival, and metastin expression was an independent prognostic factor for longer survival of pancreatic cancer patients. Our results indicate that the metastin/GPR54 signaling system acts to suppress the growth of pancreatic cancer.

Recently, the prognostic relevance of *KiSS-1* and *GPR54* has been investigated in some solid tumors [13-21]. Most of these studies have shown that the *KiSS-1/GPR54* system is negatively correlated with tumor progression. *KiSS-1* has been demonstrated to act as a suppressor in melanoma[13], thyroid cancer[14], bladder cancer[16], gastric cancer[17], esophageal cancer[18], and ovarian cancer[20].

Table 2: Comparison of the patients with pancreatic cancer who had positive immunostaining for GPR54 and those negative.

Characteristics	Positive for GPR54 (n = 30)	Negative for GPR54 (n = 23)	P value
Age	66.1 ± 8.7 (65.5, 49–86)	64.9 ± 11.5 (68.0, 32–80)	0.99
Gender			
Male	12	13	0.23
Female	18	10	
Location of tumor			
Pancreas head	21	17	0.75
Pancreas body-tail	9	6	
Size of tumor, cm	2.7 ± 1.0 (2.5, 0.8–5.0)	3.1 ± 1.2 (3.0, 1.2–6.5)	0.13
Histolopathological grading			
G1	10	4	0.19
G2-4	20	19	
pT			
pT1, pT2	6	2	0.25
pT3	24	21	
pN			
pN0	13	8	0.53
pN1	17	15	
Lymphatic invasion			
Positive	18	13	0.80
Negative	12	10	
Venous invasion			
Positive	18	12	0.57
Negative	12	11	
Perineural invasion			
Positive	15	13	0.64
Negative	15	10	
pStage			
I, II	29	20	0.18
IV	1	3	
Residual tumor			
R0	24	15	0.23
R1	6	8	

Median and range are shown in parentheses.

For example, Shirasaki et al[13] showed that downregulation of *KiSS-1* is important for the progression of melanoma in vivo. Ringel et al[14] showed that *KiSS-1* and *GPR54* mRNA were overexpressed in papillary thyroid cancer compared with follicular cancer. In bladder cancer, loss of *KiSS-1* expression is related to tumor progression[16]. In gastric cancer, lower expression of *KiSS-1* mRNA is associated with venous invasion, distant metastasis, and tumor recurrence[17]. Furthermore, *KiSS-1* is an independent prognostic marker for gastric cancer according to multivariate analysis [17]. Ikeguchi et al. [18] observed that loss of *KiSS-1* mRNA, *GPR54* mRNA, or

Table 3: The rate and site of recurrence after resection of pancreatic cancer in relation to metastin expression.

	Metastin expression Positive (n = 13)	Metastin expression Negative (n = 40)	P value
Recurrence, n (%)	5 (38.5%)	28 (70.0%)	**0.04**
Site of recurrence			
Liver, n (%)	4 (30.8%)	11 (27.5%)	0.82
Local, n (%)	2 (15.4%)	7 (17.5%)	0.86
Peritoneum, n (%)	1 (7.7%)	8 (20.0%)	0.30
Lymph nodes, n (%)	1 (7.7%)	4 (10.0%)	0.80
Lungs, n (%)	0	1 (2.5%)	0.56
Bone, n (%)	0	1 (2.5%)	0.56
Unknown*, n (%)	0	1 (2.5%)	0.56

* Confirmed by elevated tumor marker during follow-up

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],

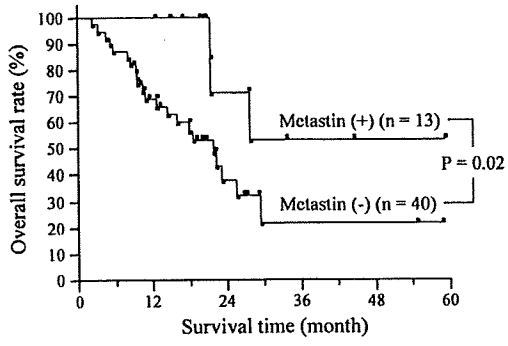

Figure 4
Impact of metastin expression on survival time of pancreatic cancer patients. Overall survival of patients whose tumors were positive (n = 13) or negative (n = 40) for metastin immunostaining. The survival of patients with positive tumors was significantly longer than that of patients with negative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

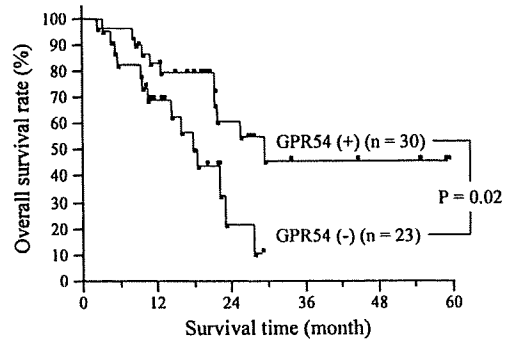

Figure 5
Impact of GPR54 expression on survival time of pancreatic cancer patients. Overall survival of patients whose tumors were positive (n = 30) or negative (n = 23) for GPR54 immunostaining. The survival of patients with tumors positive for GPR54 was significantly longer than that of those with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

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

Characteristics	Univariate analysis		Multivariate analysis	
	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].

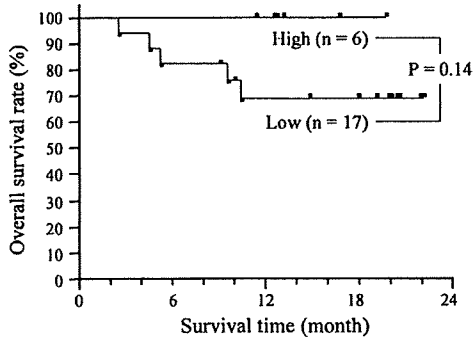

Figure 6
Impact of plasma metastin levels on survival time of pancreatic cancer patients. Overall survival of patients with high (n = 6) and low (n = 17) plasma metastin levels. There was no significant difference between the two groups (p = 0.14), but no patient with a high plasma metastin level died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

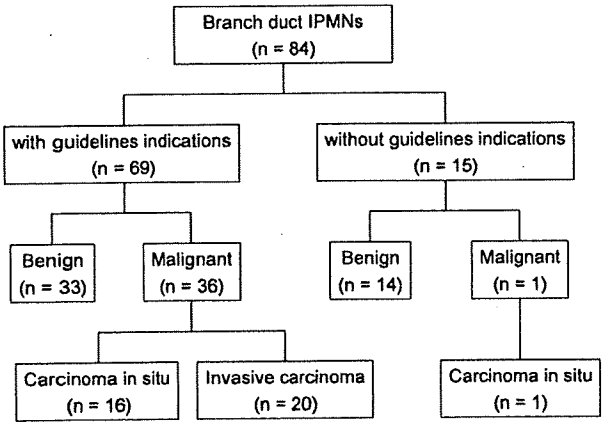

Fig. 1 Classification of 84 resected branch duct intraductal papillary mucinous neoplasms (*IPMNs*) based on the presence or absence of consensus indications for resection and the histological diagnosis. Thirty-six (52.2%) out of 69 patients with indications for resection had malignant tumors (16 carcinomas 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.

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

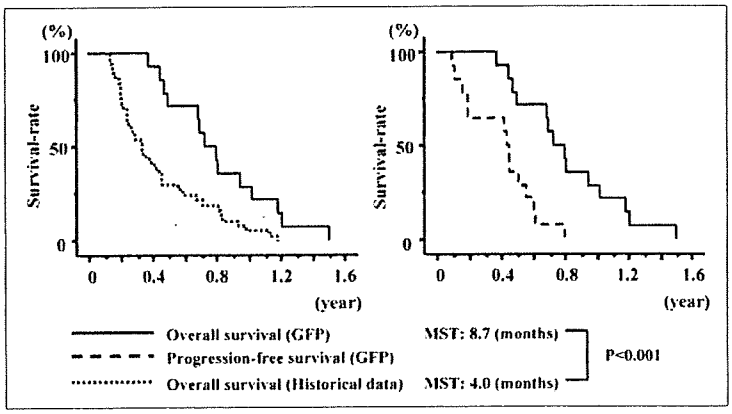

FIGURE 1 Overall and progression free survival curve; GFP chemotherapy improved overall survival curve of patients with biliary carcinoma.

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 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

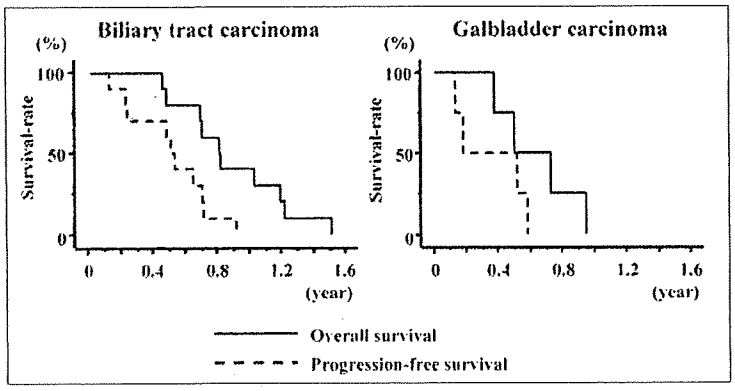

FIGURE 2 Overall and progression free survival curve according to the tumor site.

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 auguments 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

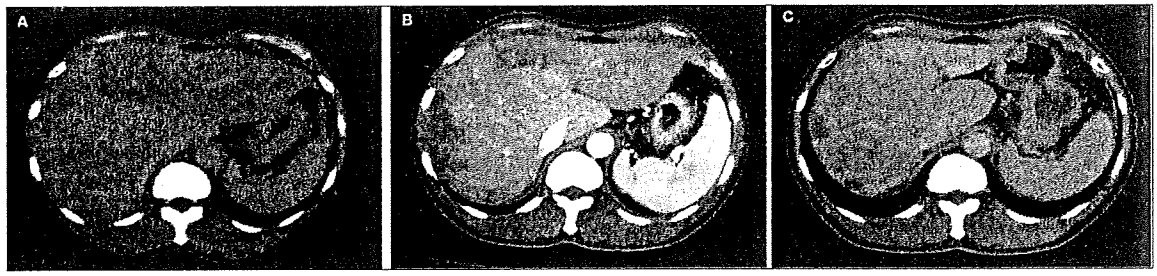

FIGURE 3 Effective case with intrahepatic biliary tract carcinoma. **A;** Most of the liver was occupied by a massive tumor and swelling, **B;** The liver tumor obviously reduced after 2cycles of GFP chemotherapy, **C;** The liver tumor almost disappeared after 4cycles of GFP chemotherapy.