with the infiltration growth pattern and that OPRT mRNA expression correlated with histological type. Furthermore, patients with pancreatic cancer who had a high TP/DPD ratio had a poor prognosis compared with patients who had a low TP/DPD ratio. On the other hand, TP, OPRT, and DPD mRNA expressions were correlated well with TP, OPRT, and DPD protein expressions, respectively. These findings suggest that TP and OPRT play an important role in pancreatic cancer and that the TP/DPD ratio has prognostic value in patients with pancreatic cancer.

TP is identical to platelet derived-endothelial cell growth factor (PD-ECGF), which has angiogenic activity. Angiogenesis facilitates rapid tumor growth, enabling the vascularized tumor to extend vertically into the deep tissue beneath the basement membrane. Several reports have demonstrated that microvessel density is correlated with the metastasis and recurrence of cancer. TP protein expression was reported to be correlated with proliferating cell nuclear antigen (PCNA) and to be a prognostic marker in human cancer. TP protein expression also suppressed apoptosis induced by hypoxia in cancer cells.[26] Furthermore, many reports indicate that cancer patients with high TP protein expression levels have a poor prognosis. Therefore, TP has received additional attention as a possible prognostic factor.[27,28] Although pancreatic cancer is well known to be a hypovascular tumor, angiogenesis may play an important role in its development. In the present study, TP mRNA expression in pancreatic cancer showed no correlation with any clinicopathological factors, except for the infiltration pattern. TP may enhance the invasion of tumor cells through the induction of matrix metallo proteinase (MMP)-1 MMP-7, and MMP-9, and TP may regulate infiltration as well as angiogenesis, possibly through regulating cell adhesion molecules. Further study is required to investigate the correlation between TP mRNA and proangiogenic and cell adhesion molecules such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and the MMP family in pancreatic cancer.

OPRT, an enzyme which also converts 5-FU to an active nucleotide, is anticipated to play a key role in the first step of the induction of the 5-FU antitumor effect, leading to DNA synthesis inhibition and RNA dysfunction. Thus, OPRT, together with TS, DPD, or TP is thought to be largely associated with the induction of the 5-FU antitumor effect. In addition, OPRT activity may be correlated positively with the activity levels of TS and TK, which are the key enzymes for DNA synthesis in the de novo and salvage pathways of. In bladder carcinoma, it was reported that OPRT activity was upregulated compared with the activity in normal bladder and that OPRT may be of prognostic value.[30] Although little is known about OPRT expression in

Table 3. Correlations between mRNA and protein expression of TP, OPRT, and DPD

(a) TP mRNA expression	Total $n = 24$	TP protein expression		P value
		High	Low	
High	13	11	2	0.015
Low	11	4	7	
(b) OPRT mRNA expression	**Total $n = 19$**	**OPRT protein expression**		**P value**
		High	Low	
High	11	8	3	0.040
Low	8	2	6	
(c) DPD mRNA expression	**Total $n = 21$**	**DPD protein expression**		**P value**
		High	Low	
High	12	11	2	0.026
Low	9	3	5	

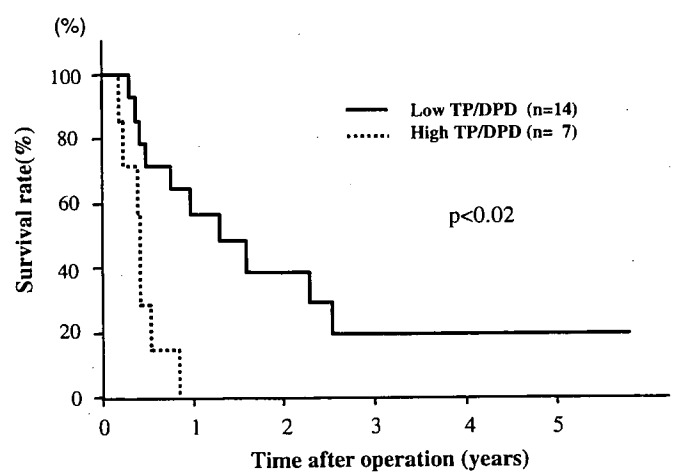

Fig. 2. Comparison of overall survival according to high and low TP/DPD ratios in tumor tissues. The survival of patients with a low TP/DPD ratio was significantly longer than that of patients with a high ratio ($P = 0.02$; log-rank test)

Table 4. Univariate and multivariate analyses of prognostic factors in pancreatic cancer

Independent factors	Univariate (P)	Multivariate (P)	Relative risk	95% confidence interval
TP/DPD ratio	0.0016	0.0126	8.786	1.593–48.470
Lymphatic invasion (ly0 versus ly1/2/3)	0.0136	0.5637		
Local advance (T2 versus T3/4)	0.0149	0.8786		
Stage (stage I/II/III versus IV)	0.0058	0.3030		

Table 5. Correlation between TP, OPRT, and DPD and chemotherapeutic outcome

Patient number	Age (years)	Sex	TP	OPRT	DPD	TP/DPD	OPRT/DPD	Postoperative chemotherapy	Survival (days)
1	65	F	Low	High	High	Low	High	5-FU	154
2	60	M	Low	–	–	–	–	TAM	110
3	78	F	High	–	High	High	–	Tegafur uracil+TAM	76
4	36	M	High	High	High	High	Low	5-FU+CDDP	193
5	70	F	High	–	High	Low	–	5FU+TAM	353
6	68	M	Low	Low	Low	High	High	TAM	307
7	53	M	Low	High	Low	Low	High	5-FU	137
8	60	F	Low	Low	High	Low	Low	5-FU+CDDP	820
9	55	M	Low	Low	Low	Low	High	5-FU+ADM+MMC	464
10	75	F	High	High	High	Low	Low	Tegafur uracil	109
11	71	F	Low	High	Low	Low	High	–	181
12	57	M	High	Low	Low	High	Low	5-FU+CDDP	152
13	65	M	Low	Low	Low	Low	High	Tegafur uracil	2084
14	58	M	Low	–	–	–	–	5-FU+CDDP	337
15	58	M	Low	High	High	Low	High	Tegafur uracil	880
16	74	M	Low	–	–	–	–	–	730
17	52	M	High	High	Low	High	High	GEM	151
18	65	F	High	High	High	Low	Low	5-FU	1163
19	53	M	High	High	High	Low	Low	Tegafur uracil, GEM	575
20	69	M	High	–	–	–	–	Tegafur uracil, GEM	270
21	59	M	High	High	High	Low	High	5-FU+CDDP+GEM	270
22	77	M	High	Low	High	High	Low	GEM	143
23	62	F	High	Low	High	High	Low	GEM	87
24	54	M	Low	High	Low	Low	High	GEM	570
25	79	F	High	Low	High	Low	Low	–	283

TAM, Tamoxifen; CDDP, cisplatin; ADM, adriamycin; MMC, mitomycin C; GEM, gemcitabine

pancreatic cancer, there was a positive correlation between OPRT mRNA expression and histological type in patients with pancreatic cancer in the present study. OPRT may play a potential role in regulating the malignant potential of pancreatic cancer.

We found that there was no relationship between DPD mRNA expression and any clinicopathological factors in pancreatic cancer. DPD is present mainly in the liver and more than 80% of a given 5-FU dose is thought to be catabolized in the liver.[1,31] As a result, the expression level of DPD influences selective cytotoxicity and is important in predicting chemosensitivity to 5-FU.[32,33] Another report has suggested that a higher expression of DPD protein was associated with advanced stage and vessel invasion in colorectal cancer.[34] Because the regulation of DPD expression and its involvement with tumor progression remains unclear, we could not reach any – even minimal – conclusions about the role of this enzyme, except in the catabolism of 5-FU.

The TP/DPD ratio shows a significant correlation with sensitivity to fluoropyrimidine analogues, such as 5′-DFUR, and to capecitabine, as it has been found that chemosensitivity to 5′-DFUR and capecitabine was reflected by differences between the catabolic and metabolic enzymes.[18,19] In addition, some reports have stated that a higher TP/DPD ratio in a tumor was associated with poorer clinical outcome,[26,35] while others reported that patients with higher TP/DPD ratios in the tumor tended to have longer recurrence-free periods with 5′-DFUR and capecitabine adjuvant chemotherapy after surgery. In particular, patients with high TP and low DPD values had long disease-free survivals with adjuvant chemotherapy after surgery.[19]

In the present study, TP and DPD alone were inadequate to predict a patient's prognosis. Therefore, we investigated the relative preponderance of TP and DPD mRNA expression with respect to survival. Considering the negative effect of TP mRNA expression and the favorable prognostic effect of DPD mRNA,[35] we calculated the TP/DPD ratio for the combined analysis of TP and DPD mRNA status. The relative risk of cancer death from tumors with a high TP/DPD ratio was greater than the risk of cancer death from tumors with only high TP mRNA expression.

Indeed, we found that a higher TP/DPD ratio in the tumor showed a poor prognosis and that the TP/DPD ratio showed no correlation with any clinicopathological features in the patients with pancreatic cancer in this study. Furthermore, although the TP/DPD ratio was related to survival and was an independent prognostic factor in our univariate and multivariate analyses, the established risk factors (lymphatic invasion, local advance, and stage) were not significant in the multivariate analysis. On this basis, the established risk factors (lymphatic invasion, local advance, and stage) would appear to be of limited value in predicting tumor progression in pancreatic cancer. At the moment, it is still unclear whether the TP/DPD ratio itself is useful as a prognostic parameter or as a predictive indicator for the efficacy of fluoropyrimidine-based chemotherapy for pancreatic cancer.

The OPRT/DPD ratio, on the other hand, has been reported to be a predictor of the antitumor effect of tegafur-uracil (UFT) and leucovorin (LV) regimens. UFT contains the 5-FU prodrug tegafur, which is converted by the P450 drug-metabolizing enzyme in the liver[36]. Recent reports have disclosed that, among the three pathways (i.e., TP,

DPD, and OPRT), the OPRT pathway was the most important for activating 5-FU. Hence, the ratio of OPRT and DPD was also evaluated in this study. The OPRT/DPD ratio was significantly higher in responding tumors than in nonresponding ones, and patients with tumors with a high OPRT/DPD ratio survived longer than those with tumors with a low OPRT/DPD ratio. Therefore, further studies are necessary to evaluate whether the OPRT/DPD ratio could be adopted as a possible predictor of the effectiveness of other fluoropyrimidine analogues.

In conclusion, using the TP/DPD ratio as a prognostic parameter for pancreatic cancer may help select patients for more intensive surgical approaches. However, the conclusions have been drawn from a retrospective study of a small number of patients. Moreover, since the time that these patients were treated, various advances have occurred, not only in surgical procedures but also in adjuvant therapies for pancreatic cancer. Further study is needed to investigate the relationship between TP and OPRT mRNA expression (and the ratio of these expressions to DPD mRNA expression) and features such as prognosis and the antitumor effect of fluoropyrimidine-based chemotherapy in pancreatic cancer.

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Original Paper

Pylorus-preserving Pancreatoduodenectomy: Preoperative Pancreatic Function and Outcome

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ABSTRACT

Background/Aims: To investigate the effects of preoperative pancreatic function on gastric emptying, body weight, and quality of life after pylorus-preserving pancreatoduodenectomy.

Methodology: Thirty-one patients who underwent pylorus-preserving pancreatoduodenectomy were divided into 2 groups according to preoperative pancreatic exocrine and endocrine function (normal *vs.* abnormal). Gastric emptying, body weight, and quality of life were evaluated before surgery, 1-2 months after surgery (short term), and 6-12 months after surgery (long term).

Results: Short-term body weight was significantly decreased in comparison to preoperative body weight regardless of preoperative exocrine and endocrine pancreatic function. Body weight returned to the preoperative level by 12 months after surgery in patients with normal preoperative pancreatic function but not in patients with abnormal pancreatic function. In both groups, gastric emptying was delayed at 1-2 months after surgery and then returned to the preoperative value by 12 months. Short-term quality of life did not differ from preoperative quality of life in either group, but long-term quality of life improved to beyond the preoperative level in both groups.

Conclusions: Preoperative pancreatic function appears to significantly influence long-term body weight after pylorus-preserving pancreatoduodenectomy.

KEY WORDS: Pancreatic function; Gastric emptying; PPPD

ABBREVIATIONS: Pylorus-Preserving Pancreatoduodenectomy (PPPD); Quality Of Life (QOL); Pancreatoduodenectomy (PD); Delayed Gastric Emptying (DGE)

INTRODUCTION

Traverso and Longmire introduced pylorus-preserving pancreatoduodenectomy (PPPD) in 1978 (1), and it is now the standard surgical procedure for treatment of periampullary lesions. It is thought that PPPD prevents long-term complications such as dumping and anorexia by preserving the reservoir function of the stomach and the duodenum-derived intestinal hormones and that, in comparison to standard pancreatoduodenectomy (PD), it improves nutritional status and quality of life (QOL) (2-5). However, complications can occur after PPPD. For example, delayed gastric emptying and impaired pancreatic function can result from the resection, and nutritional status may remain insufficient. Few studies have investigated the relation between pancreatic function and gastric emptying, nutritional status, and QOL over the long term after PPPD even though pancreatic function and gastric emptying are important indicators of postoperative nutritional status and QOL. The aim of this study was to investigate the effects of preoperative pancreatic exocrine and endocrine function on gastric emptying and recovery of body weight over the long term after PPPD.

METHODOLOGY

The present study included 31 Japanese patients who underwent PPPD in the Department of Surgery and Oncology at Kyushu University Hospital January 1994 through December 2001. The group comprised 19 men and 12 women who ranged in age from 46 to 81 years, with a mean age of 63.2 years. PPPD was performed for 19 malignant and 12 benign diseases: ampullary carcinoma, n=9; bile duct carcinoma, n=6; pancreas carcinoma, n=4; intraductal papillary adenoma of the pancreas, n=6; chronic pancreatitis, n=4; serous cystadenoma of the pancreas, n=1; and chronic cholangitis, n=1. All patients were followed up, and cancer recurrence was ruled out for more than 1 year after PPPD.

Of the 31 patients, 27 underwent gastrointestinal reconstruction by the Imanaga method (6) and 4 by the Traverso method (1). The proximal duodenum was transected 2-6cm distal to the pyloric ring. The Imanaga reconstruction procedure has been described previously (7-11): end-to-end duodenojejunostomy, end-to-side pancreatojejunostomy and hepaticojejunostomy, in that order. In Traverso reconstruction, pancreatojejunostomy is performed 5cm from the closed end of the jejunum, this is followed by hepaticojejunostomy 10cm distally and end-to-side duodenojejunostomy 30cm more distally.

Before surgery, the fecal chymotrypsin level (cut-off value: 13.2 U/g) and fasting blood sugar level (cut-

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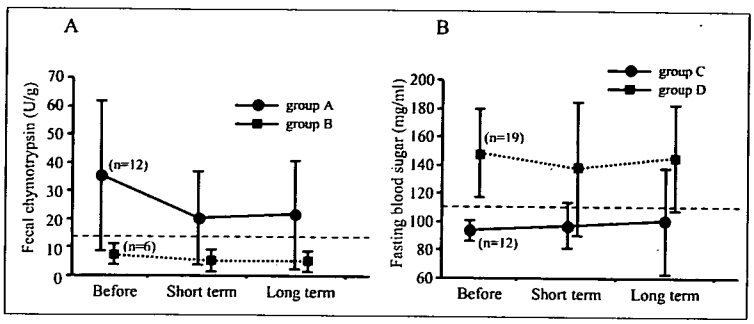

FIGURE 1 Changes in pancreatic **(A)** exocrine (fecal chymotrypsin) and **(B)** endocrine (fasting blood sugar) function.

off value: 110mg/mL) were examined in each patient for evaluation of pancreatic exocrine and endocrine function, respectively. Patients were divided into 2 groups according to preoperative pancreatic exocrine and endocrine function, and they were classified in subgroups of normal and abnormal group: group A, fecal chymotrypsin level was normal, group B, fecal chymotrypsin level was abnormal, group C, fasting blood sugar level was normal, group D, fasting blood sugar level was abnormal. Gastric emptying, body weight, and QOL were determined before surgery, 1-2 months after surgery (short term) and 6-12 months after surgery (long term). Gastric emptying was evaluated by the acetaminophen method as previously reported (7). The indices of gastric emptying were calculated from the area under the serum acetaminophen concentration curve for 90 minutes (AUC 90). Changes in each patient's body weight were calculated by referring to the preoperative level as 100%. QOL was assessed by means of a modified Kurihara questionnaire (12), which we have used previously (11,13). The questionnaire consisted of 23 items divided into 2 categories: physical (questions 1-13) and psychosocial (questions 14-23).

All values are expressed as means ± standard deviation (SD). Statistical analyses were carried out with unpaired *t*-test. A *P* value of less than 0.05 was considered significant.

RESULTS

Pancreatic Exocrine and Endocrine Function

The mean fecal chymotrypsin level in group A was decreased in the short term after surgery, but kept within normal limit in the short and long term. The level in group B with pancreatic exocrine insufficiency did not differ between time points. The mean fasting blood sugar levels in groups C and D did not differ between time points. Thus, normal or abnormal preoperative pancreatic exocrine and endocrine function did not appear to influence postoperative pancreatic function **(Figure 1A, B)**.

Influence of Preoperative Pancreatic Exocrine Function

Short-term gastric emptying was delayed in both groups (group A: before surgery, 550.8±243.9 μg·90min/mL; short term, 412.4±146.3μg· 90min/mL, and group B: before surgery, 649.8±294. 6μg·90 min/mL; short term, 478.5±348.9μg· 90min/mL). Long-term gastric emptying returned to the preoperative state in both groups (group A: long term, 702.2±284.4μg·90min/mL, and group B: long term, 761.7±345.7μg·90min/mL **(Figure 2A)**. Short-term body weight significantly decreased in both groups, while long-term body weight returned to the preoperative value in group A (short term, 90.9±4.3%; long term, 95.1±5.1%) but not in group B (short term, 89.5±5.3%; long term, 90.7±7.1%) **(Figure 2B)**. Short-term QOL was decreased in group B (group A: before surgery, 70.1±16.3%; short term, 71.0±9.5%, and group B: before surgery, 71.3±15.3%; short term, 62.5±10.2%), but long-term QOL increased to greater than the preoperative level in both groups (group A: long term, 84.5±9.6%, and group B: long term, 78.4±8.2%) **(Figure 2C)**.

Influence of Preoperative Pancreatic Endocrine Function

Short-term gastric emptying was delayed in both groups (group C: before surgery, 679.1±267.0μg· 90min/mL; short term, 456.1±220.1μg·90min/mL, and group D: before surgery, 596.8±262.2μg· 90min/mL; short term, 410.4±150.8μg·90min/mL), and long-term gastric emptying returned to the preoperative level in both groups (group C: long term, 789.4±259.4μg·90min/mL, and group D: long term, 711.3±212.2μg·90min/mL) **(Figure 3A)**. Short-term body weight was decreased significantly in both groups. Whereas long-term body weight significantly recovered and returned to the preoperative value in group C (short term, 90.6±4.4%, long term,

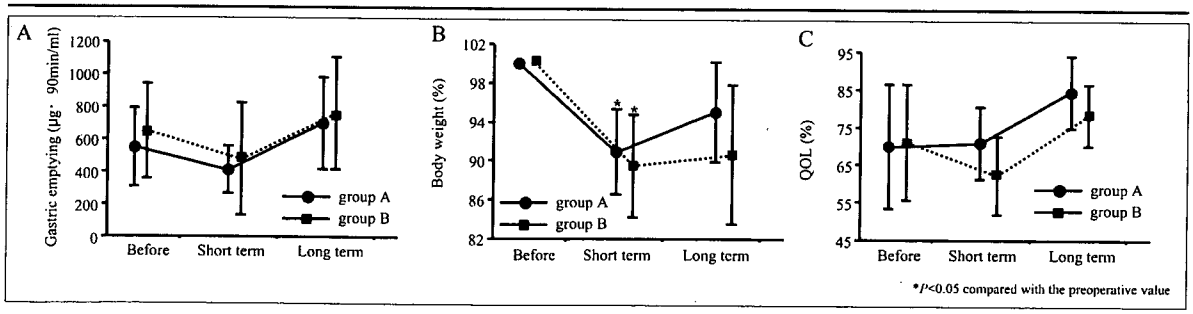

FIGURE 2 Changes in **(A)** gastric emptying (AUC 90), **(B)** body weight, and **(C)** QOL in groups A and B.

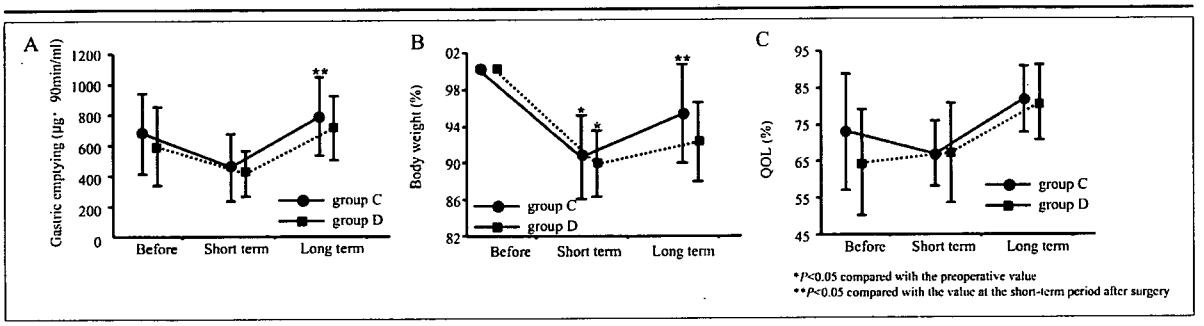

FIGURE 3 Changes in **(A)** gastric emptying (AUC 90), **(B)** body weight, and **(C)** QOL in groups C and D.

95.3±5.4%, $P<0.05$), there were no significant changes in group D (short term, 89.9±3.5%, long term, 92.2±4.2%) **(Figure 3B)**. Short-term QOL decreased in group C (before surgery, 73.0±15.8%, short term, 67.0±8.9%) and was similar in group D (before surgery, 64.5±14.4%, short term, 67.1± 13.5%). However, long-term QOL increased to greater than the preoperative level without a significant difference in both groups (group C: long term, 81.9±9.0%, and group D: long term, 80.9±10.3%) **(Figure 3C)**.

DISCUSSION

Several reports have suggested that body weight is better after PPPD than after standard PD. Kozuschek *et al.* reported that 43% of patients who underwent standard PD reached preoperative body weight after 1 year, whereas 86% of patients who underwent PPPD reached their preoperative weight (2). Braasch *et al.* reported that 28 patients who underwent PPPD reached 93% pre-illness weight and 106% preoperative weight at the time of follow-up (14). Zerbi *et al.* reported that patients who underwent PPPD had reached a mean 92% of the usual pre-illness body weight at 6 months after surgery, showing significantly better recovery of body weight than that of patients after standard PD (15). Yamaguchi *et al.* reported that postoperative loss of more than 3kg body weight was evident in 62% of patients after PPPD, that the maximum body weight loss was seen about 4.2 months after PPPD, and that body weight returned to the preoperative level 4.8 months thereafter (16). In our PPPD patients, long-term body weight was greater than 90% in all groups, and these results resembled those of previous reports. Short-term body weight decreased significantly after PPPD in patients with normal preoperative pancreatic function and in patients with abnormal preoperative pancreatic function. However, long-term body weight returned to the preoperative level in patients with normal preoperative pancreatic function, but not in patients with abnormal preoperative pancreatic function. Recovery of body weight is an important determinant of nutritional status, and our results suggest that the relation between pancreatic function and body weight is also important.

Gastric emptying is also an important determinant of nutritional status. Early delayed gastric emptying (DGE) is one of the most relevant and frequent postoperative complications and has been reported to range between 20% and 50% (17-23). The cause of DGE is not yet clear, and several factors are thought to play a role in DGE. These include gastric dysmotility after PPPD attributed to disruption of the gastroduodenal neural connection (24) and gastric dysrhythmia due to postoperative complications such as anastomotic leakage, intraabdominal abscess, and bleeding (25,26). Murakami *et al.* reported that residual pancreatic fibrosis is the most important cause of DGE after PPPD without complications (27). We previously reported that gastric emptying was delayed but returned to the preoperative level by 6 months after surgery (7). We obtained similar results in the present study, and there was no significant difference in postoperative gastric emptying between patients with normal and abnormal preoperative pancreatic function. Long-term gastric emptying was restored to the preoperative level, whereas recovery of body weight was poor in patients with abnormal preoperative pancreatic function. This suggests that preoperative pancreatic function is more important determinant of postoperative nutritional status.

Short-term QOL was the same or slightly lower than preoperative QOL. However, long-term QOL was high in comparison to preoperative and short-term QOL. It is likely that the QOL score improved with the increases in food intake and nutritional status. It is also possible that patients' anxiety over their disease state was relieved after surgery, positively influencing QOL. The difference in long-term QOL between our patients with normal and abnormal preoperative pancreatic functions suggests on indirect link between preoperative pancreatic function and improvement in QOL.

In conclusion, preoperative pancreatic function influenced the recovery of body weight after PPPD, however, it did not influence the recovery of gastric emptying or QOL.

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Fascin overexpression in intraductal papillary mucinous neoplasms (adenomas, borderline neoplasms, and carcinomas) of the pancreas, correlated with increased histological grade

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Intraductal papillary mucinous neoplasm (IPMN) is a well-established entity in pancreatic neoplasms and a precursor of infiltrating adenocarcinoma. Fascin, an actin-bundling protein involved in cellular motility, is upregulated in many human neoplasms. Its overexpression in pancreatic intraepithelial neoplasia, a precancerous lesion sharing many characteristics with IPMN, has been reported. However, fascin expression in IPMN remains unknown. The aim of this study was to investigate fascin expression in IPMNs and to elucidate its relationship to clinicopathological features, including histological grade and phenotypic subclassification. We evaluated fascin expression by immunohistochemistry in 116 surgical specimens, followed by quantitative analysis of fascin mRNA expression using a laser microdissection system and real-time reverse-transcriptase polymerase chain reaction in eight frozen samples. Fascin expression was significantly higher in borderline neoplasms (25/29, 86%) and carcinomas (37/42, 88%) than in adenomas (23/45, 51%) ($P<0.05$, respectively), but no difference was observed between borderline neoplasms and carcinomas. With regard to the subclassification, intestinal-type neoplasms (35/39, 90%) were more frequently positive for fascin than gastric-type neoplasms (36/59, 61%) ($P<0.05$). Two oncocytic-type neoplasms were both fascin-negative. Fascin mRNA expression seemed to be higher in moderately to severely dysplastic epithelium than in mildly dysplastic epithelium (not statistically significant), supporting the immunohistochemical experiments. Our findings suggest that fascin overexpression is involved in the progression of IPMN. Fascin could become a new therapeutic target for inhibition of their progression.
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Intraductal papillary mucinous neoplasm (IPMN) is a well-established entity in pancreatic neoplasms. It was first reported in 1982 as a special type of pancreatic neoplasm with a characteristic endoscopic finding of extrusion of mucin through the ampulla of Vater.[1] At present, the term is used to unify tumors characterized by intraductal proliferation of neoplastic mucinous epithelium, which usually forms papillae and leads to cystic dilation of the pancreatic ducts.[2,3] Because IPMNs show a broad spectrum of dysplasia ranging from adenoma and borderline neoplasm to carcinoma *in situ*, the existence of an adenoma–carcinoma sequence is probable. In addition, some IPMNs are associated with infiltrating adenocarcinoma. Therefore, IPMN is gaining attention as a precursor of infiltrating adenocarcinoma in the pancreas as well as pancreatic intraepithelial neoplasia (PanIN).[4–6] Investigation of factors correlated with the progression of noninvasive and/or invasive IPMNs is important.

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Several authors have proposed subclassification systems for IPMN based on histological phenotypes and/or immunohistochemical profiles of mucin core protein (MUC) expression.[7–13] Recently, a consensus on the subclassification of IPMNs was agreed among international experts on pancreatic precursor lesions, and published.[14] Subclassification makes it easier to compare studies among different institutions and understand the biological behavior, and is essential for future studies of IPMN.

Fascin-1 (also known as fascin) is a globular actin cross-linking protein. It is required for the formation of actin-based cell-surface protrusions that are essential for cellular migration and cell–matrix adhesion.[15–17] In normal epithelial cells, fascin expression is usually absent or very low, but it is significantly upregulated in transformed epithelial cells and several types of human carcinoma such as lung,[18,19] breast,[20–23] esophagus,[24,25] stomach,[26] colon,[27] pancreas,[28–31] biliary tract and ampulla,[31,32] ovary,[33] urinary bladder,[34] and skin.[35] Among the above neoplasms, fascin upregulation is most frequently observed in pancreatic infiltrating adenocarcinoma.[28,30,31] It is interesting that PanIN shows fascin expression despite being an intraepithelial neoplasia.[29,30] In general, in tumors of other organs, expression of fascin is especially strong in areas of infiltration, or is limited to such areas. Fascin expression in IPMN and its relationship with the clinicopathological features remain unclear, though IPMN has much in common with PanIN.

The aims of the present study were to analyze fascin expression using a large number of surgical IPMN specimens, to clarify its relationship with clinicopathological features including histological grade and phenotypic subtype, and to elucidate the association of fascin expression with progression of IPMNs.

Materials and methods

Patients and Tissue Specimens

A total of 116 samples of IPMN were used for the present study. All of the neoplasms were surgically resected at Kyushu University Hospital and its affiliated hospitals from 1986 to 2005. All specimens were cut into 5 mm stepwise tissue sections, and the gross features were recorded. For histopathological diagnosis, they were embedded in paraffin, and each of the serially cut sections, 4 μm in thickness, was stained with hematoxylin and eosin (H&E). On the basis of the greatest degree of dysplasia present, the lesions were classified as adenoma, borderline neoplasm, or carcinoma with or without invasion according to the World Health Organization (WHO) classification.[2] If present, invasive components were classified as tubular or mucinous noncystic (colloid) type. In accordance with the recently suggested subclassification system,[14] the lesions were also subclassified into four groups, gastric type, intestinal type, pancreatobiliary type, and oncocytic type, based on their histological phenotype and immunohistochemical expression of MUCs; gastric type MUC5AC+/MUC2−/MUC1−, intestinal type MUC5AC+/MUC2+/MUC1−, pancreatobiliary type MUC5AC+/MUC2−/MUC1+, and oncocytic type MUC5AC+/MUC2−/MUC1+. The subclassification was based primarily on histological phenotype and the immunolabeling for MUCs served as a confirmatory marker. IPMNs that could not be categorized specifically into one of the above four subtypes were segregated as unclassified type.

We also examined 10 cases of conventional pancreatic ductal adenocarcinoma for fascin expression, immunohistochemically. In addition, eight fresh-frozen samples of IPMN were obtained for quantitative analysis of fascin mRNA.

Immunohistochemistry

The primary antibodies used were follows as: anti-fascin (monoclonal, 55K-2; DAKO, CA, USA; 1:50 dilution), anti-MUC1 (Ma695; Novocastra Laboratories, Newcastle upon Tyne, UK; 1:200 dilution), anti-MUC2 (Ccp58; Novocastra Laboratories; 1:200 dilution), and anti-MUC5AC (CLH2; Novocastra Laboratories; 1:200 dilution). Sections were cut at 4 μm thickness from paraffin-embedded material, then dewaxed with xylene and rehydrated through a graded series of ethanol. After inhibition of endogenous peroxidase and antigen retrieval (microwave irradiation in citrate buffer for all the antibodies), sections were exposed to each primary antibody at 4°C overnight, and stained with a streptavidin–biotin–peroxidase kit (Nichirei, Tokyo, Japan). The sections were then finally reacted in 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted.

Evaluation of Immunohistochemical Staining of Fascin

Because IPMNs often show variability of epithelial dysplasia within the same tumor, immunohistochemical staining for fascin was evaluated within the area showing the highest degree of dysplasia in each neoplasm. The proportion of fascin positivity was measured using the following scale according to the percentage of fascin-positive tumor cells: <10%, 0; 10–30%, 1+; 30–60%, 2+; and >60%, 3+. Score 0 tumors were considered fascin-negative, whereas the others (1+ to 3+) were considered positive. All slides were evaluated independently by two investigators (HY and TI) without any prior knowledge of the patients' clinical information.

Microdissection and Extraction of Total RNA

We obtained total RNA from individual frozen samples using a laser microdissection system as

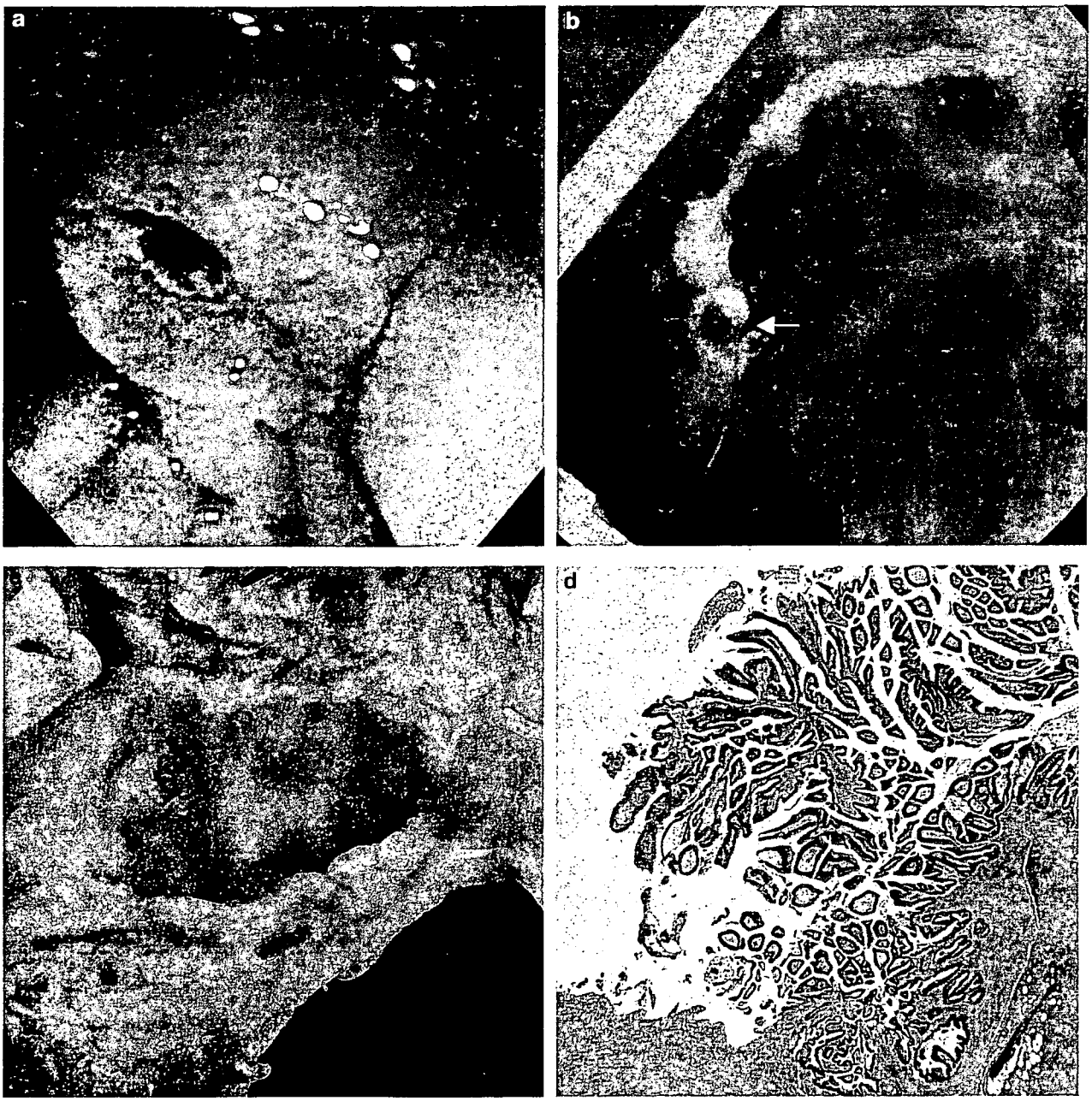

Figure 1 A representative case (75 years old, male) with typical findings of IPMN. (**a**) Endoscopic findings. Characteristic mucin extrusion through the ampulla of Vater. (**b**) Radiographic findings. Dilated main pancreatic duct (MPD) with mural nodule detected as a filling defect (arrow). (**c**) Macroscopic findings (MPD was opened at ventral side). Velvet-like appearance of the surface of a mural nodule. (**d**) Microscopic findings. Intraductal proliferation of neoplastic mucinous epithelium forming a papillary projection. Histological diagnosis is carcinoma.

described previously.[36] In brief, frozen tissue samples embedded in optimum cutting temperature compound (Sakura, Tokyo, Japan) were cut into 8-μm-thick sections. One section was stained with H&E for histological examination. IPMN cells were isolated selectively using laser microdissection and a pressure catapulting system (LMPC; Palm Microlaser Technologies AG, Bernried, Germany) in accordance with the manufacturer's protocols. After microdissection, total RNA was extracted from the selected cells according to the standard acid guanidinium thiocyanate–phenol–chloroform protocol[37] with glycogen (Funakoshi, Tokyo, Japan), and was subjected to real-time reverse-transcriptase polymerase chain reaction (RT-PCR) for quantitative measurement of fascin mRNA.

Quantitative Analysis of Fascin mRNA Expression by Real-Time RT-PCR

We designed a real-time RT-PCR protocol for the quantitative analysis of fascin mRNA and a refe-

rence gene, 18S ribosomal RNA (18S rRNA). We designed specific primers (fascin forward primer, 5′-gcaccctcaggtcaacatct-3′; reverse primer, 5′-aactccagc gtgtagccagt-3′; 18S rRNA forward primer, 5′-gatatgct catgtggtgttg-3′; reverse primer, 5′-aatcttcttcagtcgctc ca-3′), and used Basic Local Alignment Search Tool analysis to ensure the gene specificity of these primers. Quantitative one-step RT-PCR was carried out with a Quantitect SYBR Green RT-PCR kit (QIAGEN, Tokyo, Japan), and a LightCycler Quick System 350S (Roche Diagnostics, Mannheim, Germany), according to the manufacturers' instructions. In brief, the total volume of the reaction mixture was 20 μl, containing 10 μl of 2 × SYBR Green Buffer, 0.2 μl of RT mix, 1 μl of each primer (10 μmol/l), and 1 μl of total RNA. The reaction mixture was first incubated at 50°C for 15 min to allow reverse transcription. PCR was then initiated at 95°C for 10 min to activate modified Taq polymerase, followed by a 45-cycle amplification (95°C for 15 s, 55°C for 20 s, and 72°C for 10 s) and one cycle (95°C for 0 s, 65°C for 15 s, and 0.1°C/s to 99°C) for melting analysis. Each sample was run in triplicate. The mRNA expression of each gene was calculated on a standard curve constructed using total RNA from the MRC5 fibroblast cell line. For relative quantification, expression of fascin mRNA was normalized to that of 18S rRNA.

Table 1 Clinicopathologic findings of resected intraductal papillary mucinous neoplasms (IPMNs)

Parameters	*Number*
Age (years, mean ± s.d.)	66.34 ± 8.15
Sex	
Male	76 (66%)
Female	40 (34%)
Site	
Head	76 (66%)
Body and/or tail	39 (34%)
Tumor size (mm) (median (25, 75%))	30 (20, 40)
Mural nodule	
Absent	71 (61%)
Present	44 (38%)
Histological grade (WHO classification)	
Adenoma	45 (39%)
Borderline neoplasm	29 (25%)
Carcinoma	42 (36%)
Noninvasive	20 (17%)
Invasive	22 (19%)

s.d., standard deviation; WHO, World Health Organization.

Statistical Analysis

The χ^2 test was used to evaluate the association between histological grade and fascin expression. Fisher's exact test was used to assess the association between subtype and fascin expression or histological grade. After these analyses, multiple comparisons were carried out using Bonferroni's method. Spearman rank correlation analysis was used to study the relationship between histological grade and fascin score (0–3+). In the other analyses, the χ^2 test or Fisher's exact test was used for proportion, and the *t*-test, Mann–Whitney's *U*-test, analysis of variance, or the Kruskal–Wallis test was used for continuous data. *P*-values of less than 0.05 were considered statistically significant.

Results

Clinicopathological Features and Phenotypic Subclassification

A representative case showing typical endoscopic, radiographic, macroscopic and microscopic findings is shown in Figure 1. The clinicopathological findings from 116 cases are summarized in Table 1. Among the parameters, increasing tumor size ($P<0.0001$) and a presence of a mural nodule ($P<0.0001$) were significantly correlated with increased histological grade (adenoma–borderline neoplasm–carcinoma) (data not shown).

Of the 116 lesions, 59 (51%) were subclassified as gastric type, 39 (34%) as intestinal type, seven (6%)

Table 2 Subtype and histological grade (WHO classification)

	Adenoma	*Borderline*	*Carcinoma*			P
	n *(%)*	n *(%)*	n *(%)*	*Noninvasive*	*Invasive (tub:muc)*	
Sub-type						<0.0001
G-type ($n=59$)	41 (69)	13 (22)	5 (8)	1	4 (4:0)	
I-type* ($n=39$)	4 (10)	16 (41)	19 (49)	12	7 (3:4)	
PB-type* ($n=7$)	0 (0)	0 (0)	7 (100)	1	6 (6:0)	
O-type ($n=2$)	0 (0)	0 (0)	2 (100)	1	1 (1:0)	
U-type* ($n=9$)	0 (0)	0 (0)	9 (100)	5	4 (3:1)	

G-type, gastric type; I-type, intestinal type; muc, mucinous noncystic (colloid) invasive pattern; O-type, oncocytic type; PB-type, pancreatobiliary type; Tub, tubular invasive pattern; U-type, unclassified type.
*$P<0.05$ significantly increased histological grade compared with G-type, using Bonferroni's method.

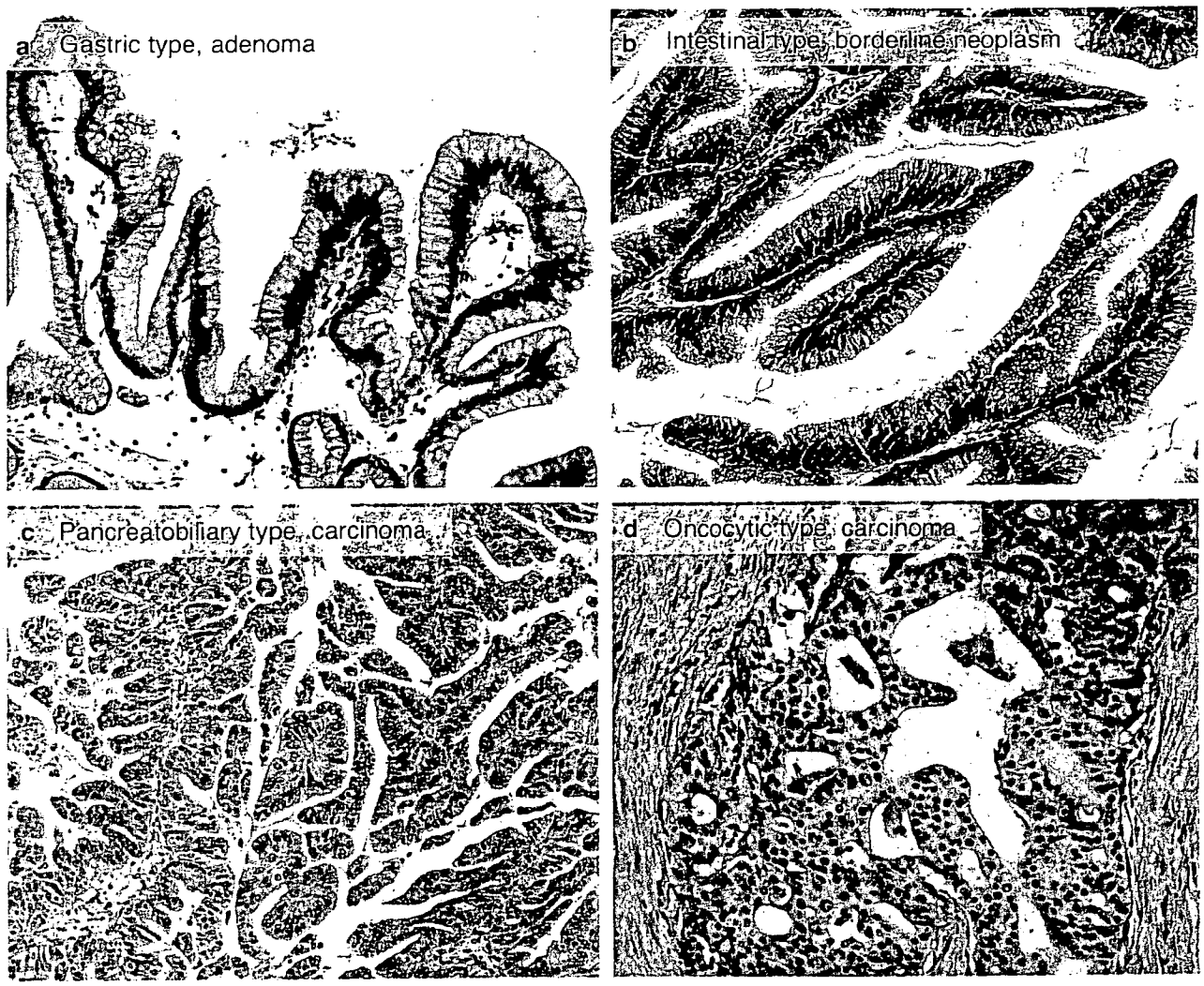

Figure 2 Representative images of the subtypes of IPMN stained with H&E. (**a**) Gastric-type IPMN consisting of cells resembling gastric foveolae with mild epithelial dysplasia. (**b**) Intestinal-type IPMN resembling intestinal villous neoplasms showing tall, columnar epithelial cells with moderate epithelial dysplasia. (**c**) Pancreatobiliary-type IPMN consisting of cells resembling cholangiopapillary neoplasms showing complex, thin, branching papillae with severe epithelial dysplasia. (**d**) Oncocytic-type IPMN consisting of cells with abundant, intensely eosinophilic cytoplasm and showing complex papillae with intraepithelial lumina and severe epithelial dysplasia.

as pancreatobiliary type, two (2%) as oncocytic type, and nine (8%) as unclassified type (Table 2). Each had characteristic papillae formation (Figure 2) and specific immunohistochemical reactivities for MUCs, as described previously.[14] All of the invasive components in the gastric-, pancreatobiliary-, and oncocytic-type neoplasms (four, six, and one neoplasms, respectively) displayed a tubular invasive pattern, whereas four of seven (57%) intestinal-type neoplasms and one of four (25%) unclassified-type neoplasms with invasive components showed a mucinous noncystic (colloid) invasive pattern. By multiple comparisons, intestinal-, pancreatobiliary-, and unclassified-type neoplasms showed significantly increased histological grade compared with gastric-type neoplasm ($P<0.05$; Table 2). Gastric-type neoplasms were significantly smaller (median diameter, 30 mm) than those of intestinal type (30 mm; $P<0.05$, data not shown).

Immunohistochemical Expression of Fascin

Of 116 IPMNs, 85 (73%) demonstrated positive immunohistochemical expression of fascin (Table 3). The immunohistochemical expression appeared as fine granular to diffuse cytoplasmic staining. In every slide prepared for immunohistochemistry, endothelial cells, lymphocytes, and stromal fibroblasts showed positive expression, and these were considered to be internal positive controls. Normal pancreatic ductal epithelium, acini, and islets of Langerhans were essentially nonreactive (Figure 3a); however, some parts of the hyperplastic ductal epithelium surrounding the IPMNs occasionally showed weak positivity for fascin. In addition, squamous metaplasia of the ductal epithelium was also weakly stained in one case. Compared with adenomas (23/45, 51%), the number of fascin-positive neoplasms was significantly higher among borderline neoplasms (25/29, 86%;

Table 3 Correlation between fascin expression and histological grade (WHO classification) or phenotypic subtype

Factor	*Fascin expression*		P
	−n (%)	*+ n (%)*	
Histological grade			<0.0001
Adenoma	22 (49)	23 (51)	
Borderline*	4 (14)	25 (86)	
Carcinoma*	5 (12)	37 (88)	
Phenotypic subtype			0.0005
G-type	23 (39)	36 (61)	
I-type**	4 (10)	35 (90)	
PB-type	2 (29)	5 (71)	
O-type	2 (100)	0 (0)	
U-type	0 (0)	9 (100)	
Total	31 (27)	85 (73)	

G-type, gastric type; I-type, intestinal type; O-type, oncocytic type; PB-type, pancreatobiliary type; U-type, unclassified type.
*$P<0.05$ compared with adenoma, using Bonferroni's method.
**$P<0.05$ compared with gastric type, using Bonferroni's method.

$P<0.05$) and carcinomas (37/42, 88%; $P<0.05$) (Table 3, Figure 3b–d). No difference was observed between borderline neoplasms and carcinomas. The fascin score was significantly raised in relation to increased histological grade (Figure 4; $P<0.001$). Within each neoplasm, high-grade-areas often showed more diffuse and intense immunoreactivity for fascin than low grade-areas (Figure 3e). All invasive components seen in the slides prepared for immunohistochemical staining were positive for fascin in both tubular and mucinous noncystic (colloid) patterns (Figure 3f). Intestinal-type neoplasms were more frequently positive for fascin (35/39, 90%) than gastric-type neoplasms (36/59, 61%) ($P<0.05$; Table 3). Two oncocytic-type neoplasms were both negative for fascin. Several parameters (age, sex, tumor site, tumor size, and absence or presence of mural nodule) had no correlation with fascin expression (data not shown).

Of the conventional pancreatic ductal adenocarcinomas, 90% (9 of 10) showed diffuse and intense fascin positivity (Figure 3h), the rate being as high as previously reported.[30,31]

Expression of Fascin mRNA

To confirm the fascin overexpression demonstrated by immunohistochemistry and to assess whether the overexpression was transcriptional or post-transcriptional, real-time RT-PCR for quantitative evaluation of fascin mRNA was performed, using frozen samples of IPMNs. A laser microdissection system was used to isolate IPMN cells for the following purposes: (1) to collect selectively the neoplastic cells showing the same degree of dysplasia within a neoplasm; and (2) to avoid contamination with stromal tissue, which contains many endothelial cells and/or fibroblasts that normally express fascin. The eight frozen samples comprised four samples of mild epithelial dysplasia (corresponding to adenoma) and four of moderate to severe epithelial dysplasia (borderline neoplasm to carcinoma).

The relative expression of fascin mRNA of each sample evaluated by real-time PCR is shown in Figure 5. A tendency was recognized whereby fascin mRNA levels in moderately to severely dysplastic epithelium were higher than those in mildly dysplastic epithelium, though the difference was not statistically significant, possibly because of the small number of samples. These data were consistent with the results of immunohistochemistry, and suggested that the overexpression of fascin in IPMNs was transcriptional.

Discussion

In the present study, we found that fascin was upregulated in the majority (73%) of IPMNs and was correlated with increased histological grade. These findings suggest that fascin overexpression is involved in the progression of IPMN. To our knowledge, this is the first report to reveal fascin overexpression and its association with clinicopathological features in IPMNs, and to evaluate quantitatively the expression of fascin mRNA in human neoplasms using laser microdissection and real time RT-PCR.

Fascin is a well-conserved actin-regulatory protein. *In vitro* experiments have indicated that it is involved in cellular processes such as motility,[27,38,39] loss of cell–cell contact in relation to adhesion molecules,[39–41] and cell proliferation.[25,27] From these observations, it may be presumed that fascin may play an important role in cellular malignant transformation. Indeed, in a variety of human carcinomas, fascin expression is consistently associated with the clinical aggressiveness of the tumor.[18,19,21,23,24,26,33]

Among the pancreatic neoplasms, Iacobuzio-Donahue *et al*[42] reported fascin upregulation for the first time in ductal adenocarcinoma using cDNA microarrays (13-fold overexpression of fascin transcripts in ductal adenocarcinoma compared with normal tissues). Immunohistochemical studies then confirmed fascin overexpression, not only in infiltrating ductal adenocarcinomas[28,30,31] but also in PanINs.[29,30] The fact that PanINs show fascin upregulation correlated with histological grade increased our interest in fascin expression in IPMNs, because PanINs and IPMNs share the following fundamental characteristics:[5,6] inherently intraductal; composed predominantly of columnar, mucin-producing cells that may grow in a flat configuration or may produce papillae; exhibit a range of cytologic and architectural atypia (mild, moderate and severe); recognized as precursors to

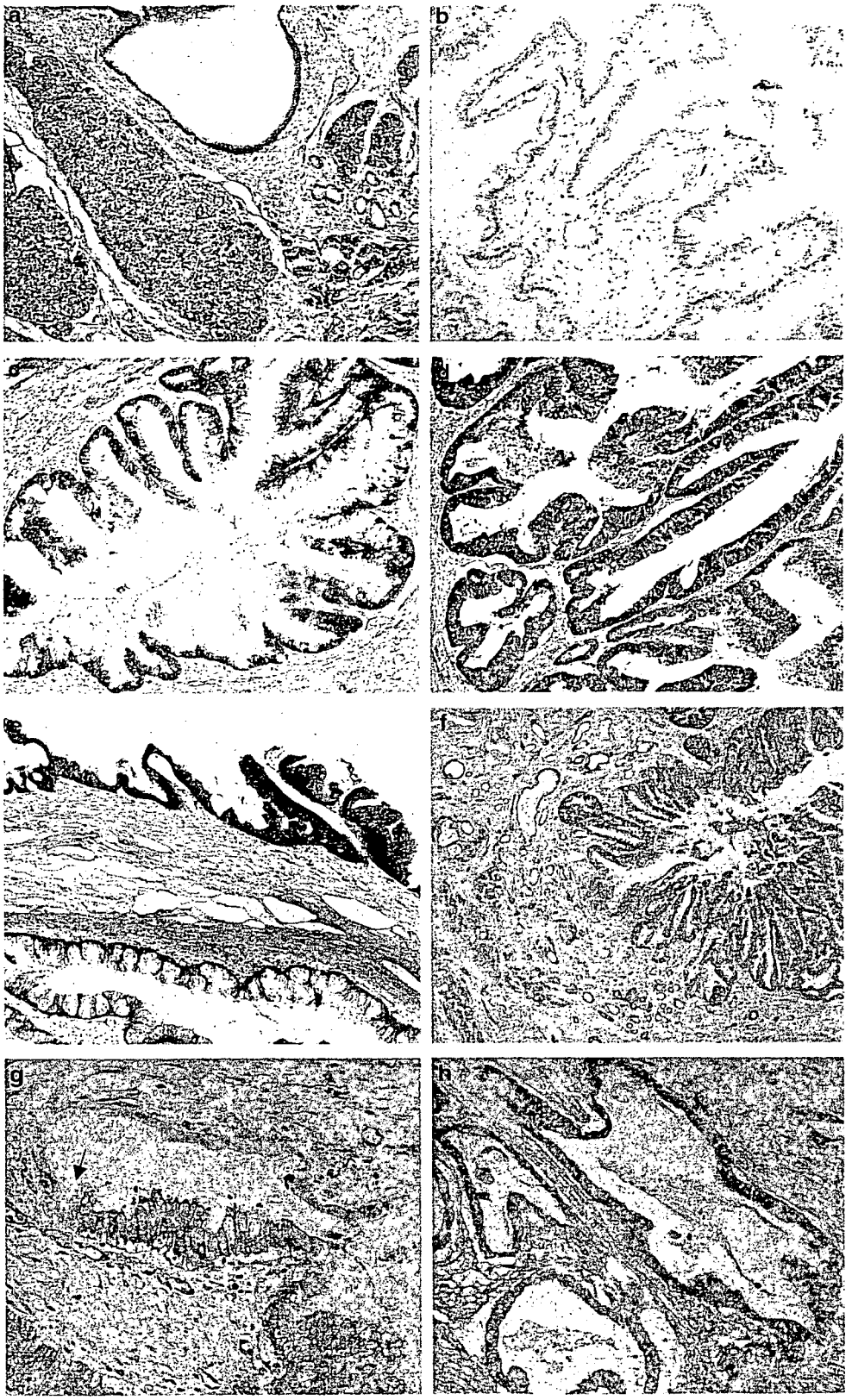

Figure 3 Fascin expression on immunohistochemistry. (**a**) Negative for normal pancreatic ductal epithelium, acini, and islets of Langerhans, whereas positive for endothelial cells and stromal fibroblasts (internal control). (**b**) Negative case of adenoma (score 0). (**c**) Positive case of borderline neoplasm (score 3+). (**d**) Positive case of carcinoma (score 3+). (**e**) Diffuse and intense reactivity in moderately to severely dysplastic epithelium (upper), with weak and basally localized reactivity in mildly dysplastic epithelium (lower) within a neoplasm. (**f**) Positive for both intraepithelial components (right upper) and invasive components (left lower). (**g**) Abrupt transition (arrow) from fascin-positive neoplastic epithelium to fascin-negative normal duct epithelium. (**h**) Diffuse and intense positivity in conventional pancreatic ductal adenocarcinoma.

invasive adenocarcinoma; and sequentially accumulate similar genetic alterations with increasing cytoarchitectural atypia.[43–45]

We showed that fascin overexpression in IPMNs was correlated with increased histological grade by immunohistochemical analysis, followed by a supporting molecular experiment that showed up-regulation of fascin mRNA. We consider that fascin upregulation would be a relatively early event in the progression of IPMN, because of the finding that fascin expression was significantly and almost equally greater in borderline neoplasms (86%) and carcinomas (88%) than in adenomas (51%). It would be interesting to compare these results with findings for PanINs. In an immunohistochemical study, Maitra *et al*[30] detected focal or diffuse cytoplasmic fascin expression in 25% of PanIN-1A, 28% of PanIN-1B, 57% of PanIN-2, and 57% of PanIN-3,

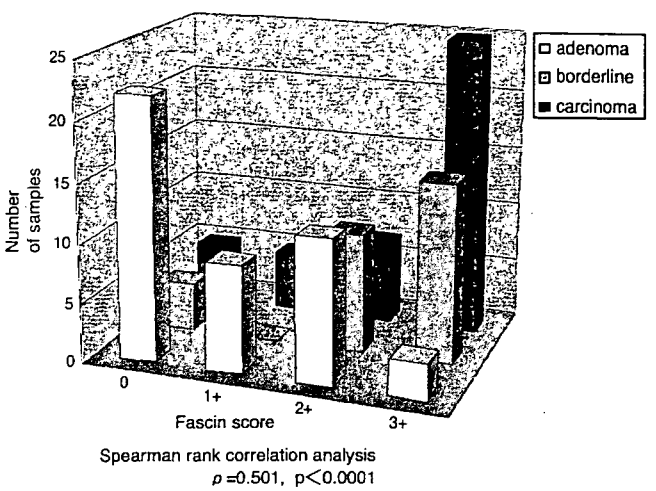

Figure 4 Correlation between histological grade and fascin score. Fascin score was significantly raised in relation to increased histological grade.

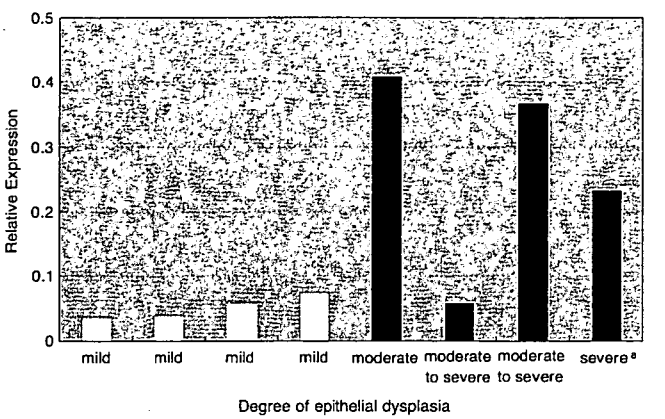

Figure 5 Relative expression of fascin mRNA evaluated by real-time RT-PCR. The relative expression of fascin in eight frozen samples was normalized to18s rRNA. Higher in moderately to severely dysplastic epithelium (black) than in mildly dysplastic epithelium (white). [a]Severe dysplasia with a little invasive components.

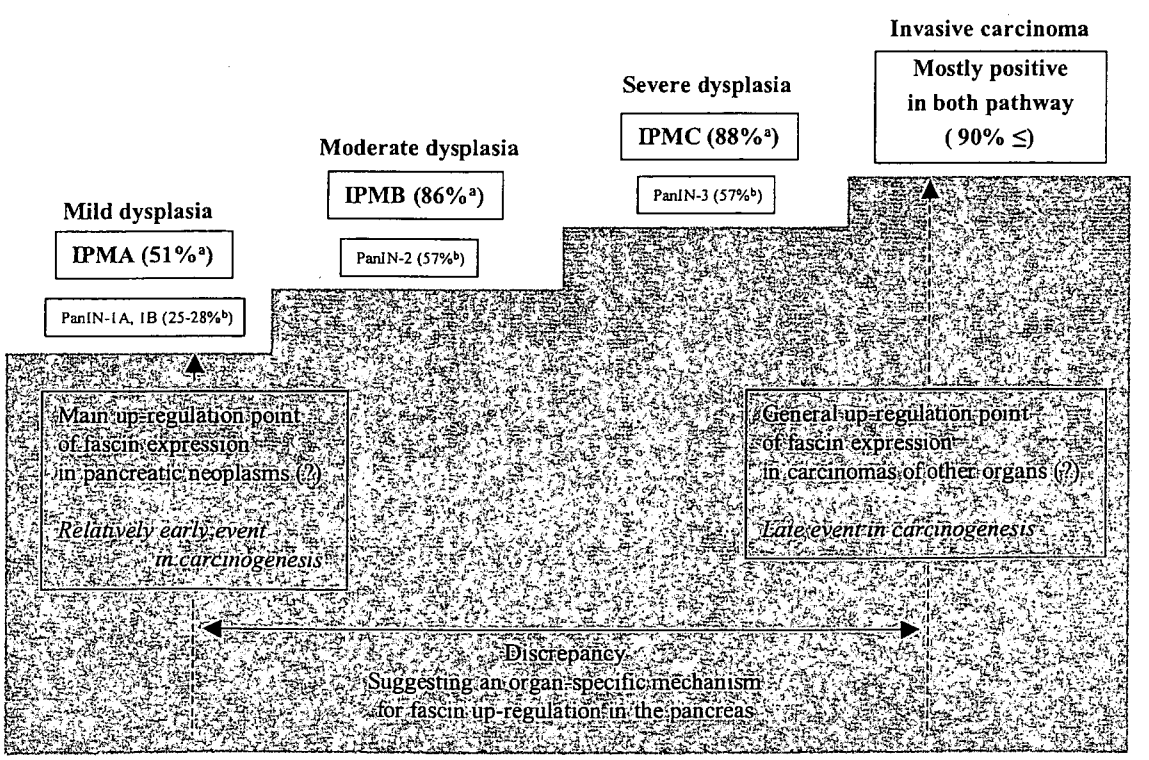

Figure 6 Hypothesis for fascin upregulation in multi-step carcinogenesis of pancreatic neoplasms. IPMA, intraductal papillary mucinous adenoma; IPMB, borderline IPMN; IPMC, intraductal papillary mucinous carcinoma. [a]Percentage of fascin-positive IPMNs documented in the present study. [b]Percentage of fascin-positive pancreatic intraepithelial neoplasms documented in Maitra *et al*.[30]

and described fascin upregulation as an 'intermediate' event in pancreatic adenocarcinoma progression. Their results and ours suggest that upregulation of fascin occurs in similar stage (relatively early phase) in tumorigenesis of both IPMNs and PanINs (Figure 6). It remains to be seen whether fascin expression represents merely a surrogate marker of histological grade, or whether it plays a pathogenic role in tumorigenesis and the progression of IPMNs. Using RNA interference, it has been shown recently that down-regulation of fascin has inhibitory effects on the migration, proliferation and invasiveness of esophageal squamous cell carcinoma cell lines.[24,25] These data suggest that fascin itself contributes to tumor progression, and raise the possibility that fascin could be a novel therapeutic target.

A previous report suggested that a pathway for fascin upregulation was dependent on amplification or overexpression of c-erbB-2/HER-2.[20] Others have shown a possible influence of Wnt signaling on fascin activity, suggesting that anomalies of this pathway may upregulate fascin expression in cancer cells.[40] However, the mechanism of fascin upregulation in IPMN is not known, because neither c-erbB-2 amplification nor Wnt signaling abnormalities are particularly common in IPMN. We consider that there is an organ-specific mechanism for fascin upregulation in the pancreas, because invasive pancreatic adenocarcinomas express prominently high levels of fascin compared with other carcinomas. In addition, IPMNs and PanINs frequently show fascin expression though they are both intraepithelial lesions. Fascin upregulation is not frequently recognized in intraepithelial neoplasms of other organs (Figure 6).

We also performed phenotypic subclassification of IPMNs, and re-confirmed the findings described previously. Gastric types usually showed mild dysplasia and intestinal types showed moderate to severe dysplasia, whereas pancreatobiliary and oncocytic types showed severe dysplasia corresponding to carcinoma *in situ*;[8,10,14] intestinal-type neoplasms were more frequently associated with a mucinous noncystic (colloid) invasive pattern compared with other types;[4,7–9,12] and the oncocytic type was a rare variant.[46] With regard to the relationship between fascin expression and the phenotypic subclassification, fascin overexpression occurred more frequently in intestinal-type neoplasms (90%) than in the gastric type (61%) by multiple comparisons. In contrast, intestinal-type neoplasms exhibited a higher histological grade than the gastric type. It remains unclear whether the difference in fascin overexpression in gastric- and intestinal-type neoplasms is affected by their histological grade, or whether there is a more essential mechanical association between fascin overexpression and subtype. For example, MUC variation may have some molecular relation to fascin expression that has not been documented. Interestingly, two oncocytic-type neoplasms in the present study were both fascin-negative, though they showed severely dysplastic epithelium corresponding to carcinoma. This may indicate that the progression pathway of oncocytic-type neoplasms differs from that of the other types in parts.

In conclusion, overexpression of fascin is correlated with increased histological grade of IPMN and occurs relatively early in the pathogenesis of IPMN. Fascin may provide a new cancer prevention strategy as a possible therapeutic molecular target to inhibit the progression of IPMNs.

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S100A6 Is Increased in a Stepwise Manner during Pancreatic Carcinogenesis: Clinical Value of Expression Analysis in 98 Pancreatic Juice Samples

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Abstract

There are few reports describing the diagnostic significance of *S100A6* expression in clinical samples obtained from patients with pancreatic disease. In the present study, we measured *S100A6* expression in pancreatic tissues and juice to evaluate its involvement in pancreatic carcinogenesis. We did quantitative real-time reverse transcription-PCR to measure mRNA expression in microdissected cells and pancreatic juice samples. Microdissected invasive ductal carcinoma and intraductal papillary mucinous neoplasm (IPMN) cells expressed significantly higher levels of *S100A6* than did microdissected pancreatitis-affected epithelial and normal cells (all comparison; $P < 0.008$). Median levels of *S100A6* in invasive ductal carcinoma were higher than those in IPMN, and those in pancreatitis-affected epithelial cells tended to be higher than those in normal cells, although these differences were not statistically significant. In analyses of pancreatic juice, IPMN and pancreatic cancer samples expressed significantly higher levels of *S100A6* than did chronic pancreatitis samples (both; $P < 0.017$), but levels in pancreatic cancer and IPMN samples did not differ form each other. Receiver operating characteristic (ROC) curve analysis revealed that measurement of *S100A6* was useful for discriminating cancer (area under the ROC curve, 0.864) or IPMN (area under the ROC curve, 0.749) from chronic pancreatitis. The present data suggest that expression of *S100A6* is increased in a stepwise manner during pancreatic carcinogenesis and may be a biomarker for evaluating malignant potential. Measurement of *S100A6* in pancreatic juice may be useful to detect early pancreatic cancer or identify individuals with high-risk lesions that may progress to pancreatic cancer. (Cancer Epidemiol Biomarkers Prev 2007;16(4):649–54)

Introduction

Pancreatic cancer is considered one of the most lethal forms of cancer because it is difficult to diagnose early and it is resistant to conventional therapies (1). The major symptoms at presentation are not sufficiently diagnostic because they are consistent with benign or inflammatory disease processes.

The importance of distinguishing pancreatic cancer and chronic pancreatitis is highlighted by reports that 5% to 10% of resected pancreatic tissues are eventually determined to be pancreatitis rather than pancreatic cancer (2). However, there is considerable overlap of the symptoms and clinical data for pancreatic cancer and pancreatitis. This overlap may in part reflect the potential for pancreatic cancer to arise in an environment of pancreatitis and the ability of pancreatic cancer to induce secondary inflammatory processes.

Cystic lesions of the pancreas are being detected with increasing frequency due to application of advanced diagnostic imaging technologies. Intraductal papillary mucinous neoplasm (IPMN) is the most common cystic neoplasm of the pancreas and was described initially by Ohhashi et al. (3) in 1982. IPMN is a precursor lesion of a subset of pancreatic cancer and is often associated with pancreatic cancer occurring as a separate lesion or as carcinoma derived from IPMN (4). Thus, it is important to distinguish pancreatic cancer from IPMN as well as from chronic pancreatitis.

Despite improvements in diagnostic modalities, it is still difficult to distinguish a small, resectable pancreatic cancer from chronic pancreatitis or IPMN. Early detection and diagnosis of pancreatic cancer would certainly improve patient survival. Therefore, novel screening and diagnostic tools are needed.

We and other investigators reported that both S100A6 mRNA and protein are overexpressed in pancreatic cancer (5-7), suggesting that S100A6 may be a promising diagnostic marker of pancreatic cancer. Although the role of S100A6 has yet to be clearly defined, it has been implicated in several cellular processes, such as cell proliferation and invasion (5, 8). In our previous study, we showed that *S100A6* is differentially expressed between normal epithelial ductal cells or pancreatic intraepithelial neoplasia (PanIN), which is a precursor lesion of pancreatic cancer (9), and invasive ductal carcinoma (IDC; ref. 5). In the present study, to refine our understanding of the involvement of *S100A6* in pancreatic carcinogenesis, we analyzed *S100A6* expression in IPMN and pancreatitis-affected epithelial (PAE) cells, only small percentage of which have the potential to progress to pancreatic cancer (10, 11). In addition, to clarify the clinical significance of *S100A6* mRNA expression in clinical samples, such as pancreatic juice, we used one-step quantitative real-time reverse transcription-PCR (RT-PCR) with gene-specific priming, short amplicons, and normalization to reference genes. It was reported recently that gene expression could be measured reliably from degraded RNA with this procedure (12-14). We successfully measured *S100A6* expression from degraded RNA in pancreatic juice samples from patients with IPMN and compared expression levels with those in pancreatic juice from patients with pancreatic cancer or chronic pancreatitis.

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Materials and Methods

Pancreatic Tissues and Pancreatic Juice. Tissue samples were obtained from the primary tumor of each resected pancreas or from peripheral tissues away from the tumor at the time of surgery at Kyushu University Hospital (Fukuoka, Japan) as described previously (15). Experienced pathologists did histologic examination of all tissues adjacent to the specimens. Pancreatic juice samples were collected from 102 patients with various pancreatic diseases and 5 patients without pancreatic disease who underwent endoscopic retrograde cholangiopancreatography for suspected malignancy of the pancreas at Kyushu University Hospital between January 1, 2002 and August 31, 2005 as described previously (16). We used pellets of cellular material from pancreatic juice for preparation of RNAs. We eventually excluded the data of nine samples in the pancreatic juice analyses. Three of these nine samples were excluded because neither signals for *S100A6* nor *β-actin* was detected with real-time RT-PCR, possibly due to improper sampling, handling, or storage of samples. Two of the remaining six samples were excluded because of DNA contamination, which was confirmed by RT-minus experiments. The remaining four samples, which were from pancreatitis-affected pancreata, were excluded because *S100A6* could not be measured quantitatively, although *β-actin* expression was detected. Therefore, the final samples included 98 pancreatic juice specimens, including 26 from patients with pancreatic cancer, 37 from patients with nonmalignant IPMNs, 30 from patients with chronic pancreatitis, and 5 from patients who underwent endoscopic retrograde cholangiopancreatography to rule out pancreatic disease and were then diagnosed with diseases other than pancreatic disease. The diagnosis of pancreatic ductal adenocarcinoma was confirmed by histologic examination of resected specimens when available, but when the case was inoperable, a clinical diagnosis was made based on imaging findings and the subsequent outcome of the patient. Staging of pancreatic cancer was done according to the Japan Pancreas Society classification (17). Chronic pancreatitis or IPMN was diagnosed based on histologic examination of resected specimens or clinical findings at the time of the initial diagnosis and during a follow-up period of at least 12 months that included conventional diagnostic imaging. Cytologic examination was done by experienced cytologists according to the classification described previously (18). Class I comprised benign and nonneoplastic epithelium with no or only slight dysplasia. Class II comprised regenerative or neoplastic epithelium with slight dysplasia. Class III included neoplastic epithelium with mild dysplasia corresponding to adenoma. Class IV contained neoplastic epithelium with moderate dysplasia highly suggestive of adenocarcinoma. Class V was unequivocal malignant epithelium corresponding to adenocarcinoma. Written informed consent was obtained from all patients, and the study was approved by our institution's surveillance committee and conducted according to the Helsinki Declaration.

Isolation of Total RNA. Total RNA was extracted from cell pellets of pancreatic juice according to the standard acid guanidinium thiocyanate-phenol-chloroform protocol (19) with glycogen (Funakoshi, Tokyo, Japan) and from cells isolated by laser microdissection with a PicoPure RNA Isolation kit (Arcturus Bioscience, Mountain View, CA) with DNase I (Roche Diagnostics, Mannheim, Germany) treatment according to the manufacturers' instructions. RNA concentrations in extracts were measured in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE), determining the absorbance at 260 and 280 nm ($A_{260/280}$). RNA integrity was assessed with an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA).

Quantitative Analysis of S100A6 mRNA Levels by One-step Real-time RT-PCR with Gene-Specific Priming. We used one-step quantitative real-time RT-PCR with gene-specific priming to examine mRNA levels in various types of clinical samples, which contain weakly or extensively fragmented RNA. A major advantage of this technology is its ability to measure gene expression reliably from fragmented RNA through synthesis of cDNA with gene-specific primers and utilization of short amplicons and normalization (12–14). We used specific primers for *S100A6* and *β-actin* as described previously (5). One-step quantitative real-time RT-PCR with gene-specific priming was done with a QuantiTect SYBR Green RT-PCR kit (Qiagen, Tokyo, Japan) with a LightCycler Quick System 350S (Roche Diagnostics). The reaction mixture was first incubated at 50°C for 15 min to allow for reverse transcription, where first-strand cDNA was synthesized with a gene-specific primer. PCR was initiated with one cycle of 95°C for 10 min to activate the modified Taq polymerase followed by 45 cycles of 94°C for 15 s, 55°C for 20 s, and 72°C for 10 s and one cycle of 95°C for 0 s, 65°C for 15 s, and +0.1°C/s to 95°C for melting analysis to visualize nonspecific PCR products because different fragments appear as separate distinct melting peaks. Each primer set used in the present study produced a single melting peak and a single prominent band of the expected size on microchip electrophoresis. To confirm the presence of DNA contamination, we did RT-PCR with or without reverse transcriptase. Each sample was run twice, and any sample showing a greater than 10% deviation in the RT-PCR value was tested a third time. Levels of mRNA in each sample were calculated from a standard curve generated with total RNA from Capan-1 human pancreatic cancer cells. Expression of *S100A6* mRNA was normalized to that of *β-actin*.

Microdissection-Based Quantitative Analysis of S100A6 mRNA. Frozen tissue samples were cut into 8-μm-thick sections. One section was stained with H&E for histologic examination. IDC cells from 21 sections, nonmalignant IPMN cells from 28 sections, PAE cells from 20 sections, and normal pancreatic epithelial cells from 19 sections were isolated selectively with a laser microdissection and pressure catapulting system (LMPC, PALM Microlaser Technologies, Bernried, Germany) per the manufacturer's protocols. After microdissection, total RNA was extracted from the selected cells and subjected to real-time RT-PCR for quantitative measurement of *S100A6* mRNA (19). Twenty-eight samples of nonmalignant IPMN cells microdissected in the present study were divided according to the WHO classification (20) into 20 samples of adenoma and 8 samples of borderline tumor (Table 1). Recently, a consensus nomenclature and criteria have been defined for classifying variants as distinctive IPMN subtypes, including gastric type, intestinal type, pancreaticobiliary type, and oncocytic type (21). The 20 samples of adenoma comprised 19 gastric-type IPMNs and 1 intestinal-type IPMN, and the 8 samples of borderline tumor comprised 4 intestinal-type IPMNs and 4 gastric-type IPMNs (Table 1). The proportion of IPMNs with a gastric versus pancreaticobiliary or intestinal phenotype in the present study was very high compared with

Table 1. Grade and type of microdissected nonmalignant IPMNs

Type*	Adenoma†	Borderline†
Gastric	19	4
Intestinal	1	4
Pancreaticobiliary	0	0
Oncocytic	0	0

*Type was according to classification of IPMN established at the international consensus meeting held in 2003 (21).
† Grade was according to the WHO classification (20).