TABLE 2 Univariate and multivariate analyses for disease-free survival

Variables	No. patients	Univariate			Multivariate		
		HR	95% CI	*P* value	HR	95% CI	*P* value
Primary location							
Head	17						
Body, tail	10	0.686	0.279–1.686	0.411			
Tumor size							
≤4.0 (cm)	14						
>4.0 (cm)	13	2.367	0.975–5.749	0.057	0.9980	0.367–2.721	0.999
pN stage							
N0	8						
N1	19	1.376	0.561–3.376	0.485			
Resection status							
R0	14						
R1	13	1.446	0.632–3.306	0.382			
hENT1 expression							
Low	11						
High	16	0.362	0.146–0.898	0.028	0.558	0.214–1.452	0.232
Histology							
Well to moderately differentiated	14						
Poorly differentiated	11	1.105	0.475–2.572	0.816			
Mucinous	2						
Microscopic vascular invasion							
None to minimal	19						
Moderate to marked	8	2.385	0.943–6.033	0.066	5.893	1.826–19.014	0.003
Microscopic lymphatic invasion							
None to minimal	18						
Moderate to marked	9	1.746	0.734–4.148	0.207			
Microscopic perineural invasion							
None to minimal	11						
Moderate to marked	16	3.028	1.140–8.041	0.026	6.014	1.877–19.268	0.003

postoperative setting. To date, there have been two studies that have investigated the prognostic and/or predictive value of hENT1 in the postoperative setting.[11,12] However, these were studies on patients who received adjuvant chemoradiation. Therefore, the clinical data from patients who received adjuvant gemcitabine alone has been lacking, and it has remained unclear whether the results seen in patients treated with adjuvant chemoradiation would translate to those who received adjuvant gemcitabine monotherapy. Our study, in addition to emerging clinical data and the previous studies, strongly suggest that intratumoral hENT1 expression may represent a prognostic and/or predictive factor for pancreatic cancer patients who undergo surgery followed by postoperative gemcitabine-based therapy.[11,12]

The existing preclinical data also support these findings. Several studies support the idea that tumor expression of hENT1 is mechanistically and biologically relevant to the tumor resistance to gemcitabine. Because gemcitabine is hydrophilic and cannot permeate the plasma membrane by passive diffusion, it requires plasma membrane nucleoside transporter proteins to efficiently enter cells and exert its cytotoxic effect.[6–8] In vitro studies have shown that gemcitabine enters pancreatic cancer cells primarily via the hENT1 transporter, and the cells lacking hENT1 are highly resistant to gemcitabine, confirming the importance of hENT1 for the activity of gemcitabine.[6,7]

An immunohistochemical analysis of hENT1 in pancreatic cancer might thus become a useful tool for determining the appropriate use of gemcitabine and other agents such as fluorouracil plus folinic acid (5-FU/LV) in patients with resected pancreatic cancer. The results of the ESPAC-3, a phase III trial that was designed to compare the survival benefit of adjuvant fluorouracil plus folinic acid (5-FU/LV) versus gemcitabine, showed that the OS was similar in both arms.[5] An in vitro study of cultured

TABLE 3 Univariate and multivariate analyses for overall survival

Variables	No. patients	Univariate			Multivariate		
		HR	95% CI	*P* value	HR	95% CI	*P* value
Primary location							
Head	17						
Body, tail	10	0.761	0.302–1.918	0.562			
Tumor size							
≤4.0 (cm)	14						
>4.0 (cm)	13	1.825	0.753–4.420	0.182			
pN stage							
N0	8						
N1	19	2.243	0.747–6.734	0.150			
Resection status							
R0	14						
R1	13	2.013	0.818–4.955	0.128			
hENT1 expression							
Low	11						
High	16	0.366	0.148–0.906	0.030	0.327	0.128–0.835	0.019
Histology							
Well to moderately differentiated	14						
Poorly differentiated	11	1.353	0.534–3.425	0.524			
Mucinous	2						
Microscopic vascular invasion							
None to minimal	19						
Moderate to marked	8	3.212	1.230–8.390	0.017	3.668	1.345–10.005	0.011
Microscopic lymphatic invasion							
None to minimal	18						
Moderate to marked	9	1.450	0.574–3.660	0.432			
Microscopic perineural invasion							
None to minimal	11						
Moderate to marked	16	1.607	0.638–4.052	0.314			

human mammary carcinoma cells (MDA-MB-435 s) showed that 5-FU is not a substrate of hENT1.[13] Therefore, patients with gemcitabine resistant tumors can still be treated with 5-FU based adjuvant regimens.

Of the 27 patients included in this study, 15 discontinued the adjuvant chemotherapy because of recurrent disease. This might be because most of the patients included in this study had relatively advanced stage cancer. Of the 27 patients, 26 patients (96%) had stage pT3 stage and 19 (70%) had pN1 disease. The validity of the predictive/prognostic value of hENT1 should be confirmed in larger prospective studies.

In summary, a high level of hENT1 expression in pancreatic cancer is significantly associated with a longer survival in patients who received adjuvant gemcitabine monotherapy after curative resection. Immunohistochemical analysis of the hENT1 expression may serve as a significant prognostic/predictive marker to appropriately select patients for gemcitabine-based adjuvant therapy or to select a more suitable drug for patients who have undergone resection of pancreatic cancer. However, the current study was a small scaled study, so our results warrant further investigation in larger prospective studies to confirm the predictive/prognostic value of hENT1. This report was presented in part to the 2011 Gastrointestinal Cancers Symposium of the American Society of Clinical Oncology, as part of General Poster Session B. San Francisco, California, January 2011.

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Use of omentum or falciform ligament does not decrease complications after pancreaticoduodenectomy: Nationwide survey of the Japanese Society of Pancreatic Surgery

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Background. *Wrapping is thought to prevent pancreatic fistula and postoperative hemorrhage for pancreaticoduodenectomy (PD), and we analyzed whether omentum/falciform ligament wrapping decreases postoperative complications after PD.*

Methods. *This is a retrospective study of wrapping using the omentum/falciform ligament in patients that underwent PD between January 2006 and June 2008 in 139 institutions that were members of the Japanese Society of Pancreatic Surgery.*

Results. *Ninety-one institutions responded to the questionnaires, and data were accumulated from 3,288 patients. The data from 2,597 patients were acceptable for analysis; 918 (35.3%) patients underwent wrapping and 1,679 patients did not. A pancreatic fistula occurred in 623 patients (37.3%) in the nonwrapping group, in comparison to 393 patients (42.8%) in the wrapping group (P = .006). The incidence of a grade B/C pancreatic fistula was lower in the nonwrapping group than the wrapping group (16.7% vs 21.5%; P = .002). An intra-abdominal hemorrhage occurred in 54 patients (3.2%) in the nonwrapping group, which was similar to the incidence in the wrapping group (32 patients; 3.5%). The mortality was 1.3% and 1.0% in nonwrapping and wrapping groups, respectively. A multivariate analysis revealed 7 independent risk factors for pancreatic fistula; male, hypoalbuminemia, soft pancreas, long operation time, extended resection, pylorus preservation, and omentum wrapping. There were 4 independent risk factors for early intra-abdominal hemorrhage and 2 independent risk factors for late intra-abdominal hemorrhage.*

Conclusion. *This retrospective study revealed that omentum wrapping did not decrease the incidence of pancreatic fistula. An additional validation study is necessary to evaluate the efficacy of wrapping for PD. (Surgery 2012;151:183-91.)*

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PANCREATICODUODENECTOMY (PD) is a major operation associated with a high incidence of mortality and morbidity, and numerous trials have been attempted to decrease the mortality and morbidity after PD.[1-4] The incidence of mortality has decreased at high-volume centers because of the progression of surgical techniques and perioperative treatment[5-7]; however, the incidence of morbidity still remains high.[1-4,8-10] Pancreatic fistula, delayed gastric emptying,[11,12] and postoperative hemorrhage after PD are the most frequent postoperative complications. Although delayed gastric emptying is not a lethal complication, both pancreatic fistula and postoperative intra-abdominal hemorrhage can lead to

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operation-related death.[13,14] In addition, a low incidence of complications is required in pancreatic surgery in order to administer postoperative adjuvant therapy quicker to improve the survival of patients with pancreatic cancer.[15] The International Study Group of Pancreatic Fistula (ISGPF) has proposed a consensus definition and clinical grading of postoperative pancreatic fistula, which made it possible to compare the incidence of pancreatic fistula associated with various surgical techniques.[16]

Wrapping with omentum/falciform ligament is one of the procedures to protect the surrounding organs against the pancreatic juice having autolytic activity, and this surgical technique is simple and easy for surgeons to perform. Several reports have so far shown the usefulness of wrapping after PD at individual institutions.[6,17-20] However, such wrapping may disturb the drainage of amylase-rich fluid, which might cause intra-abdominal adipose tissue inflammation like panniculitis, which could result in the occurrence of an intra-abdominal abscess.

The Japanese Society of Pancreatic Surgery (JSPS) decided to perform a nationwide survey to evaluate whether wrapping using the omentum/falciform ligament can help to prevent postoperative complications after PD.

MATERIAL AND METHODS

Patients. A nationwide survey of omental wrapping in patients who underwent PD between January 2006 and June 2008 was conducted at the initiative of JSPS to compare the patients' characteristics, preoperative status, preoperative treatment, surgical factors, perioperative status, and postoperative outcomes.

Postoperative complications. Pancreatic fistula was defined according to the ISGPF guidelines as an amylase level in the drainage fluid on postoperative day (POD) 3 that was >3 times the normal serum amylase level.[16] Postoperative intra-abdominal hemorrhage was defined as bleeding requiring a blood transfusion, reoperation, or interventional radiology. Early intra-abdominal hemorrhage indicates incomplete hemostasis and a failure of carrying out sufficient intra-operative management. It was defined as occurring within 3 days after PD, and it was not associated with any other postoperative complications. Late intra-abdominal hemorrhage is associated with other postoperative complications, including pancreatic fistula and intra-abdominal abscess. A biliary fistula was defined as the presence of bile in the drainage fluid that persisted to POD 4. An intra-abdominal abscess was defined as intra-abdominal fluid collection with positive cultures identified by ultrasonography or computed tomography associated with persistent fever and elevated white blood cells. Delayed gastric emptying is defined as output from a nasogastric tube of >500 mL per day that persists beyond POD 10, the failure to maintain oral intake by POD 14, or the reinsertion of a nasogastric tube. Vascular complications were defined as cerebral infarction, cerebral hemorrhage, and deep vascular embolization. Cardiac complications were defined as myocardial infarctions and heart failure. Respiratory complications were defined as pneumonia, pulmonary embolism, and respiratory distress requiring mechanical ventilation. Renal failure was defined as acute onset of hemodialysis. Mortality was defined as death within POD 30.

Statistical analyses. Comparisons between the 2 groups were carried out using unpaired t test for continuous data and the 2-tailed Chi-square or the Fisher exact test, where appropriate, for categorical data. The Tukey significant difference test was performed to evaluate the differences in postoperative drain amylase level among 3 groups. All factors with $P<.1$ in a univariate analysis were analyzed by a multivariate analysis. The analyses were performed with SPSS software for Windows (version 15.0; SPSS Inc., Chicago, IL). All statistical tests were 2-sided, and significance was defined as $P < .05$. The results are reported as the mean ± standard deviation.

RESULTS

Patients. Ninety-one institutions (65.5%) responded to the questionnaires, and the data from 2,597 patients were able to evaluate the occurrence of pancreatic fistula using the ISGPF criteria and postoperative hemorrhage and were acceptable for analysis in this study. The patients' characteristics are shown in Table I. The average number of PDs was 10.5 ± 11.5 and 7.5 ± 7.0 per year at the institutions with and without wrapping, respectively. There was no difference between the 2 groups ($P = .141$).

Postoperative outcome. The postoperative complications are shown in Table II. The incidence of pancreatic fistula in the wrapping group was significantly higher than that in the nonwrapping group. The intra-abdominal hemorrhagic site was identified in 24 patients in the nonwrapping group, and 22 patients (83.3%) experienced hemorrhage from an artery (9 common hepatic artery, 6 gastroduodenal artery, 4 superior mesenteric artery, 1 left gastric artery, 1 proper hepatic artery, and 1 splenic artery). The intra-abdominal hemorrhagic site was identified in 20 patients in the wrapping group, and 18 patients (90.0%) experienced hemorrhage from an artery (6 gastroduodenal artery, 5 common hepatic artery, 2 proper hepatic

Table I. Patients' characteristics

Parameter	*Nonwrapping group* (n = *1,679*)	*Wrapping group* (n = *918*)*	P *value*
Age, y (mean ± SD)	65.9 ± 10.1	66.5 ± 9.9	.100
Gender (male/female)	1,018/661	541/377	.402
Disease (carcinoma/other)	1,337/342	729/189	.895
Comorbidity			
Diabetes mellitus	466	268	.436
Respiratory disease	82	39	.463
Chronic pancreatitis	120	75	.344
Preoperative examination (mean ± SD)			
Hemoglobin (g/dL)	12.4 ± 1.7	12.4 ± 1.6	.887
Creatinine (mg/dL)	0.77 ± 0.41	0.79 ± 0.59	.490
Albumin (g/dL)	3.86 ± 0.49	3.81 ± 0.51	.017
Total bilirubin (mg/dL)	3.0 ± 4.8	2.2 ± 3.3	<.001
AST (IU/L)	70.3 ± 101.9	56.0 ± 79.3	<.001
ALT (IU/L)	100.0 ± 142.7	83.1 ± 211.6	.017
Amylase (IU/L)	123.7 ± 139.5	121.8 ± 181.6	.790
Preoperative biliary drainage	743 (44.3%)	478 (52.1%)	<.001
Duration of preoperative biliary drainage, days (mean ± SD)	25.8 ± 17.5	29.7 ± 21.4	.001
Pylorus preservation	1,016 (60.5%)	384 (41.8%)	<.001
Extended lymph node resection	1,399	773	.307
Pancreatic texture (hard/soft)	730/949	408/510	.635
Pancreaticoenterostomy			
Jejunum/stomach	1,523/156	792/126	.001
Duct-to-mucosal anastomosis	1,269 (75.6%)	778 (84.7%)	<.001
Usage of pancreatic stent tube	1,262 (75.1%)	779 (84.9%)	.001
Operative time, min (mean ± SD)	441 ± 137	534 ± 142	<.001

*Wrapping of pancreatic anastomosis or vessels, including hepatic artery, using either the omentum or falciform ligament.
ALT, Alanine aminotransferase; *AST*, aspartate aminotransferase.

artery, 1 superior mesenteric artery, 1 right hepatic artery, 1 left hepatic artery, 1 splenic artery, and 1 dorsal pancreatic artery). Thirty patients (75%) had late intra-abdominal hemorrhage accompanied by grade B + C pancreatic fistula and/or intra-abdominal abscess, and intra-abdominal hemorrhage was accompanied by all grades of pancreatic fistula in 32 patients (80%). Mortality was 1.3% and 1.0% in the nonwrapping and wrapping groups, respectively.

The level of amylase in the drainage fluid is shown in Table III. The amylase level of the omentum wrapping group was significantly lower than the other groups ($P = .027$) on POD 3.

Complications according to the material used for wrapping after PD. Two materials were used to wrap (Table IV). The incidence of grade B + C pancreatic fistula in the omentum group (23.9%) was significantly higher than in both the nonwrapping ($P < .001$) and falciform ligament groups ($P < .001$).

Complications according to the location of wrapping after PD. Wrapping was performed at 2 locations: wrapping of vessels, including the common hepatic artery, proper hepatic artery, stump of gastroduodenal artery, and portal vein, and wrapping of the pancreaticoenterostomy (Table V). The incidences of grade B + C pancreatic fistula in the anastomosis wrapping group and the vessel wrapping groups were also higher than those in the nonwrapping group.

Risk factors of postoperative complications. The risk factors of grade B + C pancreatic fistula and intra-abdominal hemorrhage were predicted using categorized data by a univariate analysis (Tables VI and VII). A multivariate analysis predicted 7 independent risk factors for grade B + C pancreatic fistula (Table VIII). A multivariate analysis revealed 4 independent risk factors for early intra-abdominal hemorrhage: male gender ($P = .017$; odds ratio [OR], 2.078), long operation time (≥600 minutes; $P = .020$; OR, 2.198), blood transfusion ($P = .002$; OR, 2.747), and soft pancreas ($P < .001$; OR, 4.184), and 2 independent risk factors for late intra-abdominal hemorrhage: male gender ($P = .017$; OR, 2.591) and soft pancreas ($P = .001$; OR, 4.274).

Table II. Complications after pancreaticoduodenectomy

Parameter	*Nonwrapping group* (n = *1,679*)	*Wrapping group* (n = *918*)*	P *value*
Pancreatic fistula			
All grades	627 (37.3)	393 (42.8)	.006
Grade B + C	281 (16.7)	198 (21.6)	.002
Delayed gastric empting	182 (10.8)	117 (12.7)	.146
Bile leakage	52 (3.1)	29 (3.2)	.931
Intra-abdominal abscess	179 (10.7)	111 (12.1)	.269
Intra-abdominal hemorrhage†			
Early	32 (1.9)	14 (1.5)	.482
Late	22 (1.3)	18 (2.0)	.198
Wound infection	151 (9.0)	115 (12.5)	.005
Other organ complications			
Respiratory	76 (4.6)	43 (4.7)	.859
Cardiac	25 (1.5)	28 (3.1)	.007
Vascular	24 (1.4)	20 (2.2)	.157
Renal	17 (1.0)	4 (0.4)	.117
Mortality	22 (1.3)	9 (1.0)	.459
Postoperative hospital stay, days (mean ± SD)	38.0 ± 37.9	41.3 ± 30.1	.014

*Wrapping of pancreatic anastomosis or vessels, including hepatic artery, using omentum or falciform ligament.
†Early intra-abdominal hemorrhage indicates incomplete hemostasis and a failure of carrying out sufficient intraoperative management. It was defined as occurring within 3 days after pancreaticoduodenectomy, and it was not associated with any other postoperative complications. Late intra-abdominal hemorrhage is associated with other postoperative complications, including pancreatic fistula and intra-abdominal abscess.

Table III. Postoperative drainage after pancreaticoduodenectomy

		*Wrapping group**	
Parameter	*Nonwrapping group* (n = *1,679*)	*Falciform ligament* (n = *219*)	*Omentum* (n = *699*)
Amylase level of postoperative drainage fluid (IU/l)			
POD1	4,405 ± 14,129	4,802 ± 17,644	4,950 ± 13,324
POD3	2,924 ± 2,963	2,077 ± 10,947	1,317 ± 2,963†
POD4	1,384 ± 6,876	327 ± 639	1,395 ± 8,227

*Wrapping of pancreatic anastomosis or vessels, including hepatic artery, using omentum or falciform ligament.
†$P = .027$ (nonwrapping versus omentum).
POD, Postoperative day.

DISCUSSION

This study was a report with a large number of patients on the effect of omentum wrapping or falciform ligament after a PD by a retrospective analysis after the report of ISGPF definition.[16] Each institution had their own criteria for pancreatic fistula before the publication of the definition of pancreatic fistula by an ISGPF. Therefore, it was difficult to compare the incidence of pancreatic fistula. The members of the JSPS now share the same definition of pancreatic fistula, and we can accumulate clinical data to compare the incidence of pancreatic fistula by using this common definition. These data were collected between January 2006 and June 2008. However, only 65% of the institutions could respond to the survey, because 35% of the institutions do not have database systems that can evaluate the incidence of pancreatic fistula according to the ISGPF criteria. Seven independent risk factors were identified for grade B + C pancreatic fistula, 4 factors for early intra-abdominal hemorrhage, and 2 factors for late intra-abdominal hemorrhage. Although the evaluation of delayed gastric emptying and intra-abdominal hemorrhage should be based on grading of ISGPS,[21,22] this study was conducted as a retrospective study, and it was difficult to accumulate sufficient data based on the ISGPS criteria that were reported in 2007.

The incidence of pancreatic fistula was significantly higher in the wrapping group in comparison to the nonwrapping group; moreover, the incidence of grade B + C pancreatic fistula was also higher in the wrapping group. However, the amylase level of the drainage fluid was lower in

Table IV. Complications according to the material used by wrapping

Parameter	*Nonwrapping group* (n = *1,679*)	*Wrapping group**			
		Falciform ligament, (%) (n = *219*)	P *value*†	*Omentum, (%)* (n = *699*)	P *value*†
Pancreatic fistula					
All grades	627 (37.3)	72 (32.8)	.197	321 (45.9)	<.001
Grade B + C	281 (16.7)	31 (14.2)	.332	167 (23.9)	<.001
Delayed gastric empting	182 (10.8)	25 (11.4)	.797	92 (13.2)	.106
Bile leakage	52 (3.1)	6 (2.7)	.773	23 (3.3)	.806
Intra-abdominal abscess	179 (10.7)	33 (15.1)	.051	78 (11.2)	.722
Intra-abdominal hemorrhage					
Early	32 (1.9)	3 (1.4)	.791	11 (1.6)	.580
Late	22 (1.3)	4 (0.5)	.532	14 (2.0)	.208
Wound infection	151 (9.0)	26 (11.8)	.168	89 (12.7)	.006
Other organ complications					
Respiratory	76 (4.6)	8 (3.7)	.554	35 (5.0)	.613
Cardiac	25 (1.5)	5 (2.3)	.382	23 (3.3)	.004
Vascular	24 (1.4)	8 (3.7)	.025	12 (1.7)	.601
Renal	17 (1.0)	1 (0.5)	.712	3 (0.4)	.156
Mortality	22 (1.3)	2 (0.9)	>.999	7 (1.0)	.532

*Wrapping of pancreatic anastomosis or vessels, including hepatic artery, using omentum or falciform ligament.
†Versus nonwrapping group.

Table V. Complications according to the location of wrapping

Parameter	*Nonwrapping group, (%)* (n = *1,679*)	*Wrapping group**			
		Vessels, (%) (n = *552*)	P *value*†	*Anastomosis,*‡ *(%)* (n = *366*)	P *value*†
Pancreatic fistula					
All grades	627 (37.3)	223 (40.4)	.200	170 (46.4)	.001
Grade B + C	281 (16.7)	110 (19.9)	.087	88 (24.0)	.001
Delayed gastric empting	182 (10.8)	52 (11.2)	.798	55 (15.1)	.023
Bile leakage	52 (3.1)	18 (3.3)	.848	11 (3.0)	.927
Intra-abdominal abscess	179 (10.7)	55 (9.9)	.643	56 (15.3)	.012
Intra-abdominal hemorrhage					
Early	32 (1.9)	4 (0.7)	.056	10 (2.7)	.313
Late	22 (1.3)	8 (1.4)	.806	10 (2.7)	.047
Wound infection	151 (9.0)	61 (11.1)	.153	54 (14.8)	.001
Other organ complications					
Respiratory	76 (4.6)	22 (4.0)	.591	21 (5.7)	.323
Cardiac	25 (1.5)	12 (2.2)	.274	16 (4.4)	<.001
Vascular	24 (1.4)	10 (1.8)	.525	10 (2.7)	.077
Renal	17 (1.0)	1 (0.2)	.059	3 (0.3)	.734
Mortality	22 (1.3)	6 (1.1)	.683	3 (0.8)	.602

*Wrapping of vessels, including hepatic artery, using omentum or falciform ligament.
†Versus nonwrapping group.
‡Pancreaticojejunostomy or pancreaticogastrostomy using either the omentum or falciform ligament.

patients with omental wrapping than that with other procedures. It might be suggested that the omental wrapping would disturb the drainage of oozing pancreatic juice, and that this may cause damage of the omentum. Indeed, omental wrapping is associated with complications, such as intestinal obstruction, necrosis of the omentum, and infection.[20]

A soft pancreas is susceptible to postoperative intra-abdominal hemorrhage, and a late intra-abdominal hemorrhage is a lethal complication. Omentum wrapping influenced the occurrence of intra-abdominal hemorrhage, which might be related to omentum wrapping, which is performed to protect skeletonized vessels when the surgeon considers the vessels to be fragile during an operation.

Table VI. Univariable analysis for pancreatic fistula

Parameter	*Pancreatic fistula**		P *value*
	With (n = *479*)	*Without* (n = *2,118*)	
Age, y (≥70/<70)	221/258	862/1,256	.029
Gender (male/female)	321/158	1,238/880	.010
Albumin, g/dL (≥3.5/<3.5)	354/108	1,674/383	.020
AST, IU/L (>40/<40)	211/257	807/1,261	.016
ALT, IU/L (>40/<40)	253/215	987/1,083	.013
Amylase, IU/L (>180/<180)	52/406	307/1,709	.034
Preoperative biliary drainage (yes/no)	248/231	973/1,145	.021
Pylorus preservation (yes/no)	282/197	1,118/1,000	.018
Extended resection (yes/no)	384/86	1,788/287	.013
Operation time, min (>600/<600)	116/354	378/1,693	.001
Blood loss, mL (>1,500/<1,500)	119/359	470/1,632	.233
Pancreatic texture (hard/soft)	389/90	1,070/1,048	<.001
Anastomosis (P-J/P-G)	439/40	1,876/242	.051
Duct-to-mucosal anastomosis (yes/no)	372/107	1,675/443	.491
Pancreatic stent (yes/no)	402/77	1,639/479	.002
Wrapping			<.001
Falciform ligament at pancreaticoenterostomy	5	3	
Falciform ligament at vessels	67	144	
Omentum at pancreaticoenterostomy	165	193	
Omentum at vessels	156	185	
No	627	1,052	

*Grade B + C pancreatic fistula according to the International Study Group of Pancreatic Fistula.
ALT, Alanine aminotransferase; *AST,* aspartate aminotransferase; *P-G,* pancreaticogastrostomy; *P-J,* pancreaticojejunostomy.

Table VII. Univariable analysis for intra-abdominal hemorrhage

Parameter	*Early intra-abdominal hemorrhage*			*Late intra-abdominal hemorrhage*		
	With (n = *46*)	*Without* (n = *2,551*)	P *value*	*With* (n = *40*)	*Without* (n = *2,557*)	P *value*
Age, y (≥70/<70)	25/21	1,058/1,493	.079	16/24	1,067/1,490	.826
Gender (male/female)	35/11	1,534/1,027	.025	32/8	1,527/1,030	.009
Albumin, g/dL (≥3.5/<3.5)	35/10	1,993/481	.641	29/11	1,999/480	.197
AST, IU/L (>40/<40)	20/26	998/1,492	.641	13/26	1,005/1,492	.382
ALT, IU/L (>40/<40)	25/21	1,215/1,277	.452	17/22	1,223/1,276	.507
Extended resection (yes/no)	35/10	2,137/363	.148	34/5	2,138/368	.744
Operation time, min (>600/<600)	18/30	478/2,017	.008	13/27	481/2,020	.035
Blood loss, mL (>1,500/<1,500)	15/31	574/1,960	.111	13/27	576/1,964	.142
Blood transfusion (yes/no)	27/18	776/1,642	<.001	9/27	794/1,633	.327
Pancreatic texture (hard/soft)	38/8	1,421/1,130	<.001	34/6	1,425/1,132	<.001
Anastomosis (P-J/P-G)	40/6	2,275/276	.630	35/5	2,280/277	.616
Duct-to-mucosal anastomosis (yes/no)	34/12	2,013/538	.411	31/9	2,016/541	.837
Pancreatic stent (yes/no)	38/8	2,003/548	.503	38/2	2,003/554	.011
Wrapping (yes/no)	14/32	904/1,647	<.001	22/18	1,657/900	.198
Omentum	11	688	.901	14	685	.562
At panceaticoenteric anastomosis	10	356	.109	10	356	.209

ALT, Alanine aminotransferase; *AST,* aspartate aminotransferase; *P-G,* pancreaticogastrostomy; *P-J,* pancreaticojejunostomy.

Surgeons might therefore have chosen to use wrapping for inappropriate cases or when they suspected an increased likelihood of leakage. If surgeons choose to use wrapping in worst cases, a high incidence of pancreatic fistula should be indicated in both omental wrapping and falciform ligament groups.

This study has revealed that wrapping using the omentum did not decrease the incidence of pancreatic fistula. However, this study has several

Table VIII. Risk factors for postoperative pancreatic fistula after pancreaticoduodenectomy according to a multivariable analysis

Predictor	P *value*	*Odds ratio (95% CI)*
Gender (male)	<.001	1.508 (1.200–1.896)
Albumin (<3.5 g/dL)	.035	1.332 (1.021–1.738)
Pancreas texture (soft)	<.001	4.129 (3.139–5.339)
Operation time (≥600 minutes)	.031	1.345 (1.027–1.761)
Extended resection	.013	1.461 (1.084–1.969)
Pylorus preservation	.032	1.276 (1.021–1.595)
Wrapping		
Omentum at pancreaticoenterostomy	.040	1.378 (1.104–1.871)
Omentum at vessels	.005	1.555 (1.141–2.120)

CI, Confidence interval.

limitations because it was a multicenter study using retrospective data collection, which makes it a potential source for significant bias. This study indicated that the usage of an omental flap does not reduce the occurrence of complications after PD, including the incidence of pancreatic fistula. A further validation study is therefore necessary to evaluate the efficacy of wrapping for PD.

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Chiba Cancer Center, Department of Gastroenterological Surgery
Chiba Rosai Hospital, Department of Surgery
Dokkyo Medical University, Department of Surgery II
Fujita Health University School of Medicine, Department of Biliary Pancreatic Surgery
Fukuoka University Faculty of Medicine, Department of Surgery
Fukui Red Cross Hospital, Department of Surgery
Fukui Saiseikai Hospital, Department of Surgery
Hachioji-Shokaki Hospital, Department of Surgery
Hamamatsu University School of Medicine, Department of Surgery II
Hino Municipal Hospital, Department of Surgery
Hirosaki University School of Medicine, Department of Surgery II
Hiroshima City Hospital, Department of Surgery
Hiroshima University Graduate School of Biomedical Sciences, Department of Surgery, Division of Clinical Medical Science
Hiroshima University Graduate School of Biomedical Sciences, Department of Surgery, Division of Frontier Medical Science
Hokkaido University Graduate School of Medicine, Department of General Surgery
Hyogo College of Medicine, Department of Surgery 1
Ise Municipal General Hospital, Department of Surgery
Itabashi Chuo Medical Center, Department of Surgery
Iwate Medical University School of Medicine, Department of Surgery
Jichi Medical University, Department of Surgery
Jikei University School of Medicine, Department of Surgery
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Kagoshima University, Department of Surgical Oncology
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Kumamoto University, Department of Gastroenterological Surgery
Kurume University School of Medicine, Department of Surgery

Kyorin University School of Medicine, Department of Surgery
Kyoto University, Department of Hepato-Biliary-Pancreatic Surgery and Transplantation
Kyushu University, Faculty of Medicine, Department of Surgery I
Matsunami General Hospital, Department of Surgery
Meiwa Hospital, Department of Surgery
Mie University Graduate School of Medicine, Department of Hepatobiliary Pancreatic Surgery
Miyagi Cancer Center, Department of Surgery
Miyazaki University School of Medicine, Department of Surgical Oncology and Regulation of Organ Function
Nagasaki Medical Center, Department of Surgery
Nagasaki University Graduate School of Medicine, Department of Gastroenterological Surgery
Nagasaki University Graduate School of Medicine, Department of Translational Medical Science
Nagoya City University Graduate School of Medical Sciences, Department of Gastroenterological Surgery
Nagoya University Graduate School of Medicine, Department of Gastroenterological Surgery
Nara Medical University, Department of Surgery
National Cancer Center Hospital East, Department of Upper Abdominal Surgery
Nihon University School of Medicine, Division of Digestive Surgery
Niigata Prefectural Central Hospital, Department of Surgery
Niigata University School of Medicine, Department of Surgery
Nippon Medical School, Department of Surgery I
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Okayama University Medical School, Department of Surgery
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Tokyo Medical and Dental University, Department of Hepato-Biliary-Pancreatic Surgery
Tokyo Medical and Dental University Ichikawa General Hospital, Department of Surgery
Tokyo Medical University, Department of Surgery
Tokyo Women's Medical University Medical Center East, Department of Surgery
Tokyo Women's Medical University, Institute of Gastroenterology, Department of Gastroenterological Surgery
University of Occupational and Environmental Health, Department of Surgery I
University of Yamanashi Faculty of Medicine, Department of Surgery I
Wakayama Medical University, Second Department of Surgery
Yamagata University Faculty of Medicine, Department of Gastroenterological and General Surgery
Yamaguchi University Graduate School of Medicine, Department of Digestive Surgery and Surgical Oncology
Yame General Hospital, Department of Surgery
Yokohama City University, Department of Gastroenterological Surgery.

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Coexpression of MUC16 and mesothelin is related to the invasion process in pancreatic ductal adenocarcinoma

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The invasion process is a crucial step for pancreatic ductal adenocarcinoma (PDAC); however, the genes related to invasion remain unclear. To identify specific genes for the invasion process, we compared microarray data for infiltrating cancer and PanIN-3, which were harvested from an individual PDAC patient by microdissection. Furthermore, immunohistochemical, coimmunoprecipitation and invasion analyses were performed to confirm the biologic significance of molecules identified by expression profile. In the present study, we focused on MUC16 and mesothelin among 87 genes that were significantly upregulated in infiltrating components compared to PanIN-3 in all PDAC patients, because MUC16 was the most differently expressed between two regions, and mesothelin was reported as the receptor for MUC16. Immunohistochemical analysis revealed that MUC16 and mesothelin were expressed simultaneously only in infiltrating components and increased at the invasion front, and binding of MUC16 and mesothelin was found in PDAC by immunoprecipitation assay. The downregulation of MUC16 by shRNA and the blockage of MUC16 binding to mesothelin by antibody inhibited both invasion and migration of pancreatic cancer cell line. MUC16 high/mesothelin high expression was an independent prognostic factor for poor survival in PDAC patients. In conclusion, we identified two specific genes, MUC16 and mesothelin, associated with the invasion process in patients with PDAC. (*Cancer Sci* 2012; 103: 739–746)

For most patients with pancreatic ductal adenocarcinoma (PDAC), the diagnosis is made at an advanced stage;[1] the survival rate for these patients is dismal because PDAC has a propensity for early local invasion and vascular dissemination.[2] The genetic and biochemical determinants of the process of invasion and metastasis in PDAC are still largely unknown.

Pancreatic ductal adenocarcinoma appears to arise from histologically well-defined precursor lesions in the ducts of the pancreas, called pancreatic intraepithelial neoplasms (PanIN).[3,4] PanIN are graded based on their degree of architectural and nuclear atypia and are categorized into a four-tier classification, including PanIN-1A, 1B, 2 and 3.[5] PanIN-3 lesions demonstrate widespread loss of nuclear polarity, nuclear atypia and frequent mitoses, and whereas cancerous cells break through the basement membrane, they evolve into infiltrating adenocarcinoma. The invasion process is the crucial step in PDAC because cancer cells that invade the vasculature, or lymphatic or neural vessels, can progress further to metastasis only after obtaining infiltrating status. In the present study, we identified specific molecular markers associated with invasion in PDAC, which might be useful not only as early diagnostic markers but also as new therapeutic targets for patients with PDAC.

Several molecular markers, including tissue plasminogen activator,[6] artemin[7] and RhoGDI2,[8] have been reported to be associated with invasion in PDAC. However, some of these molecular markers are of little clinical value as therapeutic targets for patients with PDAC because these genes are also expressed in normal pancreatic tissues or other normal organs.[6–8] In this study, we first used a gene expression profiling technique to identify the specific genes that are differentially expressed between infiltrating cancer cells and PanIN-3 cells, which were harvested from an individual patient by laser microdissection. Based on our gene expression array data, clinical and biologic implications of MUC16 and mesothelin expression were further explored.

Material and Methods

Patients. Our study population included 103 patients with PDAC who underwent curative resection between January 2004 and December 2007 at Wakayama Medical University Hospital (WMUH). Informed consent was obtained from all patients in accordance with the guidelines of the Ethical Committee on Human Research of WMUH. Patient characteristics are presented in Table 1. The TNM staging criteria of the International Union Against Cancer was used for histologic classification.[9] None of the patients had received neoadjuvant chemotherapy or radiation therapy before surgery. The median follow-up duration after resection was 16.8 months (range: 1.6–67.3 months).

Laser microdissection and RNA extraction. Tissue samples including cancer cells and adjacent normal cells were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA) by freezing tissue blocks in liquid nitrogen immediately after surgical resection for expression profiling. We used the tissues obtained from five patients with PDAC who had coexisting infiltrating cancer cells and PanIN-3 cells, and used the tissues from three patients as controls, including two patients with pancreatitis and one patient with bile duct cancer.

The specimens were cut into 9-μm sections at −20°C with the use of a LEICA cryostat (model 3050S; Leica, Tokyo, Japan) and then fixed on slides in 70% ethanol and stained with hematoxylin. The infiltrating cancer cells and PanIN-3 cells were harvested separately from an individual PDAC tissue using laser microdissection. As a control, the normal pancreatic duct cells were also obtained by laser microdissection, because PDAC originates from pancreatic ductal epithelial cells. Before laser microdissection, two pathologists (YS and

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Table 1. Patient characteristics (n = 103)

Age, median (range)	69 (31–87)
Gender, male/female	54/49
Tumor site, Ph/Pbt/Phbt	71/30/2
Surgical technique, PD/DP/TP	71/30/2
Differentiation, well/moderate/poor	42/51/10
Tumor size	
≤20mm	18
>20 but ≤40mm	69
>40 but ≤60mm	14
>60mm	2
UICC stage	3
IA	3
IB	5
IIA	24
IIB	63
III	1
IV	7
Postoperative recurrence, yes/no	79/24

DP, distal pancreatectomy; Pbt, pancreatic body and tail; PD, pancreatoduodenectomy; Ph, pancreatic head; TP, total pancreatectomy; UICC, Union for International Cancer Control.

YN) diagnosed infiltrating cancer regions and PanIN-3 regions in the PDAC tissues, and normal pancreatic epithelium in normal pancreatic tissues. We estimated that the proportion of infiltrating cancer cells, PanIN-3 cells, or normal pancreatic ductal cells in the laser microdissected purified samples was at least 95%. Hence, we required more than 30 specimens (range, 35–78 specimens) in each sample for infiltrating cancer cells, more than 110 specimens (range, 111–414 specimens) for PanIN-3 cells and more than 450 specimens (range, 450–520 specimens) for normal pancreatic ductal epithelium cells to obtain enough RNA volume to use for our expression analysis. Total RNA was extracted from the harvested cells using the RNeasy Micro Kit (Qiagen, Hilden, Germany). The concentration of each total RNA sample was measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The integrity of the RNA was determined by capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and the extracted RNA was accepted for experiments if the RNA integrity reading was >7.0.

Genome-wide transcriptional profiling. The gene expression was analyzed with Human Genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA, USA). The manufacturer's instructions regarding the protocols and the use of reagents for hybridization, washing and staining were followed (as previously described).[10] Data were collected using an Affymetrix GeneChip Scanner 3000 instrument. The cell intensity data files were obtained using the Affymetrix Suite 5.0 software program; then, the array data were imported into a DNA-Chip Analyzer (dChip, http://www.dchip.org) for high-level analysis.

Immunohistochemistry. Pretreatment was performed in a microwave using citrate buffer (pH 6.0) for 5 × 3 min at 700 W. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol, and nonspecific binding sites were blocked with 10% normal goat serum. Primary antibodies were diluted in PBS: MUC16 (1:1000, mouse monoclonal, X325, Abcam, Cambridge, UK) and mesothelin (1:20, mouse monoclonal, 5B2, Novacastra, Newcastle upon Tyne, UK). Diluted primary antibodies were added, and samples were incubated overnight at 4°C. Antibody binding was then immunodetected using the avidin–biotin–peroxidase complex, as described by the supplier (Nichirei, Tokyo, Japan). Finally, the reaction products were demonstrated using a DAB substrate, and then counterstained with hematoxylin, dehydrated with ethanol and fixed with xylene.

To investigate the localization of the MUC16 and mesothelin, fluorescence immunohistochemistry was performed for paraffin-embedded tissue slides. Double labeling of the two mouse monoclonal antibodies (MUC16 [X325] and mesothelin [5B2]) was done using a Zenon kit (Molecular Probes, Eugene, OR, USA) to directly label the antibodies with either Alexa Fluor 488 or 594 according to the manufacturer's instructions.

Evaluation of immunohistochemistry. For scoring assessment, 200 cells were counted in each of the five different fields with high magnification, ×400, on the maximum cut surface of the tumor. We used ovarian cancer tissue and mesothelioma tissue as positive controls for MUC16 and mesothelin expression, respectively. The staining intensity was defined as follows: 0, no staining; 1+, weak; 2+, moderate; 3+, strong, based on the intensity levels of positive control being taken as 3+ (Fig. 1A).[11–13] If there were areas with a variety of staining intensities, the predominant intensity was chosen. The quantification of positivity (0–100%) was based on an estimate of the percentage of stained cancer cells in the lesion. The final immunostaining scores were calculated by multiplying the staining intensity and percentage positivity, thereby giving immunostaining scores ranging from 0 to 300.[14–17] The cut-off values of immunostaining scores were set as the median value, in accordance with previous reports.[18,19] The immunostains were scored by three investigators (SH, YN and HY) blinded to the clinical and pathologic data. If differences of opinion arose, a consensus was achieved by discussion.

Cell lines and RNA interference. Human pancreatic cancer cell line PK9 was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Tohoku University (Miyagi, Japan).

Short hairpin RNA (shRNA) plasmids designed to target MUC16 were synthesized by SA Biosciences (Frederick, MD, USA) as follows: insert sequence ACAGCAGCATCAAGAGTTATT and ggaatctcattcgatgcatac (negative control). Each plasmid (0.8 μg) was mixed with 1 μL Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) in a final volume of 100 μL of Opti-MEM medium and was added to PK9 cells grown to 40% confluence in 24-well plates. Forty-eight hours after transfection, G418 solution (Roche, Basel, Switzerland) was added in the appropriate concentration. The stably transfected cells were maintained in RPMI-1640.

Coimmunoprecipitation assay. To address binding between MUC16 and mesothelin, we performed coimmunoprecipitation assays using pancreatic cancer cell line PK9 and two surgical specimens obtained from 2 PDAC patients. The coimmunoprecipitation assays were performed using the Universal Magnetic Co-IP Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's protocol. Monoclonal antibody against CA125 (OC125, Abcam, Cambridge, UK), monoclonal antibody against mesothelin (MN-1, Rockland, Gilbertsville, PA, USA) or rabbit IgG control (Abcam) were used for immunoprecipitation and immunoblotting.

***In vitro* invasion and migration assay in PK9 cell line transfected with MUC16 shRNA.** To investigate the effect of MUC16 expression on invasion and migration of pancreatic cancer cells, *in vitro* invasion and migration assays were performed in the membrane culture system using an 8-μm pore size PET membrane coated with or without Matrigel (24-well, BD Biosciences, San Diego, CA, USA). Parental PK9 cells, vector control-PK9 cells and PK9 cells transfected with MUC16 shRNA were seeded into 5 × 10^4 cells/500 μL growth medium on the Matrigel layer. The following procedures were performed (as previously described).[20]

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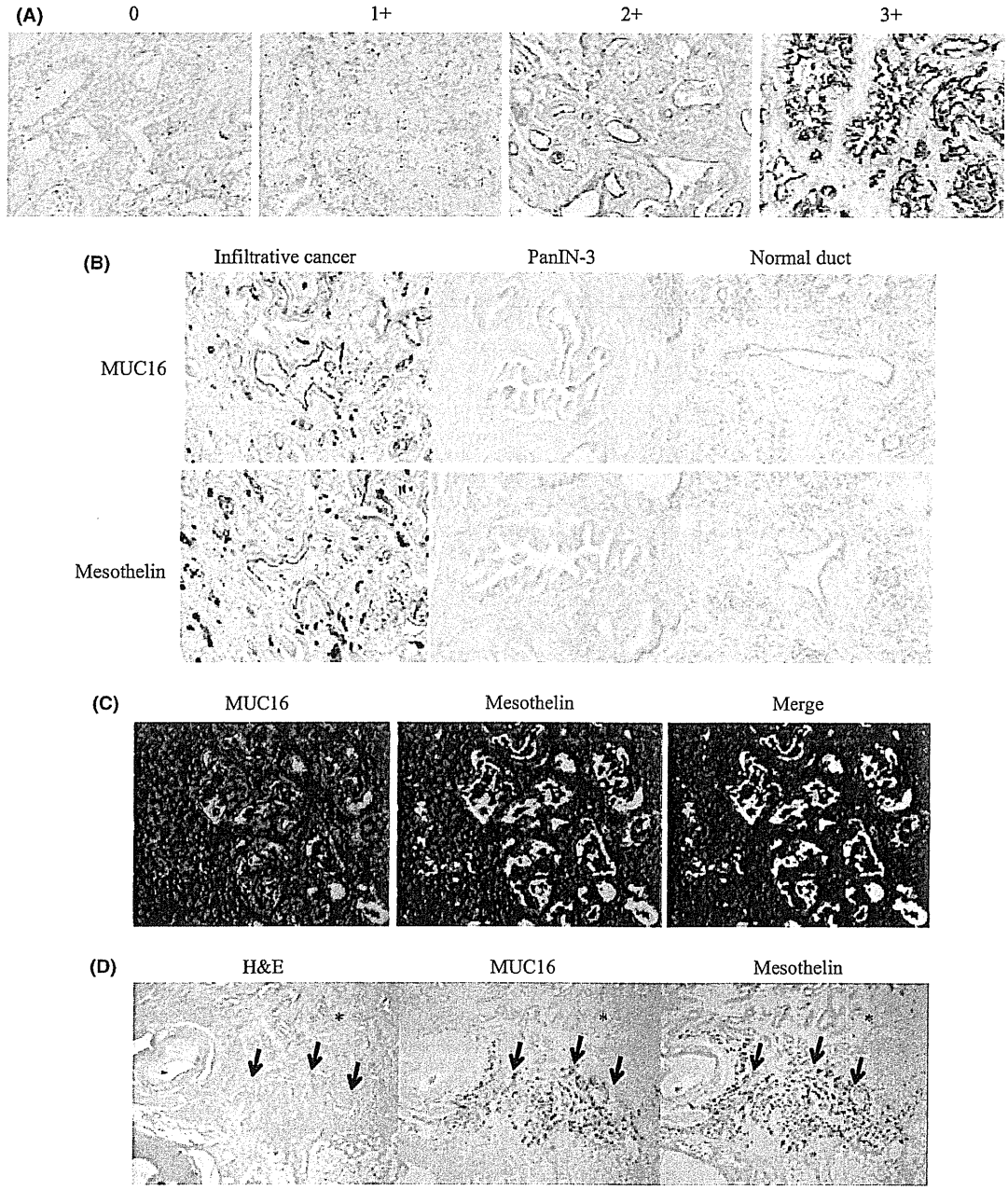

Fig. 1. (A) Image of staining intensity grade. (0) no staining, (1+) weak, (2+) moderate, (3+) strong intensity. (B) MUC16 and mesothelin were stained at the apical membrane or cytoplasm only in infiltrative cancer, whereas no staining appeared in PanIN-3 cells and normal ductal cells. (C) MUC16 and mesothelin expressed at the apical cancer cell surface in invasive ductal cancer cells labeled with Zenon Alexa Fluor 594 and 488. The merged image shows MUC16 and mesothelin expressed in the same cancer cells simultaneously. (D) The expression of MUC16 and mesothelin was higher at the invasion front (arrow) than in the main tumor (*). H&E, hematoxylin and eosin stain.

***In vitro* invasion and migration assays with blocking antibodies for MUC16 and mesothelin.** To investigate the binding between MUC16 and mesothelin, we evaluated the effect of blocking antibodies against interaction between MUC16 and mesothelin on invasion and migration of pancreatic cancer cell PK9 by using *in vitro* invasion and migration assay. Because OC125 (DAKO, Carpinteria, CA, USA) and M11 (DAKO) are known to block the interaction between MUC16 and mesothelin,[21] each antibody was used for blocking the interaction. Sodium azide was removed using the AbSelect Antibody Purification System (Innova Biosciences, Cambridge, UK).

Statistical analysis. The association between MUC16/mesothelin expression and clinicopathologic factors in the patients with PDAC was assessed using the χ^2-test or the Fisher exact test. The survival curves were calculated using the Kaplan–Meier method and then compared by means of the log-rank test. The prognostic significance of clinicopathologic features and MUC16/mesothelin expression was determined using univariate Cox regression analysis. Cox proportional hazards models were fitted for multivariate analysis. Statistical procedures were performed using SPSS version 13.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Identification of the transcriptional biomarkers associated with the invasion of pancreatic ductal adenocarcinoma by gene expression profiling. Microarray data for the infiltrating cancer and PanIN-3, which were harvested from an individual PDAC patient, were compared on the basis of the following criteria: (i) a ≥ 1.5-fold change in the expression levels between the infiltrating cells and PanIN-3 cells; (ii) a >100 absolute difference between the expression levels of the infiltrating cells and PanIN-3 cells; and (iii) $P < 0.05$.[22,23] A total of 109 genes were differentially expressed between infiltrating cancer and PanIN-3 cells in PDAC, including 87 genes that were upregulated and 22 that were downregulated in the infiltrating PDAC, and then 18 genes, which were expressed more in both infiltrating cancer and PanIN3 than in normal pancreatic epithelium, were listed (see Table 2), to focus on more significant genes related to carcinogenesis in PDAC. Among the upregulated genes identified by expression profiling, we focused on MUC16 because MUC16 expression in the infiltrating cancer was substantially higher than that of the PanIN-3 cells in all five PDAC patients and normal pancreatic duct epithelium (Table 2), indicating that MUC16 is specifically expressed in invasive PDAC. We also focused on mesothelin in the upregulates genes list, because it had been previously reported to be a ligand receptor of MUC16.[24,25]

Immunohistochemical staining of MUC16 and mesothelin in pancreatic ductal adenocarcinoma. The immunohistochemical analyses were performed in the paraffin-embedded tissues from 103 patients with PDAC. MUC16 and mesothelin were stained by immunohistochemistry at the tumor apical membrane or cytoplasm (or both) in PDAC samples (Fig. 1B). Both MUC16 and mesothelin were expressed only in the infiltrating cancer cells and not in the PanIN-3 cells ($n = 30$) or normal pancreatic epithelial cells ($n = 103$) (Fig. 1B). Furthermore, we found that these genes were not expressed in any non-epithelial cells, including stromal cells, acinar cells and islet cells. Fluorescence immunohistochemistry using the merge technique showed that MUC16 and mesothelin were stained in the same cancer cells simultaneously (Fig. 1C). We observed that MUC16 and mesothelin were more highly expressed at the invasion front than in the main tumor in 48 of the 103 patients (47%) with PDAC (Fig. 1D).

The scores of MUC16 and mesothelin expression were calculated for each sample. The median scores of MUC16 and mesothelin were 150 (range, 0–300) and 180 (range, 0–300), respectively. The binarization of the score data for these markers was performed as "high expression" versus "low expression" at the median level. We categorized all samples into two groups to analyze the association of MUC16 and mesothelin expression with the clinicopathologic features in the patients with PDAC: the MUC16 high/mesothelin high expression group ($n = 41$) versus the other group ($n = 62$), which included the patients with MUC16 high/mesothelin low expression ($n = 11$), those with MUC16 low/mesothelin high expression ($n = 11$) and MUC16 low/mesothelin low expression ($n = 40$).

Association of MUC16 and mesothelin expression with pathologic factors. The correlation of pathologic factors and MUC16/mesothelin expression was analyzed (Table 3). These pathologic factors were evaluated in accordance with the second English edition of the Classification of Pancreatic Carcinoma, proposed by the Japan Pancreas Society.[26] The analysis indicated that a tumor size >4.0 cm, serosal invasion, invasion of other organs, and lymphatic permeation occurred significantly more often in the MUC16 high/mesothelin high expression group than in the other groups ($P = 0.0041$, $P = 0.0131$, $P = 0.0356$ and $P = 0.0250$, respectively).

Binding of MUC16 and mesothelin in pancreatic cancer cell PK9 and surgical specimens from patients with pancreatic ductal adenocarcinoma. The coimmunoprecipitation assays between MUC16 and mesothelin using pancreatic cancer cell line PK9 and surgical specimens obtained from two PDAC patients (number 1: stage IIB, number 2: stage IV) showed that the whole cell lysates or tissue homogenates were immunoprecipitated and immunoblotted with anti-MUC16 and anti-mesothelin antibody (Fig. 2A), indicating that MUC16 and mesothelin can bind in PDAC.

Role of MUC16 and mesothelin in invasion, migration and cell growth of pancreatic cancer cell line. PK9 cells express MUC16 and were transfected with shRNA targeted to MUC16. Stable

Table 2. Upregulated genes in the infiltrating cancer compared to PanIN-3 component of pancreatic ductal adenocarcinoma as determined by expression profiling

Probe ID	Gene name	Gene symbol	Fold change, mean	Mean expression level	
				IC/PanIN-3	IC/normal
220196_at	Mucin 16	MUC16	26.7	14.6	31.6
206884_s_at	Sciellin	SCEL	17.4	3.8	4.7
205388_at	Troponin C type 2	TNNC2	10.1	4.1	10.0
204416_x_at	Apolipoprotein C-I	APOC1	6.7	5.9	7.2
213524_s_at	G0/G1switch 2	G0S2	5.4	4.3	13.9
202504_at	Tripartite motif-containing 29	TRIM29	4.5	2.6	8.8
204070_at	Retinoic acid receptor responder 3	RARRES3	3.7	3.4	5.4
242625_at	Radical S-adenosyl methionine domain containing 2	RSAD2	3.6	2.4	12.1
204885_s_at	Mesothelin	MSLN	3.0	2.2	2.2
201564_s_at	Fascin homolog 1, actin-bundling protein	FSCN1	3.0	2.7	3.1
205483_s_at	Interferon, alpha-inducible protein	IFI	3.0	2.5	7.6
228640_at	BH-protocadherin	PCDH7	2.7	2.5	7.5
239979_at	Epithelial stromal interaction 1	EPSTI1	2.5	2.1	6.5
231956_at	KIAA1618	KIAA1618	2.4	2.4	3.8
204285_s_at	Phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1	2.2	2.1	3.4
222810_s_at	RAS protein activator like 2	RASAL2	2.2	2.2	2.3
243271_at	Sterile alpha motif domain containing 9-like	SAMD9L	2.1	1.9	5.7
200736_s_at	Glutathione peroxidase 1	GPX1	2.0	1.9	2.0

IC, infiltrating cancer; PanIN, pancreatic intraepithelial neoplasms.

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Table 3. The association of MUC16 and mesothelin expression with pathologic factors in patients with pancreatic ductal adenocarcinoma

	Number	MUC16 high/ mesothelin high group	Other group	*P*
		41	62	
Differentiation				
Well/ moderate	93	35	57	0.1908
Poor	10	6	4	
Tumor size				
>40mm	16	12	4	0.0041
≤40mm	87	29	58	
Local progression				
Intrapancreatic common bile duct invasion				
Positive	22	6	16	0.1757
Negative	81	35	46	
Duodenal invasion				
Positive	40	12	28	0.1052
Negative	63	29	34	
Serosal invasion				
Positive	74	35	39	0.0131
Negative	29	6	23	
Retropancreatic tissue invasion				
Positive	85	35	50	0.5369
Negative	18	6	12	
Portal venous system invasion				
Positive	25	13	12	0.1523
Negative	78	28	50	
Arterial system invasion				
Positive	5	4	1	0.0803
Negative	98	37	61	
Extrapancreatic nerve plexus invasion				
Positive	33	16	17	0.2166
Negative	70	25	45	
Invasion of other organs				
Positive	6	5	1	0.0356
Negative	97	36	61	
Lymphatic permeation				
Positive	88	39	49	0.0250
Negative	15	2	13	
Vascular permeation				
Positive	64	28	36	0.2948
Negative	39	13	26	
Perineural invasion				
Positive	76	29	47	0.5665
Negative	27	12	15	
Lymph node metastasis				
Positive	69	32	37	0.0523
Negative	34	9	25	

MUC16–shRNA-transfected PK9 cells showed downregulation of MUC16 protein expression compared to the vector control (data not shown). Invasion chamber experiments revealed that MUC16–shRNA-transfected PK9 cells had significant suppression of cell invasion (Fig. 2B). Migration assays also demonstrated that downregulation of MUC16 significantly reduced migration (Fig. 2C). The blockage of MUC16 binding to mesothelin with the neutralizing antibodies against MUC16 (OC125 or M11) significantly suppressed invasion and migration of pancreatic cancer cells (Fig. 2D,E). In terms of the effect of MUC16 on cell growth, parental PK9 cells, vector control-PK9 cells and MUC16–shRNA-transfected PK9 were seeded in concentration of 10×10^4/mL, and the cell numbers were counted on day 1, 3 and 5 using a hemocytometer. As a result, the cell growth was significantly suppressed after inhibition of MUC16 expression (Fig. 2F).

Association of MUC16 and mesothelin expression with survival in patients with pancreatic ductal adenocarcinoma. The overall survival of the MUC16 high/mesothelin high expression group was significantly worse than in the other group (median 11.9 *vs* 22.8 months, $P = 0.0006$; Fig. 3A). The 1-, 3- and 5-year survival rates of the MUC16 high/mesothelin high group versus the other group were as follows: 51.2 *vs* 72.6%, 8.0 *vs* 25.6% and 0 *vs* 11.5%, respectively. The disease-free survival of the MUC16 high/mesothelin high expression group was also worse than the other group (median 6.7 *vs* 10.9 months, $P = 0.0002$; Fig. 3B). The 1-, 3- and 5-year disease-free survival rates of the MUC16 high/mesothelin high group versus the other group were as follows: 12.2 *vs* 48.4%, 2.5 *vs* 20.3% and 0 *vs* 11.5%, respectively. In the univariate analysis of the overall survival of the patients with PDAC, a tumor size > 4.0 cm, duodenal invasion, portal venous system invasion, lymphatic permeation, vascular permeation, lymph node metastasis and MUC16 high/mesothelin high expression were potential factors for predicting poor survival (Table 4). According to a multivariate analysis of overall survival, vascular permeation and MUC16 high/mesothelin high expression were independent factors for predicting short survival for the patients with PDAC ($P = 0.0025$, HR, 2.241; 95% CI, 1.364–4.310; $P = 0.0158$, HR, 1.936; 95%CI, 1.132–3.310, respectively; Table 4). Similarly, in the multivariate analysis of disease-free survival, a tumor size > 4.0 cm, lymphatic permeation and MUC16 high/mesothelin high expression were independent prognostic factors for a poorer disease-free survival ($P = 0.0167$, HR, 2.141, 95% CI, 1.148–4.000; $P = 0.0202$, HR, 3.984, 95% CI, 1.241–12.821; $P = 0.0131$, HR, 1.985, 95% CI, 1.155–3.412, respectively; Table 5).

Discussion

We first identified genes specific to the invasion process in PDAC using microdissection and gene expression profiling techniques. In this study, we compared microarray data of infiltrating cancer and PanIN3, which were harvested from an individual PDAC patient, to exclude the difference in original gene expression among individuals. Then, we were able to identify similar genes that were differently expressed between infiltrating cancer and PanIN-3 in all five patients.

Among the identified upregulated genes, we focused on MUC16 because its expression in the infiltrating cancer was substantially higher than that in the PanIN-3 cells. We also focused on mesothelin in the list, because it was reported to be a ligand receptor of MUC16. Their interaction has been postulated to play an important role during tumorigenesis and metastasis in ovarian cancer.[24,25] Rump and colleagues reported that the binding of MUC16 and mesothelin expressed by cancer cells mediates heterotypic cell adhesion and might contribute to the metastasis and invasion of ovarian cancer.[24]

In the present study, immunohistochemical analysis revealed that MUC16 and mesothelin were expressed in the infiltrating cancer cells but not in the PanIN-3 cells or normal pancreatic tissues, consistent with the results of gene expression profiling. Furthermore, fluorescence immunohistochemistry showed that MUC16 and mesothelin were expressed simultaneously in the PDAC cells.

MUC16 encodes the CA125 antigen and is a membrane-bound mucin protein with a high molecular weight between 2.5 and 5.0 million daltons.[27] Its proposed structure comprises an N-terminal domain of >22 000 amino acid residues that are presumably heavily glycosylated, a central domain containing up to 60 glycosylated repeat sequences constituting

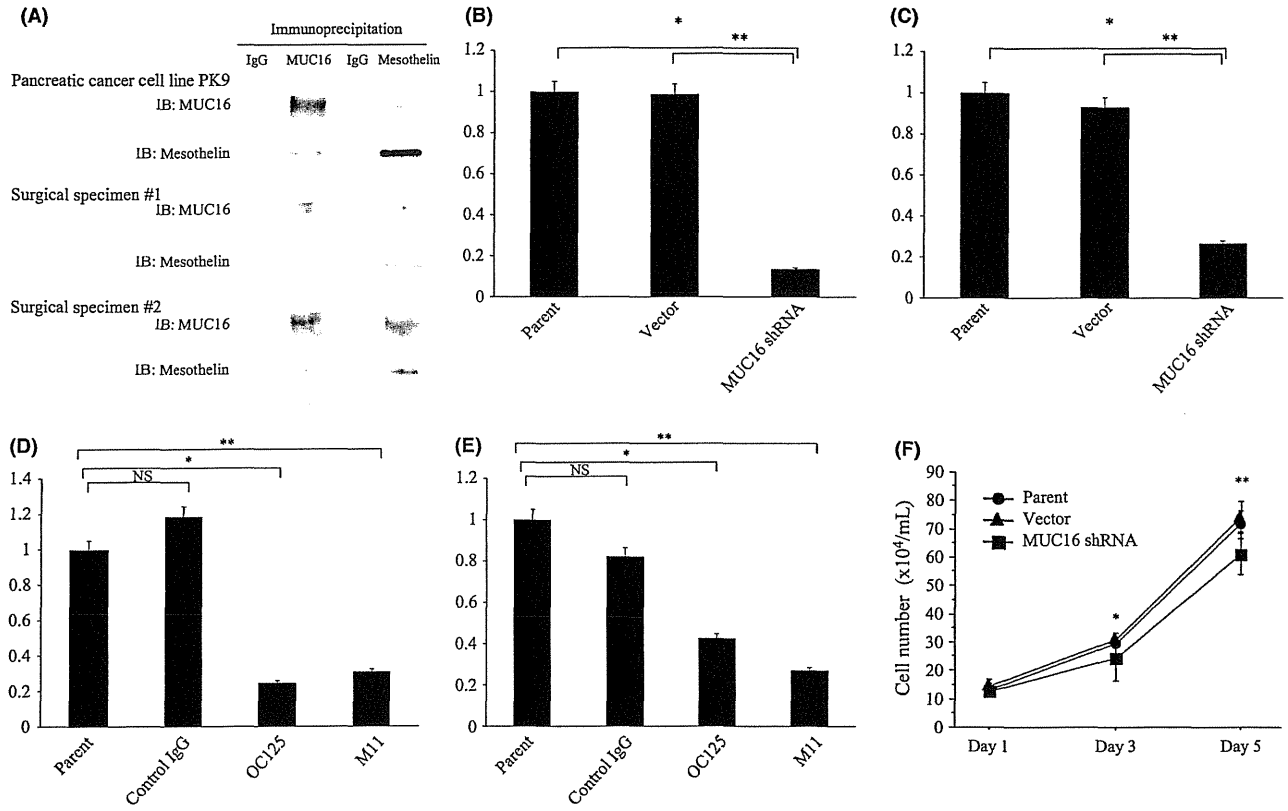

Fig. 2. (A) The results of coimmunoprecipitation assay in pancreatic cancer cell line PK9 and clinical samples from the patients with pancreatic ductal adenocarcinoma. The whole cell lysates extracted from cell line or tissue homogenates extracted from two surgical specimens were immunoprecipitated and immunoblotted with anti-MUC16 and anti-mesothelin antibody. IB, immunoblotting. (B) Invasion chamber experiments in PK9 transfected with MUC16 shRNA. The invasion was significantly suppressed after inhibition of MUC16 expression (*P = 0.0009, **P = 0.0067). (C) Migration assays in PK9 transfected with MUC16 shRNA. The migration was significantly suppressed after downregulation of MUC16 expression (*P = 0.0005, **P = 0.0055). (D) Invasion assay with the blockage of MUC16 binding to mesothelin with the neutralizing antibodies against MUC16 (OC125 or M11, *P = 0.0014, **P = 0.0043). (E) Migration assay with the blockage of MUC16 binding to mesothelin with OC12 5 or M11 (*P = 0.0020, **P = 0.0003). (F) Cell growth assay in PK9 transfected with MUC16 shRNA. The cell growth was significantly suppressed after inhibition of MUC16 expression (*P = 0.0469, **P = 0.0036). NS, not significant.

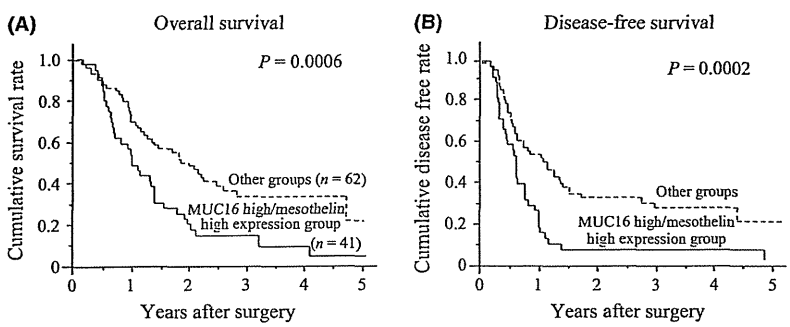

Fig. 3. The overall survival (A) and disease-free survival (B) of the MUC16 high/mesothelin high expression group was worse than that of the other groups (median, 11.9 *vs* 22.8 months, P = 0.0006; 6.7 *vs* 10.9 months, P = 0.0002, respectively).

the tandem repeats characteristic of mucins, and a C-terminal domain composed of a transmembrane domain and a short cytoplasmic tail with possible phosphorylation sites.[28] Few reports have described the expression of MUC16 in cancers. In this study, using immunohistochemistry, we detected the expression of MUC16 in 94 of 103 PDAC cases (91%).

The mesothelin gene encodes a 71-kDa precursor protein that is processed into the 40-kDa glycosylphosphatidylinositol-anchored membrane glycoprotein, mesothelin and a 31-kDa fragment called megakaryocyte potentiating factor.[29,30] Mesothelin expression in normal human tissues is limited to mesothelial cells lining the pleura, pericardium and peritoneum,[29] and the protein is also expressed by a variety of solid tumors, including ovarian cancer, malignant mesothelioma, lung cancer and PDAC.[31,32] Mesothelin expression reportedly conferred chemoresistance and a poorer clinical outcome in ovarian cancer patients.[33]

We found that the coexpression of MUC16 and mesothelin was also increased at the invasion front (n = 48), compared to that in the main tumor in several PDAC tissues, and, then, MUC16 high/mesothelin high expression in PDAC was significantly associated with large tumors, serosal invasion, invasion of other organs and lymphatic permeation. These results indicate that these molecules seem to be involved in invasion and

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Table 4. Univariate and multivariate analysis using the Cox proportional hazards regression model of overall survival in 103 patients with pancreatic ductal adenocarcinoma

	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	*P*	HR	95% CI
Age, ≥70	0.2692	0.906	0.962–1.011	–	–	–
Gender, male	0.7711	1.026	0.678–1.689	–	–	–
Differentiation, poor	0.9228	1.043	0.451–2.410	–	–	–
Tumor size, > 40 mm	0.0070	2.203	1.241–3.906	0.3294	1.340	0.743–2.421
Local progression						
CH, positive	0.1651	1.458	0.856–2.481	–	–	–
DU, positive	0.0465	1.595	1.007–2.525	0.0782	1.575	0.950–2.604
S, positive	0.3320	1.297	0.767–2.188	–	–	–
RP, positive	0.0715	1.848	0.948–3.610	–	–	–
PV, positive	0.0203	1.818	1.098–3.012	0.6830	1.119	0.653–1.916
A, positive	0.6183	1.259	0.507–3.135	–	–	–
PL, positive	0.0666	1.543	0.971–2.451	–	–	–
OO, positive	0.4899	1.342	0.581–3.101	–	–	–
Lymphatic permeation, positive	0.0034	3.937	1.575–9.804	0.1190	2.375	0.801–7.042
Vascular permeation, positive	< 0.0001	3.155	1.859–5.348	0.0025	2.421	1.364–4.310
Perineural invasion, positive	0.1345	1.527	0.877–2.660	–	–	–
Lymph node metastasis, positive	0.0043	2.151	1.272–3.636	0.8436	1.067	0.561–2.033
MUC16/mesothelin expression, high	0.0008	2.206	1.392–3.495	0.0158	1.936	1.132–3.310

A, arterial system invasion; CH, intrapancreatic common bile duct invasion; CI, confidence interval; DU, duodenal invasion; HR, hazard ratio; OO, invasion of other organs; PL, extrapancreatic nerve plexus invasion; PV, portal venous system invasion; RP, retropancreatic tissue invasion; S, serosal invasion.

Table 5. Univariate and multivariate analysis using the Cox proportional hazards regression model of disease-free survival in 103 patients with pancreatic ductal adenocarcinoma

	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	*P*	HR	95% CI
Age, ≥70	0.5105	1.161	0.743–1.815	–	–	–
Gender, male	0.9862	0.996	0.638–1.555	–	–	–
Differentiation, poor	0.5830	0.792	0.344–1.825	–	–	–
Tumor size, > 40 mm	0.0001	3.257	1.770–5.988	0.0167	2.141	1.148–4.000
Local progression						
CH, positive	0.6377	1.138	0.664–1.953	–	–	–
DU, positive	0.0105	1.805	1.148–2.833	0.0633	1.590	0.975–2.591
S, positive	0.0864	1.605	0.935–2.755	–	–	–
RP, positive	0.1104	1.689	0.887–3.205	–	–	–
PV, positive	0.0410	1.675	1.021–2.755	0.6492	1.136	0.656–1.965
A, positive	0.8599	1.095	0.397–3.021	–	–	–
PL, positive	0.2523	1.316	0.822–2.110	–	–	–
OO, positive	0.7087	1.189	0.479–2.959	–	–	–
Lymphatic permeation, positive	0.0034	3.937	2.370–18.181	0.0202	3.984	1.241–12.821
Vascular permeation, positive	0.0012	2.198	1.362–3.546	0.1429	1.506	0.871–2.604
Perineural invasion, positive	0.0452	1.736	1.012–2.985	0.1162	1.577	0.894–2.778
Lymph node metastasis, positive	< 0.0001	3.778	1.938–5.917	0.2388	1.484	0.770–2.857
MUC16/mesothelin expression, high	0.0002	2.378	1.497–3.777	0.0131	1.985	1.155–3.412

A, arterial system invasion; CH, intrapancreatic common bile duct invasion; CI, confidence interval; DU, duodenal invasion; HR, hazard ratio; OO, invasion of other organs; PL, extrapancreatic nerve plexus invasion; PV, portal venous system invasion; RP, retropancreatic tissue invasion; S, serosal invasion.

migration of pancreatic cancer cells. Recent reports show the role of MUC16 in ovarian cancer tumorigenesis,[34,35] and it has been noted that MUC16 regulates cell growth, invasion and metastasis in epithelial ovarian cancer.[34] However, another report indicates the opposite concept, that downregulation of MUC16 inhibits invasion and migration due to the suppression of epithelial to mesenchymal transition in ovarian cancer cells.[35] Thus, the role of MUC16 in ovarian cancer cell invasion and migration is still controversial and no report regarding the role of MUC16 on pancreatic cancer cell invasion and migration has yet appeared.

To examine the role of interaction of MUC16 and mesothelin on pancreatic cancer invasion and migration, we investigated whether shRNA and blocking antibodies for MUC16 suppress invasion and migration of pancreatic cancer cells. We investigated the expression of MUC16 and mesothelin by RT-PCR, western blotting and immunocytochemistry in eight pancreatic cancer cell lines (PK9, PANC1, MIAPaCa2, AsPC1, BxPC3, Capan-1, Capan-2 and PK1). By RT-PCR, both MUC16 and mesothelin mRNAs were detected in five cell lines, including PK9, AsPC1, BxPC3, Capan-2 and PK1. Using western blotting and immunocytochemistry, the strongest positive

expressions of both MUC16 and mesothelin were found in PK9. Therefore, in the present study, we used only PK9 cell line for biological experiments. The blockage of the interaction between MUC16 and mesothelin suppressed invasion and migration of pancreatic cancer cells, suggesting that MUC16 binding to mesothelin is important for cell invasion and migration in pancreatic cancer cells.

Furthermore, we focused on the survival of patients with MUC16 high and mesothelin high expression because coexpression of these two genes is obviously correlated to the invasion of PDAC, and MUC16 high/mesothelin high expression was an independent prognostic factor for poor survival. We examined whether there are any differences in survival between the MUC16 high/mesothelin high group and the MUC16 high/mesothelin low group or MUC16 low/mesothelin high group. However, these groups were very small ($n = 11$), and larger groups of patients are necessary for further study.

The mechanism of overexpression of MUC16 and mesothelin in PDAC has not yet been clarified yet. It is also unclear whether the coexpression of MUC16 and mesothelin was coincidental or the increased expression of MUC16 was associated with an upregulation of mesothelin expression. These issues should be clarified in further studies. Moreover, other molecules in Table 2 besides MUC16 and mesothelin might potentially contribute to the invasion process. In the future, we analyze the roles of other upregulated genes in infiltrating cancer than in PanIN-3 for PDAC patients.

In conclusion, MUC16 and mesothelin are involved in pancreatic cancer cell invasion and migration, and MUC16 and mesothelin clinically represent new prognostic biomarkers for PDAC and might be new therapeutic targets for patients with PDAC, including immunotherapy using a peptide vaccine or monoclonal antibody therapy.

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Disclosure Statement

The authors have no conflict of interest.

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