[16, 17]. A recent prospective randomized controlled trial showed that DGE occurred in 50% of patients in whom the retrocolic route was used but in only 5% in whom the antecolic route was used [18]. These data suggest that the antecolic route is better. However, the 50% incidence of DGE associated with the retrocolic route seems high. We have shown that vertical retrocolic duodenojejunostomy, by which the stomach and duodenum are brought down through the left side of the transverse mesocolon in a straight, vertical manner, reduces the incidence of DGE [19].

Thus, a prospective randomized controlled trial was conducted to compare the incidence of clinical DGE and gastric emptying variables assessed by the ^{13}C-acetate breath test between patients who underwent antecolic duodenojejunostomy and those who underwent vertical retrocolic duodenojejunostomy. The aim of the study was to establish the superiority of the vertical retrocolic route with respect to gastric emptying after PpPD.

Patients and methods

Of 50 patients underwent pancreaticoduodenectomy at Miyazaki University Hospital between March 2005 and July 2007, 46 patients were scheduled to undergo PpPD. Patients were recruited into the study before surgery, on the basis of whether PpPD was anticipated and informed consent was obtained. Specific exclusion criteria included tumor infiltration into the duodenal bulb or presence of lymph node metastasis of the prepylorus ($n = 3$), failure to provide informed consent including the ^{13}C-acetate breath test ($n = 4$) were then excluded. Thus, 35 patients who underwent PpPD and consented to the protocol were enrolled in the study.

This prospective randomized controlled trial was approved by the ethical committee of our university hospital and informed consent was obtained from all patients. The randomization protocol involved assignment of patients to one of two reconstruction methods, the antecolic route and the vertical retrocolic route. Randomization took place during surgery before reconstruction. Gastric emptying was evaluated by means of the ^{13}C-acetate breath test just before surgery and on postoperative day (POD) 30.

Operative technique

The area resected during PpPD included the gallbladder, common hepatic duct, head of the pancreas, duodenum (except for the first portion), and 10 cm of the proximal jejunum. A few arcades of the right gastric artery and right gastroepiploic artery to the stomach were divided along the wall of the antrum (approximately 2–3 cm from the pyloric ring) for dissection of the peripyloric lymph nodes. The duodenum was freed from the surrounding tissue and transected approximately 4–5 cm distal to the pyloric ring. The lymph nodes in the hepatoduodenal ligament, the para-aortic lymph nodes, and those along the common hepatic artery and the right side of the superior mesenteric artery were dissected. The right gastric artery was divided at its origin in all patients. The left gastric artery and vein were carefully preserved. The lesser omentum close to the liver was dissected to allow free movement of the stomach. The vagal nerve, with the exception of the hepatic and pyloric branches, was preserved. These procedures allowed the stomach and the duodenum to be mobilized to the left in a straight, vertical manner.

As the first step in reconstruction, the proximal jejunum was brought through the right side of the transverse mesocolon by the retrocolic route. An end-to-side pancreaticojejunostomy was performed with duct-to-mucosal anastomosis. The pancreatic duct was anastomosed to the whole layer of the small opening in the jejunum to approximate the duct to the jejunal mucosa with the use of eight interrupted 5-0 PDS-II sutures (polydioxanone, Johnson & Johnson Co.), regardless of the size of the pancreatic duct. A 5-Fr polyethylene pancreatic drainage tube with a small knob (Sumitomo Bakelite Co., Japan) was placed in the pancreatic duct and exteriorized through the jejunal limb. The cut surface of the pancreas was then anastomosed to the jejunal seromuscular layer, and the end-to-side pancreaticojejunostomy was completed. A one-layer end-to-side hepaticojejunostomy with interrupted 5-0 PDS sutures was then performed 5–10 cm distal to pancreaticojejunostomy.

The final step was randomized to either to the antecolic route or vertical retrocolic route. For vertical retrocolic duodenojejunostomy, the left side of the transverse mesocolon (left side of the middle colic vessels) was opened, and the duodenum was brought down together with the gastric antrum in a straight, vertical manner. A retrocolic end-to-side duodenojejunostomy was performed at the caudal side of the transverse mesocolon and the antrum was fixed to the transverse mesocolon with a few 4-0 silk sutures. For antecolic duodenojejunostomy, the stomach was brought down antecolically. Braun anastomosis was added in both groups. Finally, the opening of the old ligament of Treitz and the jejunum brought up for pancreaticojejunostomy and hepaticojejunostomy were fixed to the mesocolon, and two or three closed drains were placed around the pancreatic and biliary anastomosis. All patients were given prophylactic antibiotics and H2 blocker postoperatively; none were given prokinetic drugs such as erythromycin.

Data collection and study endpoints

Clinicopathological data were collected prospectively for all patients. Data included postoperative mortality and morbidity, including pancreatic fistula, intraabdominal bleeding, pancreaticojejunostomy or hepaticojejunostomy leakage, intraabdominal abscess, and wound infection. Pancreatic fistula was defined when an amylase level in the fluid from the closed drains was >10,000 IU/l.

The first endpoint was clinical DGE defined as (1) the need for nasogastric tube decompression for more than 10 days (DGE 10), (2) the need for reinsertion of the nasogastric tube, or (3) an inability to take in an appropriate amount solid food orally by POD 14 (DGE 14), as described elsewhere [18].

The secondary endpoint was recovery of gastric emptying as assessed by ^{13}C-acetate breath test [20]. For at least 4 days before this test, all drugs, including H_2 blocker, were withdrawn. All patients ingested a liquid meal (200 Kcal/200 ml, RACOL, Ohtsuka Pharmaceutical Co., Tokyo, Japan) labeled with 100 mg sodium ^{13}C-acetate (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) in the morning after an overnight fast before surgery and on POD 30. Breath samples were collected in the collection bag (1.3 l) before and after ingestion of the test meal, i.e., before and at 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 105, 120, and 180 min after ingestion of the ^{13}C-acetate. The recovery of ^{13}C in the breath samples was analyzed by isotope-selective infrared spectrometry (UBiT IR 300, Otsuka Electronics Co., Ltd, Osaka, Japan). The time when $^{13}CO_2$ reached maximum excretion (Tmax) and half-emptying time (T1/2) were calculated by using analysis software (Microsoft Office Excel, Microsoft Japan, Tokyo, Japan).

Statistical analysis

All values are expressed as mean ± SD. Differences between groups were examined for statistical significance by chi-square test, unpaired or paired Student's t-test, Wilcoxon signed-rank test, or Mann–Whitney U test. Statistical analysis was performed by the statistician who was blind to the study group.

Results

Patient characteristics

Clinical characteristics of the enrolled patients are shown in Table 1. There were no statistical differences between the two groups in age, sex ratio, type of disease, percentage of patients with malignant disease, preoperative laboratory data including *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (BT-PABA) test value, percentage of patients with diabetes mellitus, HbA1c, operation time, or length of the remaining duodenum.

Table 1 Patient characteristics

	Duodenojejunostomy reconstruction route		
	Antecolic $n = 17$	Vertical retrocolic $n = 18$	*P* value
Age (years)	69.7 ± 11.0	66.9 ± 12.9	0.50
Male/female ratio	11/6	9/9	0.38
Hemoglobin (g/dl)	11.8 ± 1.3	12.3 ± 1.5	0.30
Serum albumin (g/dl)	3.67 ± 0.31	3.71 ± 0.46	0.76
Total cholesterol (mg/dl)	171.6 ± 37.4	179.4 ± 38.4	0.55
Diabetes mellitus (+/−)	5/12	2/16	0.23
BT-PABA test (%)	52.3 ± 18.2	49.2 ± 16.1	0.60
HbA1c (%)	5.7 ± 1.6	5.5 ± 0.9	0.67
Soft pancreas	9	10	0.88
Operation time (min)	602.6 ± 93.5	581.7 ± 76.5	0.48
Blood loss (ml)	1619.4 ± 914.9	1535.0 ± 877.7	0.78
Residual duodenum (cm)	3.7 ± 0.7	3.8 ± 0.5	0.54
Division of right gastric artery	17	18	0.54
Final diagnosis			
Benign/malignant disease	5/12	2/16	0.23
Bile duct cancer	8	6	
Pancreatic cancer	4	6	
Ampullary cancer	0	2	
Duodenal cancer	0	1	
IPMN	2	1	
Chronic pancreatitis	2	2	
Benign bile duct tumor	1	0	

Values are mean ± SD or number of patients

IPMN intraductal papillary mucinous neoplasm, *BT-PABA* *N*-benozoyl-L-tyrosyl-*p*-aminobenzoic acid

Postoperative complications

As shown in Table 2, postoperative morbidity was observed in 9 of 17 patients (53%) in the antecolic group and 6 of 18 patients (33%) in the vertical retrocolic group. Intra-abdominal bleeding associated with pancreatic fistula and/or intra-abdominal abscess was observed in one patient in each group, and both patients were treated successfully by interventional transarterial embolization. Intra-abdominal abscess was the main complication and were treated successfully by drainage. No operative death or hospital death was observed.

Table 2 Postoperative outcomes

	Duodenojejunostomy reconstruction route		
	Antecolic $n = 17$	Vertical retrocolic $n = 18$	P value
Postoperative morbidity	9	6	0.24
Major P-J leakage	0	0	
Pancreatic fistula	1	1	0.97
H-J leakage	0	0	
Intra-abdominal bleeding	1	1	0.97
Intra-abdominal abscess	6	5	0.63
Wound infection	3	3	0.94
Respiratory dysfunction	0	0	
G-1 bleeding	1	0	0.49
D-J leakage	0	0	
Mortality	0	0	
NG tube removed (POD)	1.2 ± 0.4	1.1 ± 0.3	0.59
DGE10	0	0	
Reinsertion of NG tube	0	0	
Liquid meal begun (POD)	5.4 ± 2.7	5.7 ± 2.4	0.72
Solid foods begun (POD)	8.4 ± 3.0	10.2 ± 5.1	0.21
DGE14	1	4	0.34
Postoperative stay (days)	40.8 ± 12.3	39.4 ± 11.1	0.74

P-J pancreaticojejunostomy, *H-J* hepaticojeunostomy, *G-I* gastrointestinal, *D-J* duodenojejunostomy, *NG* nasogastric

Clinical DGE

DGE clinically defined as DGE10 or DGE14 and the length of postoperative hospital stay are shown in Table 2. The nasogastric tube was removed on POD 1.2 ± 0.4 in the antecolic group on POD 1.1 ± 0.3 in the vertical retrocolic group. No patient needed a nasogastric tube for more than 10 days (DGE10), and reinsertion of a nasogastric tube was not necessary in any patient. The number of days to the start of liquid diet was similar between the two groups (5.4 days in the antecolic group and 5.7 days in the vertical retrocolic group). With respect to DGE14, one patient in the antecolic group and four in the vertical retrocolic group failed unlimited solid food oral intake by POD 14. Thus, the incidence of DGE defined as DGE14 was 6% (1 of 17 patients) in the antecolic group and 22% (4 of 18 patients) in the vertical retrocolic group. Although the rate was higher in the vertical retrocolic group, the difference did not reach statistical significance ($P = 0.34$). The overall incidence of DGE after PpPD was 14% (5 of 35 patients).

^{13}C-acetate gastric emptying test

Tmax did not differ between the vertical retrocolic group and the antecolic group before or on POD 30 ($P = 0.56$ before surgery and $P = 0.31$ on POD 30). Similarly, T1/2 did not differ between the two groups ($P = 0.99$ before surgery, $P = 0.26$ on POD 30) (Table 3). Neither reconstruction route had a significant effect on gastric emptying on POD 30 after PpPD.

Table 3 ^{13}C-Acetate gastric emptying test results

	Duodenojejunostomy reconstruction route		
	Antecolic $n = 17$	Vertical retrocolic $n = 18$	P value
Before surgery			
Tmax (h)	1.11 ± 0.25	1.08 ± 0.29	0.56
T1/2 (h)	1.78 ± 0.31	1.92 ± 0.81	0.99
After surgery (POD 30)			
Tmax (h)	1.54 ± 1.22	2.12 ± 2.14	0.31
T1/2 (h)	3.63 ± 3.15	6.21 ± 8.62	0.26

Tmax the time when $13CO_2$ reached maximum excretion, *T1/2* half emptying time

The ^{13}C-acetate gastric emptying test values before and on POD 30 were compared in each group. In the vertical retrocolic group, Tmax was significantly prolonged on POD 30 compared to that before surgery (2.12 ± 2.14 h versus 1.08 ± 0.29 h, $P < 0.02$), whereas no significant difference was found in the antecolic group (1.54 ± 1.22 h versus 1.11 ± 0.25 h, $P = 0.29$). However, T1/2 was significantly longer in each group on POD 30 compared to the corresponding preoperative value ($P = 0.0023$ in the antecolic group, $P = 0.0002$ in the vertical retrocolic group (Table 3). Gastric emptying was not completely restored to the preoperative level in either group by POD 30. Mean Tmax on POD 30 in the antecolic group was increased 1.39-fold, and that in the vertical retrocolic group was increased 1.96-fold. Similarly, T1/2 was increased 2.04-fold in the antecolic group and 3.23-fold in the vertical retrocolic group. Greater increases in Tmax and T1/2 were observed in the vertical retrocolic group than in the antecolic group.

Tmax before and after surgery in each patient is shown in Fig. 1. Individual changes in Tmax before and after surgery were similar to individual changes in T1/2. A greater than twofold increase in Tmax was observed in 3 (18%) of 17 patients in the antecolic group, and in 4 (22%) of 18 patients in the vertical retrocolic group. Tmax of all patients before surgery ($n = 35$) was 1.09 ± 0.26 h, ranging from 0.7 to 1.5 h. Tmax greater than 1.5 h on POD 30 was found in four patients in each group. Tmax on POD 30 remained similar to the preoperative level in most patients (approximately 80%) in both groups.

Discussion

The present study showed that the incidence of clinical DGE was lower with the antecolic route than with the vertical

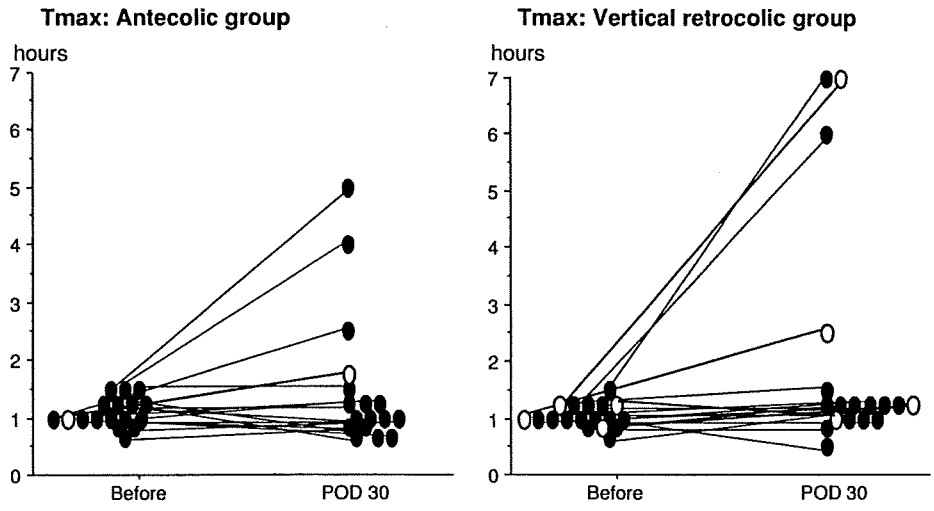

Fig. 1 Changes in Tmax of individual patients (before surgery versus POD 30). *Open circles* represent patients who were not able to tolerate appropriate solid food by POD 14 (DGE14)

retrocolic route, but the difference was not significant (6% with the antecolic route versus 22% with the vertical retrocolic route, $P = 0.34$). Moreover, gastric emptying (Tmax, T1/2) as assessed by the ^{13}C-acetate breath test did not differ significantly between the antecolic route and the vertical retrocolic route before or on day 30 after PpPD. T1/2 was significantly prolonged in both groups after PpPD, indicating that gastric emptying remained impaired on POD 30, regardless of the reconstruction route. The degree of impairment was greater in patients in whom vertical retrocolic reconstruction was performed. An analysis of individual patients revealed that on POD 30, gastric emptying was similar to the preoperative level in approximately 80% of patients, regardless of the reconstruction route.

Since Traverso and Longmire [1] first reported PpPD in 1978, the procedure has been accepted as a standard procedure for periampullary diseases. This is because it yields better quality of life, nutritional status, and weight gain without any difference in postoperative survival than the Whipple procedure [1–4, 21]. The postoperative mortality rate has fallen recently, but complications associated with pancreaticoduodenectomy remain, the most troublesome of which are pancreatic fistula, intra-abdominal infection, intra-abdominal bleeding, wound infection, and DGE. DGE was first reported by Warshaw and Torchiana [5]. Postoperative DGE decreases patient comfort, increases the risk of aspiration pneumonia, prolongs hospital stay, and increases medical costs.

DGE is considered a specific complication of PpPD, because it is specifically attributed to pylorus-sparing resection of the pancreatic head [5–7, 10]. Several underlying mechanisms have been proposed: (1) gastric atony or gastroparesis caused by vagotomy, resection of the duodenal pacemaker, or disruption of the gastroduodenal neural connections [11], (2) local ischemic injury of the antrum and pylorus [7], (3) gastric atony in response to a reduced circulating levels of motilin [12], (4) torsion or angulation of the reconstructed alimentary tract [7], (5) gastric dysrhythmia or gastroparesis secondary to an intraabdominal complication such as anastomotic leakage, abscess, or local inflammation [15, 21]. Recent studies have shown that DGE does not occur as a result of pylorus preservation but rather as a consequence of postoperative complications [17, 22, 23]. Although the exact mechanism underlying DGE is not clear, our results suggest that DGE is related to clinical or even subclinical local inflammation caused by postoperative complications; three of our five patients with DGE (DGE14) had abscess or pancreatic fistula.

DGE has been generally defined as DGE10 (need for a nasogastric tube for more than 10 days) and DGE14 (failure to tolerate solid food by POD 14). The reported incidence of DGE ranges from 20 to 60% [5–13]. In the present study, no patient needed nasogastric decompression for more than 3 days. The nasogastric tube was removed on POD 1 in 30 (86%) of the 35 patients and on POD 2 in the remaining five. None required reinsertion of a nasogastric tube. With respect to DGE14, failure to tolerate solid food was observed in 5 of 35 patients, for an overall incidence of 14%.

A difference in DGE with respect to the reconstruction route, whether antecolic or retrocolic duodenojejunostomy, has been reported. In a retrospective study, Park et al. [23]. found that the incidence of DGE was 31.7% in the retrocolic group, but only 6.5% in the antecolic group. Hartel et al. [24]. reported an incidence of 24% with the retrocolic route and 5% with the antecolic route. Sugiyama et al. [25]. reported that DGE occurred in 1 of 12 patients (8%) in the antecolic group, but in 13 of 18 patients (72%) in the retrocolic group. These retrospective studies have suggested that the incidence of DGE is lower with the antecolic route than with the retrocolic route. A recent prospective randomized study by Tani et al. [18]. yielded an incidence of 50% for the retrocolic route, but 5% for the antecolic route. In the current prospective randomized

controlled trial, the incidence of DGE was 22% with the vertical retrocolic route and 6% with the antecolic route, but the difference was not statistically significant. Although the purpose of this study was to show the superiority of the vertical retrocolic route, an interim analysis did not show any advantage of the vertical retrocolic route; hence, we decided to terminate the study.

In addition to clinically defined DGE, the ^{13}C-acetate gastric emptying test showed that gastric emptying on POD 30 did not differ between the antecolic route and the vertical retrocolic route. Moreover, the gastric emptying did not recover to the preoperative level by 30 days in approximately 20% of patients, regardless of the reconstruction route. A greater increase in Tmax and T1/2 was observed with the vertical retrocolic route than with the antecolic route. These results suggest that the vertical retrocolic route offers no advantage. An analysis of the individuals showed that gastric emptying variables (Tmax, T1/2) had recovered to the preoperative level in approximately 80% of patients on POD 30, regardless of the reconstruction route. The day of analysis and type of meal selected (POD 30, liquid meal) should be reconsidered in another study.

In conclusion, the incidence of DGE and gastric emptying variables (Tmax, T1/2) after PpPD were similar between patients in whom reconstruction was performed by the antecolic route and those in whom it was performed by the vertical retrocolic route. On POD 30, gastric emptying was impaired in both groups compared to the preoperative level, but an analysis of individuals showed that it had recovered to the preoperative level in most patients, regardless of the reconstruction route.

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ORIGINAL ARTICLE

Significance of RRM1 and ERCC1 expression in resectable pancreatic adenocarcinoma

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The identification of molecular markers, useful for therapeutic decisions in pancreatic cancer patients, is crucial for advances in disease management. Gemcitabine, although a cornerstone of current therapy, has limited efficacy. *RRM1* is a key molecule for gemcitabine efficacy and is also involved in tumor progression. We determined *in situ RRM1* and *excision repair cross complementation group 1* (*ERCC1*) protein levels in 68 pancreatic cancer patients. All had R0 resections without preoperative therapy. Protein levels were determined by automated quantitative analysis (AQUA), a fluorescence-based immunohistochemical method. The relationship between protein expressions and clinical outcomes, including response to gemcitabine at the time of disease recurrence, was determined. Patients with high *RRM1* showed significantly better overall survival than patients with low expression ($P=0.0196$). There was a trend toward better overall survival for patient with high ERCC1 ($P=0.0552$). When both markers were considered together, patients with both high *RRM1* and *ERCC1* faired the best in terms of overall and disease-free survival ($P=0.0066$, $P=0.0127$). In addition, treatment benefit from gemcitabine in patients with disease recurrence was observed only in patients with low *RRM1*. The combination of *RRM1* and *ERCC1* expression is prognostic in pancreatic cancer patients after a complete resection. On disease recurrence, only patients with low *RRM1* derive benefit from gemcitabine.
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Keywords: pancreatic cancer; *RRM1*; *ERCC1*; AQUA; prognosis; gemcitabine

Introduction

Pancreatic cancer is one of the leading causes of tumor-related mortalities. The prognosis of patients after complete resection is poor, and more than 50% of patients develop tumor recurrence at distant or locoregional sites, with an estimated 5-year survival of only 20% (Kayahara *et al.*, 1993; Nitecki *et al.*, 1995; Staley *et al.*, 1996; Sener *et al.*, 1999; Li *et al.*, 2004). The addition of chemotherapy and radiotherapy to surgical resection is important, and gemcitabine, a pyrimidine nucleotide analogue, has become the standard chemotherapeutic agent in such programs (Burris *et al.*, 1997; Oettle *et al.*, 2007) (Rothenberg *et al.*, 1996). However, the clinical response rate to gemcitabine remains modest, mainly because of the profound chemoresistance inherent in pancreatic cancer. The selection of patients who derive a true benefit from gemcitabine could be an important stepping stone toward improvement of outcome of pancreatic cancer.

RRM1, the gene that encodes the regulatory subunit of ribonucleotide reductase, is a key determinant of gemcitabine efficacy. In various cancers, we and others have described that overexpression of the *RRM1* gene is strongly associated with gemcitabine resistance (Cao *et al.*, 2003; Rosell *et al.*, 2004; Bergman *et al.*, 2005; Bepler *et al.*, 2006; Nakahira *et al.*, 2007). However, there is no clinical study that investigated the correlation between *RRM1* protein expression and gemcitabine resistance.

On the other hand, the expression of *RRM1* was also reported to correlate with the tumorigenic and metastatic potential of lung cancer (Gautam *et al.*, 2003), and an oncogenic ras-transformed cell line with high expression of an *RRM1* transgene had reduced metastatic potential (Fan *et al.*, 1997). Furthermore, high expression of *RRM1* in transgenic mice is associated with resistance to carcinogen-induced lung tumorigenesis (Gautam and Bepler, 2006). Recently, overexpression of *RRM1* and the *excision repair cross-complementation group 1* (*ERCC1*) gene product was reported to correlate with favorable prognosis in non-small-cell lung cancer (Zheng *et al.*, 2007).

The present study was designed to evaluate the protein expression of *RRM1* and *ERCC1* in pancreatic cancer by automated quantitative analysis (AQUA). We describe the relationship between *RRM1* and *ERCC1* expression, the association between the expression of these proteins and prognosis, as well as the response to

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gemcitabine therapy. To our knowledge, this study is the first to examine both the prognostic and predictive aspects of *RRM1* in the same clinical samples.

Results

RRM1 and ERCC1 expression characteristics

We constructed a tissue microarray using triplicate 0.6-mm cores from formalin-fixed and paraffin-embedded specimens of the primary tumor. Immunostaining showed a granular nuclear pattern for *RRM1*, and a fine granular pattern for *ERCC1* (Figure 1). Next, we used AQUA to analyse the expression levels of *RRM1* and *ERCC1* in specimens obtained from 68 patients. The scores of *RRM1* ranged from 116 to 1644 (median, 539; mean, 546) for all specimens, and the scores of *ERCC1* ranged from 55 to 1469 (median 382, mean 412).

The average score of triplicate tissues from each patient was used for analysis of the association between staining and clinical parameters. The AQUA scores for *RRM1* did not correlate significantly with those of *ERCC1* ($r=0.172$, $P=0.1610$) (Figure 2). The median values of *RRM1* and *ERCC1* expression levels were used to divide the patients into high and low expression groups. There were no significant differences between patients with high and low tumoral *RRM1* expression or high and low tumoral *ERCC1* expression with respect to age, sex, histopathological type (well/mod/poor), tumor size, tumor location (head/body/tail), pathological depth of tumor (pT1/T2/T3), the total number of resected lymph nodes, pathological lymph node metastasis (negative/positive) and the number of metastatic

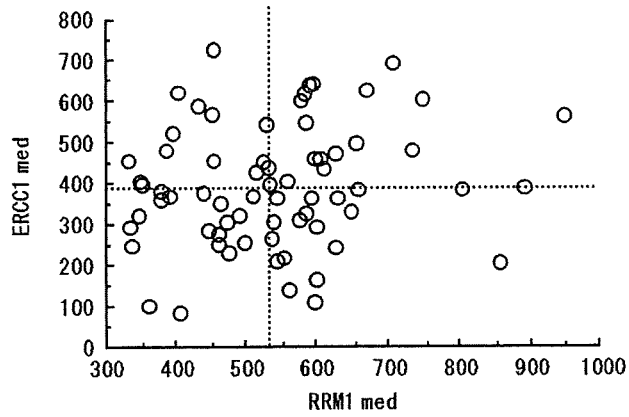

Figure 2 Relationship between automated quantitative analysis (AQUA) scores of *RRM1* and *excision repair cross-complementation group 1* (*ERCC1*) expression. *RRM1* expression did not correlate with that of *ERCC1* ($r=0.172$, $P=0.161$).

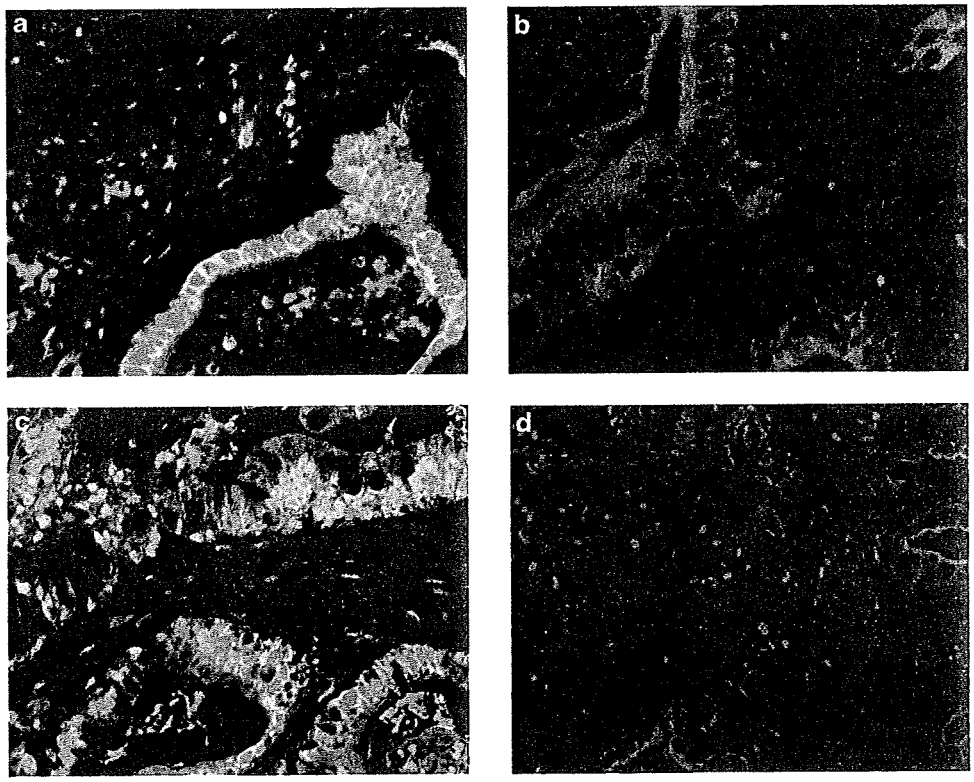

Figure 1 Staining for *RRM1* and *excision repair cross-complementation group 1* (*ERCC1*) proteins. (**a**) *RRM1*-positive sample. Note the granular nuclear pattern. Nucleus, blue; cytoplasm, red; *RRM1*, green; and merged, light blue to light green. (**b**) *RRM1*-negative sample. Nucleus, blue; and cytoplasm, red. (**c**) *ERCC1*-positive sample. Note the fine granular pattern in the nucleus. Nucleus, blue; cytoplasm, red; *ERCC1*, green; and merged, light blue to light green. (**d**) *ERCC1*-negative sample. Nucleus, blue; and cytoplasm, red.

Table 1 Relationship between protein expression levels and clinicopathological factors

	RRM1 expression level			*ERCC1 expression level*		
	High	*Low*	P-*value*	*High*	*Low*	P-*value*
Age (years) (mean ± s.d.)	66.8 ± 7.6	64.4 ± 7.9	0.220	64.6 ± 7.7	66.6 ± 7.8	0.283
Sex (male/female)	15/19	18/16	0.628	15/19	18/16	0.628
Histopathology (well/mod/poor)	17/14/3	9/18/7	0.102	12/19/3	14/13/7	0.237
Tumor size (cm) (mean ± s.d.)	27.4 ± 9.3	26.7 ± 8.2	0.752	25.2 ± 8.2	28.9 ± 8.9	0.077
Tumor location (head/body/tail)	27/6/1	27/4/3	0.497	27/4/3	27/6/1	0.497
pT (T1/T2/T3)	1/1/32	1/0/33	0.602	1/1/32	1/0/33	0.602
Total number of resected lymph node	34.4 ± 12.9	30.3 ± 13.6	0.243	30.8 ± 10.6	34.3 ± 15.7	0.330
PN (positive/negative)	12/22	17/17	0.327	18/16	11/23	0.141
Total number of metastatic lymph node	1.6 ± 1.9	1.0 ± 1.7	0.202	1.1 ± 1.7	1.5 ± 1.9	0.315
Gem therapy (+/−)	14/20	14/20	0.999	13/21	15/19	0.806

Abbreviation: *ERCC1, excision repair cross-complementation group 1.*

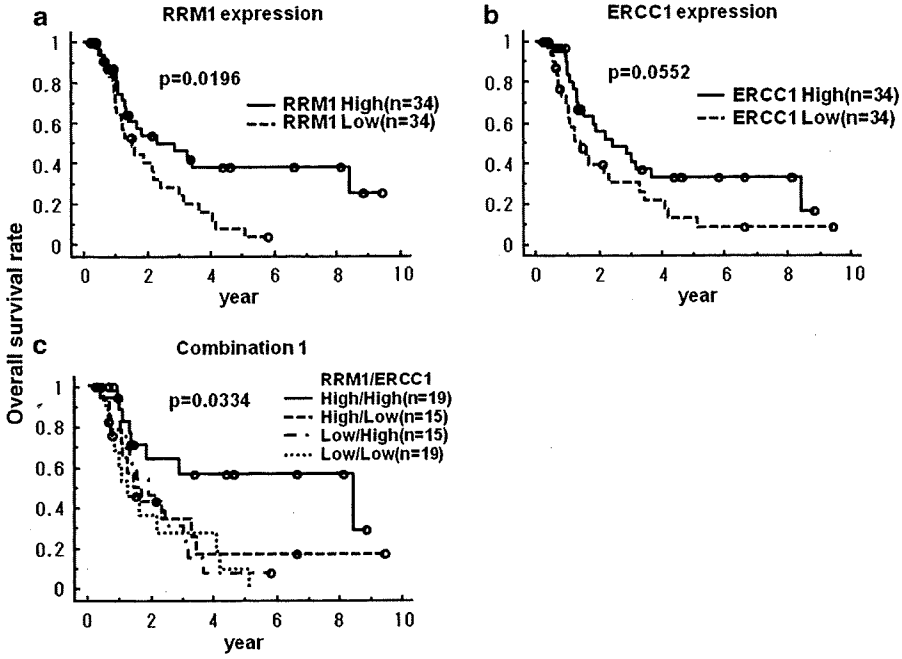

Figure 3 Relationship between *RRM1* and *excision repair cross-complementation group 1* (*ERCC1*) expression levels and overall survival rate. (a) Relationship between *RRM1* and overall survival is significant (3-year survival; 46.3 versus 28.6%, $P=0.0196$). (b) Relationship between *ERCC1* and overall survival is marginal ($P=0.0552$). (c) Relationship between the combination of *RRM1* and *ERCC1* expression levels in the same tumor and overall survival rate. Only high expression levels of *RRM1* and *ERCC1* in the same tumor related with the improvement of overall survival rate ($P=0.0334$).

lymph nodes, and whether or not gemcitabine was used as chemotherapy (Table 1).

Relationship between RRM1/ERCC1 expression and prognosis

The median overall survival of all patients was 16.3 months (4.3–113) and the median disease-free survival was 10.3 months (2–106). The Kaplan–Meier overall survival estimates were significantly better for patients with high *RRM1* expression compared with those having low *RRM1* expression levels (3-year survival; 46.3 versus 28.6%, $P=0.0196$) (Figure 3a). Likewise, patients with high *ERCC1* expression had a better overall survival than those with low levels of expression; although this difference was only marginally significant ($P=0.0552$) (Figure 3b). When we divided the 68 patients into four groups; that is, high tumoral expression of both proteins (High/High, $n=19$), high expression of only *RRM1* (High/Low, $n=15$), high expression of only *ERCC1* (Low/High, $n=15$) and low expression of both proteins (Low/Low, $n=19$); only patients of the High/High group had a significantly better prognosis than the others (3-year survival; 56.7 versus 30.5%, $P=0.0066$) (Figure 3c, Supplementary Figure 1).

With regard to disease-free survival, high *ERCC1* expression levels were significantly associated with better outcome (3-year survival; 30.2% for high versus

23.1% for low, $P=0.0454$). There was no significant difference in disease-free survival between the high and low *RRM1* expression groups (Supplementary Figures 2A and B). With respect to the combination of *RRM1* and *ERCC1*, only the High/High group showed a significantly better disease-free survival compared with the other groups (3-year survival, 43.2 versus 19.2%, $P=0.0127$) (Supplementary Figures 2C and D).

Univariate and multivariate analysis of factors associated with prognosis

We investigated the prognostic significance of various clinicopathological factors in pancreatic cancer patients who underwent radical resection. Univariate analysis showed that only the pathological type and absence or presence of lymph node metastases, were prognostically significant for disease-free survival ($P=0.034$, 0.025, respectively), and both parameters had marginal significance for overall survival ($P=0.078$, 0.084, respectively) (Table 2). Multivariate analysis identified the *RRM1* expression level as the only independent determinant of overall survival (hazard ratio (HR) 1.89, $P=0.046$), and none of the parameters tested was selected by the analysis as a significant prognostic factor in disease-free survival.

RRM1 expression and response to gemcitabine

Of all the 68 patients, 28 received therapy with single-agent gemcitabine. In 23 patients, this treatment was initiated at the time of tumor recurrence. To elucidate the relationship between *RRM1* expression level and gemcitabine therapy, we used survival after recurrence, which represented the period from starting gemcitabine therapy or other therapies in 50 patients with relapse, until death. First, we examined the survival benefit of gemcitabine. The 23 patients who were treated with gemcitabine had a significantly better survival than those who did not ($P=0.0074$) (Supplementary Figure 3). After dividing patients that were treated with gemcitabine into high and low *RRM1* expression groups, only patients with low *RRM1* expression benefited from gemcitabine therapy ($P=0.0010$) (Figure 4b). The survival of patients with high *RRM1* expression treated with gemcitabine was not significantly better than of those not treated with gemcitabine ($P=0.3309$) (Figure 4a). The interaction term between *RRM1* expression and gemcitabine treatment was significant for survival after recurrence ($P=0.0109$).

Discussion

Ribonucleotide reductase, composed of the regulatory subunit *RRM1* and the catalytic subunit *RRM2*, is a key enzyme involved in DNA synthesis, catalyzing the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides (Wright *et al.*, 1990; Hurta and Wright, 1992). *ERCC1*, a structure-specific DNA repair endonuclease responsible for the 5′ incision, has a key role in the removal of adducts from genomic DNA

Table 2 Prognostic factors for postoperative survival by Cox's proportional hazard model

	Univariate analysis				*Multivariate analysis*			
	DFS		*OS*		*DFS*		OS	
	HR	P-*value*	*HR*	P-*value*	*HR*	P-*value*	*HR*	P-*value*
Histology (poor, mod/well)	1.91	0.034	1.75	0.078	1.77	0.066	1.56	0.172
PN (positive/negative)	2.00	0.025	1.76	0.084	1.73	0.107	1.50	0.256
RRM1 expression (low/high)	1.55	0.129	2.04	0.022	1.39	0.265	1.89	0.046
ERCC1 expression (low/high)	1.75	0.048	1.78	0.059	1.42	0.265	1.54	0.194

Abbreviations: DFS, disease-free survival; *ERCC1*, excision repair cross-complementation group 1; HR, hazard ratio and OS, overall survival.

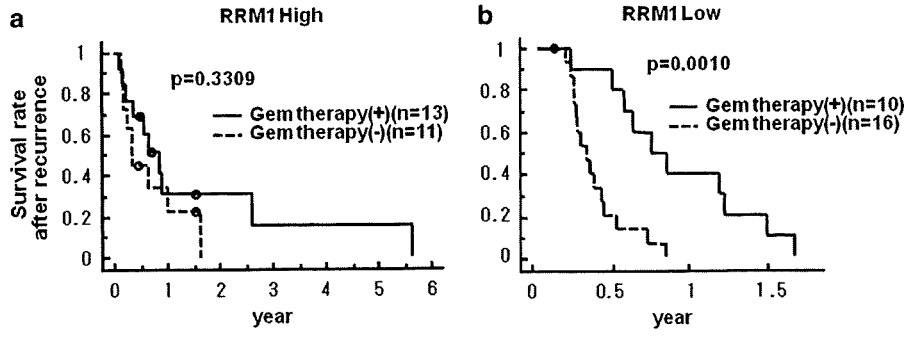

Figure 4 Relationship between survival after recurrence and patients treated with or without gemcitabine (**a**) in high *RRM1* expression group, and (**b**) in low expression group. Only patients with low *RRM1* expression benefited from gemcitabine therapy ($P=0.0010$).

through the nucleotide excision repair pathway (Reardon *et al.*, 1999; Niedernhofer *et al.*, 2004; Ceppi *et al.*, 2006). *RRM1* is reported to influence cell survival, probably through interaction with the *phosphatase and tensin homolog* (*PTEN*), which is an inhibitor of cell proliferation, and suppresses cell migration and invasion by reducing the phosphorylation of focal adhesion kinase (Gautam *et al.*, 2003; Bepler *et al.*, 2004). In lung cancer, the expression levels of *RRM1* and *ERCC1* are significantly correlated (Bepler *et al.*, 2006; Ceppi *et al.*, 2006).

Gemcitabine is the first line cytotoxic agent for treatment of patients with advanced pancreatic cancer, and it is the only agent with proven benefit in a large adjuvant clinical trial (Oettle *et al.*, 2007). However, it is estimated that only 25% of patients benefit from gemcitabine (Burris *et al.*, 1997). *RRM1* expression appears to be the key determinant of gemcitabine resistance (Dumontet *et al.*, 1999; Goan *et al.*, 1999; Jung *et al.*, 2001). This is partially due to expansion of the dNTP pool, which competitively inhibits the incorporation of gemcitabine triphosphate into DNA (Plunkett *et al.*, 1996). Another mechanism is the direct interaction between *RRM1* and gemcitabine with *RRM1* acting as a 'molecular sink' for gemcitabine (Davidson *et al.*, 2004; Bergman *et al.*, 2005). *ERCC1* is reported to be associated with the repair of cisplatin-induced DNA adducts in ovarian cancer (Li *et al.*, 2000), gastric cancer (Metzger *et al.*, 1998), colorectal cancer (Shirota *et al.*, 2001), lung cancer (Olaussen *et al.*, 2006) and esophageal cancer (Joshi *et al.*, 2005; Kim *et al.*, 2008).

Quantitative analysis of gene expression in pancreatic cancer is challenging because it contains more stromal tissue than other cancers (Sato *et al.*, 2004; Bachem *et al.*, 2005; Infante *et al.*, 2007), which makes laser microdissection a necessity to obtain gene expression of tumor tissue (Giovannetti *et al.*, 2006). Quantitative analysis of the *RRM1* protein had been difficult because of technical limitations. However, an automated, quantitative *in situ* assessment of protein expression was developed recently (Camp *et al.*, 2002), and applied for objective and practical evaluation of *RRM1* and *ERCC1* protein expression levels in tumor specimens (Zheng *et al.*, 2007). In this study, we used the above mentioned technology for gene expression analysis in pancreatic cancer specimens.

We found that the expression levels of *RRM1* and *ERCC1* affected the clinical outcome similar to that described in non-small-cell lung cancer (Zheng *et al.*, 2007). Patients with high levels of expression of both proteins had the best prognosis, including both disease-free survival and overall survival. However, once treatment with gemcitabine was initiated at the time of recurrence, it was only the group of patients with low levels of *RRM1* that benefited significantly from this intervention. In other words, patients with high tumoral *RRM1* levels may as well be treated with other agents, such as S-1 or oxaliplatin plus 5-fluorouracil plus leukovorin (CONKO-003), instead of gemcitabine (Ueno *et al.*, 2005; Okusaka *et al.*, 2008; Saif, 2008). In contrast, patients with low tumoral *RRM1* levels showed improved survival following treatment with gemcitabine (Moore *et al.*, 2007; Boeck and Heinemann, 2008). Many clinical trials of anticancer drugs, including molecular targeting agents, did not result in the improvement of outcome when conducted in unselected groups of patients (Heinemann *et al.*, 2006; Herrmann *et al.*, 2007; Cascinu *et al.*, 2008). However, if patients can be divided into groups with high or low likelihood of benefit from gemcitabine, a more rational design of future trials becomes available (Simon *et al.*, 2007). We believe that future treatment strategies for pancreatic cancer should be more precise and tailored to individual patients, and *RRM1* may be one of the candidate molecules for the stratification. We found that *RRM1* and *ERCC1* were not significantly coexpressed in pancreatic cancer, which is different from several previous reports in non-small-cell lung cancer (Ceppi *et al.*, 2006; Zheng *et al.*, 2007). This discrepancy may be explained by differences in tissue of origin and mechanisms of carcinogenesis between pancreatic cancer and lung cancer.

It is important to carry out prospective tailored therapeutic trials in pancreatic cancer with the goal of improving the clinical outcome, and it is our opinion that *RRM1* and *ERCC1* could play an important role in the design of such trials.

Materials and methods

Patients

Between January 1992 and March 2008, 166 patients underwent surgery for pancreatic cancer at Osaka University Hospital. We excluded 84 patients for the following reasons: (1) tumors were not resectable in 26 patients because of liver metastases or peritoneal carcinomatosis, (2) surgery resulted in R1 (residual microscopic cancer) or R2 (residual macroscopic cancer) resections in 21 patients, (3) chemotherapy or chemoradiotherapy was provided preoperatively to 37 patients and (4) lack of neutral-buffered formalin-fixed and paraffin-embedded tumor blocks or/and clinical follow-up information for study purposes in 14 cases. As the natural history of variant pancreatic neoplasms differs from the usual pancreatic ductal adenocarcinoma, patients with intraductal papillary mucinous neoplasms, mucinous cystic adenocarcinomas and medullary adenocarcinomas were excluded from this study. Supplementary Table 1 summarizes the characteristics of the 68 patients who were enrolled in this study. They included 33 men and 35 women with a mean age of 60.7 ± 7.8 years ($\pm$ s.d.). All patients had R0 (no residual cancer) resections by pancreaticoduodenectomy in 54 patients, distal pancreatectomy in 12 patients and other resections in 2 patients. The histopathological grading showed poorly, moderately, and well-differentiated adenocarcinoma in 10, 32 and 26 patients, respectively. The UICC-TNM classification was 2, 1 and 65 patients with pT1, pT2 and pT3; 29, 33 and 6 patients with pN0, pN1 and pM1lym; and 1, 1, 27, 33 and 6 patients with stage IA, IB, IIA, IIB and IV, respectively. None of the patients had received neoadjuvant therapy preoperatively. All 68 patients were followed until disease recurrence and/or death. The median follow-up period was 16.3 months (range, 4.3–113), the 5-year survival rate was 23.4%, and the recurrence of disease was observed in 50 patients. Treatment with gemcitabine was carried out in 28 patients; 5 patients

received it as adjuvant chemotherapy and 23 patients received it after disease recurrence. Radiation therapy was not carried out during all the follow-up period.

Immunofluorescence and automated quantitative analysis
We carried out immunostaining after constructing a tissue microarray. Immunofluorescence combined with AQUA was used to assess *in situ* expression of the target molecules as described previously (Zheng *et al.*, 2007). Antigens were retrieved by incubating the tissue in a microwave oven. Optimal concentrations of antisera and antibodies were used to detect *RRM1*, *ERCC1* and cytokeratin. The antiserum to *RRM1* was generated from rabbits and affinity-purified (R1AS-6) as described previously (Zheng *et al.*, 2007). Commercially available antibodies were used for the analysis of *ERCC1* (Ab-2 clone 8F1, MS-671-R7, Laboratory Vision Corporation, Fremont, CA, USA) and cytokeratin (antihuman pancytokeratin AE1/AE3, M3515 and Z0622, Dako Cytomation, Glostrup, Denmark) (Zheng *et al.*, 2007). They were visualized with the use of fluorochrome-labeled secondary antibodies. The final slides were scanned with SpotGrabber (HistoRx, New Haven, CT, USA), and images were analysed with AQUA (version 1.6, PM-2000, HistoRx). The AQUA scores ranged from 0 (no expression) to 3000 (maximal observed expression).

Statistical analysis and ethical issues
Data are expressed as mean ± s.d. Differences in continuous values were evaluated by the Student's *t*-test (Table 1). The Fisher's exact probability test was used to compare discrete variables (Table 1). We evaluated correlations between AQUA scores of *RRM1* and *ERCC1* by Pearson's correlation coefficient (Figure 2). Disease-free and overall survival rates were estimated by the Kaplan–Meier method and compared using the log-rank test (Table 1, Figures 3 and 4). Cox's proportional hazard regression model with stepwise comparisons was used to analyse independent prognostic factors (Table 2). The predictive value of *RRM1* was studied by testing the interaction between *RRM1* expression and gemcitabine treatment in the same Cox model. A P-value <0.05 was used to indicate statistical significance.

This study was analysed by the statistical expert in our laboratory and the study protocol was approved by the Human Ethics Review Committee of Osaka University, and a signed consent form was obtained from each subject.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Molecular markers associated with lymph node metastasis in pancreatic ductal adenocarcinoma by genome-wide expression profiling

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Lymph node metastasis (LNM) is the most important prognostic factor in patients undergoing surgical resection of pancreatic ductal adenocarcinoma (PDAC). In this study, we aimed to identify molecular markers associated with LNM in PDAC using genome-wide expression profiling. In this study, laser microdissection and genome-wide transcriptional profiling were used to identify genes that were differentially expressed between PDAC cells with and without LNM obtained from 20 patients with PDAC. Immunohistochemical staining was used to confirm the clinical significance of these markers in an additional validation set of 43 patients. In the results, microarray profiling identified 46 genes that were differently expressed between PDAC with and without LNM with certain significance. Four of these biomarkers were validated by immunohistochemical staining for association with LNM in PDAC in an additional validation set of patients. In 63 patients with PDAC, significant LNM predictors in PDAC elucidated from multivariate analysis were low expression of activating enhancer binding protein 2 (*AP2α*) ($P = 0.012$) and high expression of mucin 17 (*MUC17*) ($P = 0.0192$). Furthermore, multivariate analysis revealed that *AP2α*-low expression and *MUC17*-high expression are independent prognostic factors for poor overall survival ($P = 0.0012$, 0.0001, respectively). In conclusion, *AP2α* and *MUC17* were independent markers associated with LNM of PDAC. These two markers were also associated with survival in patients with resected PDAC. We demonstrate that *AP2α* and *MUC17* may serve as potential prognostic molecular markers for LNM in patients with PDAC. (*Cancer Sci* 2010; 101: 259–266)

Pancreatic ductal adenocarcinoma (PDAC) has the worst survival rate of all cancers, with a 5-year survival rate of <5%. To date, the only curative treatment for PDAC is surgery, but <20% of patients who undergo surgery are alive after 5 years.[1,2] Numerous studies have demonstrated that the presence of LNM is the most important prognostic factor for patients undergoing surgery for PDAC.[1–5] Understanding the molecular events involved in the development of LNM in PDAC could aid researchers in the identification of biologic determinants, and will aid in the identification of diagnostic biomarkers and development of more effective therapies.

Gene expression profiles provide a lot of important information about the molecular characteristics of the cancers and can be used to distinguish related cancer subtypes. Recently, several studies have used gene expression profiling technologies to identify differentially expressed genes in PDAC compared with normal pancreas.[6–8] In the present study, we focused on and identified the genes associated with LNM, which is the most important prognostic factor in patients who undergo surgical resection for PDAC. Gene identification was accomplished by comparison of gene expression profiles between PDAC with and without LNM.

Most microarray studies of PDAC were performed in cell lines partly representing the whole character of PDAC or the whole resected tissues of pancreatic cancer, which contained a number of different cell types including normal ductal, acinar, islet, inflammatory, and nerve cells, because of the characteristics of PDAC.[6,9,10] Therefore, the expression profiles for the whole resected tissues represent characteristics of both tumor and adjacent non-neoplastic cells. In this study, we performed gene expression profiling using pure PDAC cells obtained selectively by microdissection to elucidate molecular profiles of PDAC more accurately.[11,12]

In this study, we identified the genes associated with LNM in PDAC using gene expression profiling, and validated their usefulness as diagnostic and prognostic biomarkers for PDAC by protein expression analysis using immunohistochemical staining.

Materials and Methods

Patients and tissue samples. The ethical committee of the chamber of physicians in the Center Institute of Japanese Foundation for Cancer Research Hospital and Wakayama Medical University Hospital approved this study. Informed consent was obtained from all patients before their inclusion in the study. Our study population consisted of 63 patients with resected PDAC who had undergone radical operations between January 2004 and May 2007, had available stored frozen tissue blocks, and had tumor-free resection margins on microscopic examination of the surgical specimen. None of the patients had received neoadjuvant chemotherapy or radiation therapy before surgery. The patients characteristics were: males/female = 25/38; age range, 49–87 years (mean, 70 years). The tumors were located in head of the pancreas in 45 patients and in body or tail in 18 patients, and 19 patients had tumors of more than 4.0 cm whereas 44 patients had tumors of <4.0 cm. Histologically, there were 25 patients with well differentiated adenocarcinoma, 27 with moderate, and 11 with poor differentiated adenocarcinoma. The TNM staging criteria of the International Union Against Cancer (UICC) (6th edition) were used for histologic classification:[13] T1 in two patients, T2 in 11 patients, and T3 in 50 patients. The patients included two with stage IA, seven with stage IB, 19 with stage IIA, 19 with stage IIB, and 16 with stage IV. Among them, 35 patients had histologically confirmed LNM, and 28 had no LNM. Median follow-up duration after surgery was 475 days (range, 18–1792 days).

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Table 1. Underexpressed and overexpressed genes in pancreatic ductal adenocarcinoma with lymph node metastasis identified by expression profile

Probe ID	Gene name	Fold
A. Underexpressed genes in pancreatic ductal adenocarcinoma with lymph node metastasis		
204124_at	Solute carrier family 34 (sodium phosphate), member 2	−5.27
1559072_a_at	Leucine rich repeat containing 62	−3.84
203404_at	Armadillo repeat containing, X-linkedn2	−3.36
208063_s_at	Calpain 9	−3.32
229041_s_at	Homo specimens, clone IMAGE:5205388, mRNA	−3.11
212776_s_at	Obscuring-like 1	−2.90
240633_at	Docking protein 7 (DOK7)	−2.77
205129_at	Nucleophosmin/nucleoplasmin, 3	−2.74
226344_at	Zinc finger, matrin type 1	−2.74
204284_at	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	−2.65
205541_s_at	G1 to S phase trandition 2	−2.63
221869_at	Zinc finger protein 512B	−2.56
55872_at	Zinc finger protein 512B	−2.53
212775_at	Obscuring-like 1	−2.49
238751_at	CDNA clone IMAGE:4791597	−2.40
204653_at	Transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha) (AP2α)	−2.39
228384_s_at	Chromosome 10 open reading frame 33	−2.38
243409_at	Forkhead box L1 (FOXL1)	−2.09
225484_at	Testis specific, 14	−1.99
225485_at	Testis specific, 14	−1.93
B. Overexpressed genes in pancreatic ductal adenocarcinoma with lymph node metastasis		
220639_at	Transmembrane	25.16
1553296_at	G protein-coupled receptor 128	6.86
228974_at	CDNA FLJ42233 fis, clone THYMU3000420	5.56
209847_at	Cadherin 17, LI cadherin (liver–intestine) (LI cadherin)	5.17
204607_at	3-hydroxy-3-methylgultalyl-Coenzyme A synthase 2 (mitochondrial)	4.77
224355_s_at	Membrane-spanning 4-domains, subfamily A, member 8B	4.72
207259_at	Chromosome 17 open reading frame 73	4.29
205488_at	Granzyme A	4.24
232321_at	Mucin 17, cell surface associated (MUC17)	4.19
240110_at	3-hydroxy-3- methylgultalyl-Coenzyme A synthase 2 (mitochondrial)	4.09
223303_at	UNC-112 related protein 2	4.05
235301_at	KIAA 1324-like	3.78
206084_at	Protein thyrosine phosphatase, receptor type, R	3.54
244771_at	Kelch domain containing 6	3.44
242447_at	Hypothetical gene supported by AK091454	3.44
243774_at	Mucin 20, cell surface associated	3.38
220421_at	Butyrophilin-like 8 similar to Butylphilin-like protein 8 precursor	3.24
208029_s_at	Complement component 4 binding protein beta (C4BPB)	3.04
239294_at	Transcribed locus	3.01
206698_at	X-linked Kx blood group (McLeod syndrome) (XK)	2.96
210675_s_at	Protein thyrosine phosphatase, receptor type, R	2.89
223960_s_at	Chromosome 16 open reading frame 5	2.86
218510_x_at	Family with sequence similarity 134, member B	2.81
208170_s_at	Tripartite motif-containing 31	2.71
231941_s_at	Mucin 20, cell surface associated	2.59
224480_s_at	Lung cancer metastasis-associated protein	2.47
209668_x_at	Carboxylesterase 2 (intensine, liver)	2.40
238032_at	Transcribed locus	2.29
235256_s_at	Galactose mutarotase (aldose 1-epimerase)	2.28
1555897_at	Amine oxidase (flavin containing) domain 2 (LSD1)	2.09
238851_at	Ankyrin repeat domain 13A	2.07

Immediately after surgical resection, tissue samples including tumor and adjacent normal cells were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA) by freezing tissue blocks in liquid nitrogen; the blocks were then stored at −143°C until further processing.

Laser microdissection and RNA extraction. The specimens of PDAC were cut into 9-μm sections at −20°C with the use of the Leica cryostat (model 3050S; Leica, Tokyo, Japan). We prepared more than 30 specimens of PDAC, ranging from 30 to 120 specimens, for gene expression profiling. Specimens containing only cancer cells of the pancreas were then obtained from the primary tumors by laser microdissection. Total RNA was extracted from the harvest cells with 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).

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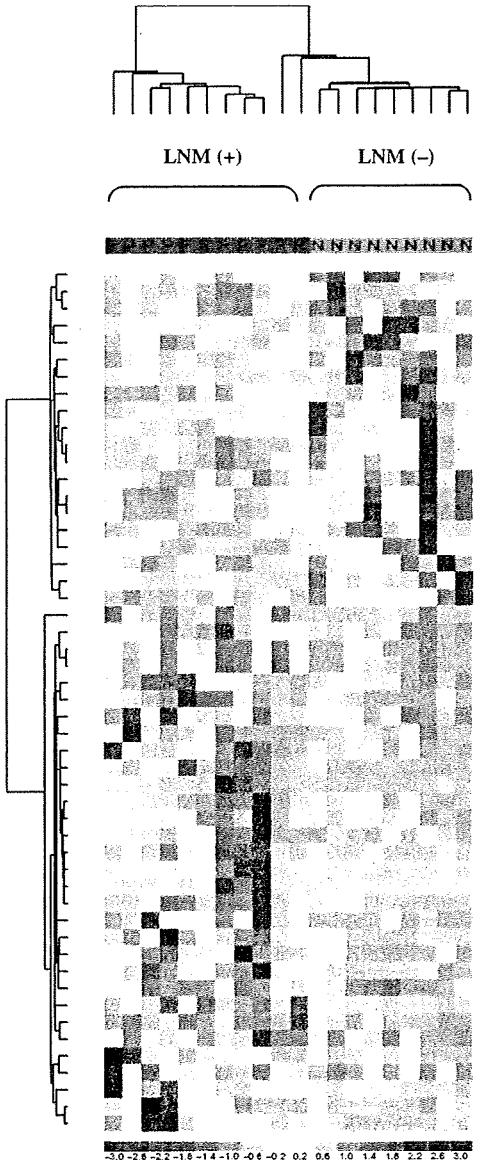

Fig. 1. Supervised hierarchical clustering of pancreatic ductal adenocarcinoma with and without lymph node metastasis using the selected 46 genes expressed differentially between two groups. Red, overexpressed genes; blue, underexpressed genes. LNM (+), positive lymph node metastasis; LNM (–), negative lymph node metastasis.

RNA integrity was determined by capillary electrophoresis with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), and the extracted RNA was accepted for experiments if the RNA integrity number was over 7.0. Eventually, we selected 20 PDAC (59%) among consecutive 34 surgical resections, because the RNA integrity number of PDAC of 14 other patients was <7.0. The pathological characteristics of the 20 patients with PDAC were: 10 patients with well differentiated adenocarcinoma, nine with moderate, and one with poor differentiated adenocarcinoma. Two patients had tumors of more than 4.0 cm and 18 patients had tumors of <4.0 cm. Among them, 11 patients had histologically confirmed LNM, and nine had no LNM. According to UICC TNM staging, the 20 patients included three with stage IB, six with stage IIA, four with stage IIB, and seven with stage IV.

Table 2. Accuracy for lymph node metastasis in pancreatic ductal adenocarcinoma by immunohistochemical staining intensities of 7 genes using all available cut-off points in the training set (n = 20)

Marker	Accuracy in scoring criteria		
	Score 0 *vs* 1, 2, 3	Score 0, 1 *vs* 2, 3	Score 0, 1, 2 *vs* 3
DOK7	75%	65%	55%
AP2α	60%	65%	85%
LI-cadherin	85%	85%	70%
Granzyme A	55%	65%	75%
MUC17	95%	85%	70%
C4BPB	80%	70%	70%
XK	60%	75%	70%

AP2α, activating enhancer binding protein 2; *C4BPB*, complement component 4 binding protein, beta; *DOK7*, docking protein 7; *LI cadherin*, liver–intestine cadherin; *MUC17*, mucin 17; *XK*, X-linked Kx blood group.

Table 3. Immunohistochemical analysis between pancreatic ductal adenocarcinoma patients with and without lymph node metastasis

Marker	Lymph node metastasis (±) *vs* (–)		
	Training set (n = 20)	Validation set (n = 43)	
	P-values	*P*-values	Accuracy (%)
DOK7	0.0241	0.1073	63
AP2α	0.0012	<0.0001	81
LI cadherin	0.0017	0.0046	70
Granzyme A	0.0277	0.1386	61
MUC17	<0.0001	0.0005	74
C4BPB	0.0030	0.1434	53
XK	0.0171	0.0223	91

AP2α, activating enhancer binding protein 2; *C4BPB*, complement component 4 binding protein, beta; *DOK7*, docking protein 7; *LI cadherin*, liver–intestine cadherin; *MUC17*, mucin 17; *XK*, X-linked Kx blood group.

Gene expression profile. Gene expression of 20 RNA samples (11 positive and nine negative LNM patients) of pancreatic cancer cells was analyzed with Human Genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA, USA). The manufacturer's instructions for protocols and use of reagents for hybridization, washing, and staining were followed. Briefly, 100 ng of total RNA of each sample was reverse transcribed with a poly(T) primer containing a T7 promoter, and the cDNA was generated as a double strand. An *in vitro* transcription was performed to produce unlabeled cRNA. Next, first-strand cDNA was produced from a random primed reaction. cDNA was converted to a double strand in a reaction with a poly(T) primer containing a T7 promoter. Finally, an *in vitro* transcription was performed with biotinylated ribonucleotides to produce biotin-labeled cRNA. Labeled cRNA was then hybridized with the GeneChips for 16 h at 45°C. The chips were washed and stained with streptavidin–phycoerythrin with the use of an Affymetrix FS-450 fluidics station. Data were collected with an Affymetrix GeneChip Scanner 3000. The CEL files were obtained with Affymetrix Suite 5.0 software; then the array data was imported into DNA-Chip Analyzer (dChip, http://www.dchip.org) for high-level analysis.

Immunohistochemistry. The choice of antibody was empirical and was based on availability and suitability for frozen tissues. Each antibody was titrated three to five different dilutions, according to the manufacturer's recommendation. If the signal-to-background ratio was not acceptable for the dilution tested, the incubation time was readjusted. First, 9-μm cryosections were fixed in 4% paraformaldehyde solution for 10 minutes, and

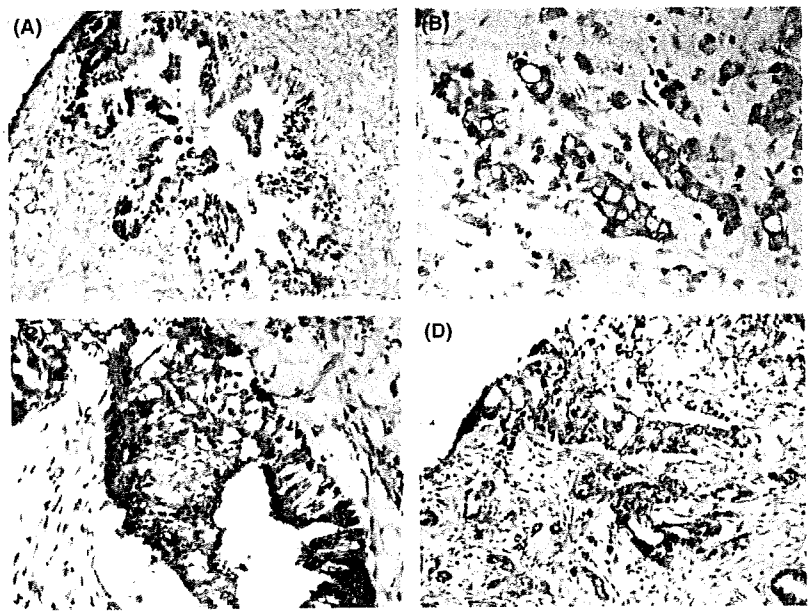

Fig. 2. Immunohistochemical staining of four genes associated with lymph node metastasis in pancreatic ductal adenocarcinoma patients (A–D). Activating enhancer binding protein 2 (*AP2α*) expressed in nucleus of pancreatic cancer cells (A). Liver–intestine cadherin (*LI-cadherin*) (B), mucin 17 (*MUC17*) (C), and X-linked Kx blood group (*XK*) (D) expressed in membrane of pancreatic cancer cells.

then washed in 1% PBS. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol, and nonspecific binding sites were blocked with 10% normal rabbit or goat serum. Primary antibodies diluted in PBS as follows: *DOK7* (1:100, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA), *AP2α* (1:50, mouse monoclonal; Santa Cruz Biotechnology), *FOXL1* (1:1000, rabbit polyclonal; CeMines, Evergreen, CO, USA), *LI-cadherin* (1:150, goat polyclonal; Santa Cruz Biotechnology), *Granzyme A* (1:50, mouse monoclonal; Abcam, Cambridge, UK): *MUC17* (1:150, goat polyclonal; Santa Cruz Biotechnology), *C4BPB* (1:25, goat polyclonal; Santa Cruz Biotechnology), *XK* (1:100, goat polyclonal; Santa Cruz Biotechnology), and *LSD1* (1:1000, mouse monoclonal; Abcam). Diluted primary antibodies were added and samples were incubated overnight at 4°C. Antibody binding was then immunodetected with the avidin–biotin–peroxidase complex, as described by the supplier (Nichirei, Tokyo, Japan). Finally, the reaction product was demonstrated by a DAB substrate, and then counterstained with hematoxylin, dehydrated with ethanol, and fixed with xylene. Immunostains were scored semiquantitatively by two independent pathologists blinded to clinical and pathologic data.

Statistical analysis. The association between lymph node status and each protein's immunoreactivites and clinicopathological characteristics was tested by means of a χ^2-test or the Mann–Whitney *U*-test. Logistic regression was performed for multivariate analysis of parameters potentially associated with LNM. Overall survival was defined as the time interval between the date of resection and the date of death from any cause, or censoring based on the date of last contact. Survival curves were calculated by the Kaplan–Meier method and then compared by the log-rank test. Cox's proportional hazards regression model with stepwise analysis was used to analyze the independent prognostic factors. Statistical procedures were performed with SPSS version 17.0 (SPSS, Chicago, IL, USA). A *P*-value <0.05 was considered statistically significant.

Results

Identification of transcriptional biomarkers for PDAC with LNM. Using microdissection, we obtained cancer tissues from surgical specimens from 11 PDAC patients with LNM and from nine without LNM. To identify transcriptional gene expression changes associated with lymph node status, we performed microarray profiling of PDAC using Human Genome U133 Plus 2.0 GeneChips. Genes with altered expression levels were determined by the comparison of PDACs with and without LNM on the basis of the following criteria: (i) a 1.5-fold or greater change in the expression levels between the means of the two groups; (ii) a >100 of absolute difference between the means of the expression levels of the two groups; and (iii) a *P*-value <0.05. From the results, the 46 genes expressed differentially between two groups were selected, including 17 genes that were down-regulated, whereas 29 were up-regulated in the PDAC with LNM group (Table 1).

Using the selected 46 genes, we performed hierarchical clustering on the samples from 20 patients by Pearson's correlation distance metric and average linkage. In the results, the dendrogram contained two main branches, one of which contained only PDAC samples with LNM; the other branch contained all PDAC samples without LNM and two with LNM, suggesting the potential significance of these genes as transcriptional biomarkers for PDAC with LNM (Fig. 1).

Evaluation of biomarker candidate gene product by immunohistochemical analysis. First, to validate the data obtained by transcriptional gene expression profile at the protein level, we investigated the expression of nine gene products (*DOK7*, *AP2α*, *FOXL1*, *LI-cadherin*, *Granzyme A*, *MUC17*, *C4BPB*, *XK*, and *LSD1*) for which antibodies were found to be available by preliminary immunohistochemical screenings. Immunoreactivities of *DOK7*, *LI-cadherin*, *MUC17*, and *XK* were located in the plasma membrane; those of *AP2α*, *FOXL1*, and *LSD1* were located in the nucleus; and those of *Granzyme A* and *C4BPB* were located in the cytoplasm. We performed immunohistochemical analysis of these nine genes identified by expression analysis in samples from 20 PDAC patients, which were used in expression profiling (training set). *FOXL1* and *LSD1* proteins were expressed in more than 95% of tumor nuclei in all 20 samples, showing no significant difference between the two groups; therefore, these proteins were excluded as biomarker candidates. The results of the immunohistochemical staining of the remaining seven gene products were evaluated. The percentage of positively stained tumor nuclei (*AP2α*) was scored as follows: score 0, <10%; score 1, ≧10% to 20%; score 2, ≧20% to 50%; score 3, ≧50%. The intensity and percentage of positively stained tumor membrane or cytoplasm (*DOK7*,

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Table 4. Univariate and multivariate analysis of factors associated with lymph node metastasis in pancreatic ductal adenocarcinoma (n = 63)

Factors ratio	Lymph node metastasis (–)		Lymph node metastasis (±)		Univariate analysis	Multivariate analysis
	No	%	No.	%	*P*-values	*P*-values, odds ratio (95% CI)
Clinicopathological features						
Age						
≧ 70	15	54	19	54	0.9549	0.2642
<70	13	46	16	46		
Sex						
Male	16	57	9	26	0.0113	0.905
Female	12	43	26	74		
Location of tumor						
Head	18	64	27	77	0.2617	0.2038
Body and/or tail	10	36	8	23		
Tumor size (cm)						
≧ 4	8	29	11	31	0.8060	0.3607
<4	20	71	24	69		
T stage†						
T1/2	9	32	4	11	0.0435	0.4889
T3/4	19	68	31	89		
Differentiation						
Well/moderate	24	86	30	86	>0.9999	0.8649
Poor	4	14	5	14		
Biomarkers						
AP2α						
Low expression 20.9 (1.95–223)	19	68	2	6	<0.0001	0.0120
High expression	9	32	33	94		
LI-cadherin						
Low expression	4	14	23	66	<0.0001	0.0650
High expression	24	86	12	34		
MUC17						
Low expression 12.2 (1.50–98.5)	3	11	26	74	<0.0001	0.0192
High expression	25	89	9	26		
XK						
Low expression	7	25	23	66	0.0013	0.9867
High expression	21	75	12	34		

†UICC on TNM staging criteria, 6th edition.[12] *AP2α*, activating enhancer binding protein 2; *C4BPB*, complement component 4 binding protein, beta; CI, confidence interval; *DOK7*, docking protein 7; *LI cadherin*, liver–intestine cadherin; moderate, moderately differentiated adenocarcinoma; *MUC17*, mucin 17; poor, poorly differentiated adenocarcinoma; well, well-differentiated adenocarcinoma; *XK*, X-linked Kx blood group.

LI-cadherin, *Granzyme A*, *MUC17*, *C4BPB*, and *XK*) were as follows: score 0, stain, <10%; score 1, weak stain, ≧ 10% to 50%; score 2, weak stain, ≧ 50%; or strong stain, ≧ 10% to 50%; score 3, strong stain, ≧ 50%. We calculated the accuracy for lymph node status by immunohistochemical staining intensities of each gene product using all available cut-off points (i.e. score 0 *vs* 1, 2, 3; score 0, 1 *vs* 2, 3; score 0, 1, 2 *vs* 3) in the training set. Then, as shown in Table 2, the binarization of score data for these seven markers was performed as "low expression" *versus* "high expression" at the binary score cut-off points at which the accuracy value was the highest in the training set.[14]

Next, immunohistochemical analysis was also performed in other samples from 43 patients including 24 patients with LNM and 19 patients without LNM in PDAC for further confirmation (validation set). We compared the immunohistochemical staining intensities of each gene product in PDAC between with and without LNM. For protein expression of *AP2α*, *LI-cadherin*, *MUC17*, and *XK*, immunohistochemical analysis resulted in significant differences between PDAC with and without LNM in both training and validation sets (Table 3). The expression of these four marker proteins was significantly related to lymph node status, which was consistent with the results of transcriptional expression profiling, and moreover, these four marker proteins were only expressed in PDAC but not in normal pancreas tissues (Fig. 2).

Factors related to LNM. The median number of lymph nodes examined was 21 (range, 3–63). There were no significant differences concerning to the number of lymph nodes examined between the patients with LNM and without LNM (median, 25 *vs* 21.5; $P = 0.0617$).

The univariate analysis for 63 patients with PDAC indicated that LNM was significantly higher for female patients ($P = 0.0113$) and patients with T3 or 4 disease ($P = 0.0435$), and for PDAC with low expression of *AP2α* ($P < 0.0001$), or with high expression of *LI-cadherin* ($P < 0.0001$), *MUC17* ($P < 0.0001$), and *XK* ($P = 0.0013$) (Table 4). On multivariate analysis, however, expression of *AP2α* and *MUC17* was shown to be the only significant independent factors associated with LNM of PDAC (Table 4).

Furthermore, for the patients with LNM, both the metastatic lymph node number and the lymph node ratio, determined by dividing the number of lymph node metastasis by the total

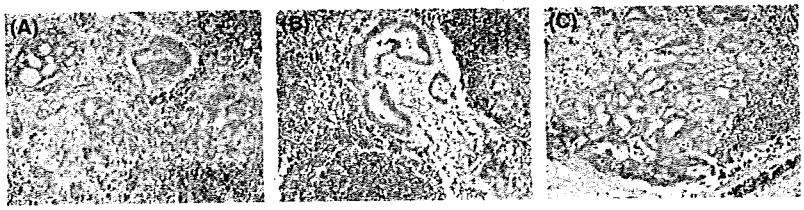

Fig. 3. Immunohistochemical staining in metastatic lymph node tissues from pancreatic ductal adenocarcinoma (A–C). High expression of liver–intestine cadherin (*LI-cadherin*) (A), mucin 17 (*MUC17*) (B), and X-linked Kx blood group (*XK*) (C) are shown in metastatic adenocarcinoma in lymph node.

number of examined lymph nodes, were significantly higher in patients with *MUC17*-high expression than in patients with *MUC17*-low expression (metastatic lymph node number, 4 *vs* 1, $P = 0.0027$; lymph node ratio, 0.16 *vs* 0.06, $P = 0.0062$). However, there were no different significances between those for patients with *AP2α*-low expression and with *AP2α*-high expression.

Expression of molecular markers in metastatic lymph node tissues from PDAC. Protein expression of *AP2α*, *LI-cadherin*, *MUC17*, and *XK* in 11 metastatic lymph node tissue samples of PDAC patients was examined by immunohistochemical staining. Among 11 metastatic lymph node tissues of PDAC, low expression of *AP2α* was shown in 11 (100%) metastatic lymph nodes, and high expression of *LI-cadherin*, *MUC17*, and *XK* was shown in eight (73%), 11 (100%), and 11 (100%), respectively (Fig. 3).

Prognostic factor for patients with PDAC. The overall survival period of patients without LNM ($n = 28$) was better than that of patients with LNM ($n = 35$) (median, 844 *vs* 470 days, $P = 0.0174$, log-rank test; Fig. 4A). The survival of patients with *AP2α*-low expression was significantly worse than for those with *AP2α*-high expression ($P = 0.0015$, log-rank test; Fig. 4B). In addition, the survival of patients with *MUC17*-high expression was significantly worse than for those with *MUC17*-low expression (median, 451 *vs* 567 days, $P = 0.0368$, log-rank test; Fig. 4C). In the combined evaluation of *AP2α* and *MUC17* expression, patients with *AP2α*-low and *MUC17*-high expression had a worse survival than those with *AP2α*-high and *MUC17*-low expression; a significant difference for survival was found between the two groups ($P = 0.0009$, log-rank test; Fig. 4D).

Multivariate analysis with factors proven to be significant in the univariate analysis revealed that poor differentiation, *AP2α*-low expression, *MUC17*-high expression, and *AP2α*-low and *MUC17*-high expression were independent prognostic factors for poor overall survival (Table 5).

Discussion

Recent clinical studies have revealed that the most important prognostic factor in PDAC is the presence of LNM in patients with PDAC who have undergone surgery.[1–5] In the post-genomic era, the search for novel prognostic and therapeutic targets for PDAC has been extensively performed;[6–8,15] however, there remain no effective molecular markers of clinical utility in PDAC. In this study, we focused on and identified specific genes that have characteristics of lymphatic metastasis in PDAC, and that may be used as diagnostic and prognostic markers.

Some large studies using genome-wide expression profiling revealed that metastases of human cancer arose from primary cancer tissues in which the vast majority of cancer cells had already obtained the ability to metastasize,[16–18] suggesting that comparison between primary pancreatic cancer cells with and without LNM by expression profiling could lead to identifying the genes associated with LNM in PDAC, because the differences of gene expression between PDAC with and without LNM depend on the differences of biological nature of the tumor, but not the stage of tumor progression. Therefore, we decided to identify the genes related to LNM using the primary tissues of PDAC. Some studies using gene expression profiling have assayed and described the data by using the whole tissues of pancreatic cancer.[6–8] One should consider the limitation of these previous studies in terms of the component heterogeneity in PDAC, because the stromal portion in PDAC usually exceeds the cancer cell proportion. Therefore, we obtained highly purified cancer cells by microdissection for a genome gene expression analysis. A few studies, which identified the genes associated with LNM in PDAC by gene expression analysis

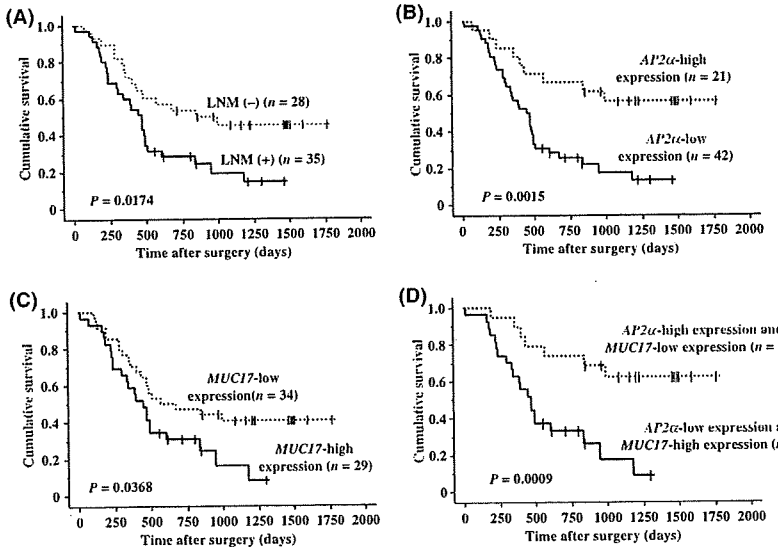

Fig. 4. (A) Overall survival (OS) without lymph node metastasis was better (median, 844 *vs* 470 days). (B) OS with *AP2α*-low expression was worse than that of high expression. (C) OS with *MUC17*-high expression was worse than that of low expression (median, 451 *vs* 567 days). (D) OS with *AP2α*-low and *MUC17*-high expression was worse than that of *AP2α*-high and *MUC17*-low expression. LNM (+), positive lymph node metastasis; LNM (–), negative lymph node metastasis.

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Table 5. Multivariate analysis using Cox's proportional hazards regression model to determine prognostic parameters in patients with pancreatic ductal adenocarcinoma (n = 63)

Factors	*P*-values	Relative risk	95% CI
Lymph node status	0.6489	0.342	0.342–1.950
Histologic differentiation	0.0037	1.435	1.435–6.415
AP2α-low expression	0.0012	5.412	1.944–15.06
MUC17-high expression	0.0001	42.07	6.355–278.5
AP2α-low/*MUC17*-high expression	<0.0001	46.57	6.953–312.0

AP2α, activating enhancer binding protein 2; CI, confidence interval; *MUC17*, mucin 17; poor, poorly differentiated adenocarcinoma.

using microdissection, have been reported.[12] However, the genes associated with LNM in PDAC identified in this study are not included in these studies, and the differences in the results probably may depend on the samples collected in each study. Furthermore, for effective utilization of the vast amount of information gathered through microarray studies, we performed protein expression analysis using immunohistochemical staining to validate the nine genes associated with LNM in PDAC that were identified by expression profiling and had available antibodies. In the results, we could identify four molecular markers (*AP2α*, *LI-cadherin*, *MUC17*, and *XK*) associated with LNM in PDAC. Indeed, *AP2α* had low expression and *LI-cadherin*, *MUC17*, and *XK* had high expression in PDAC of patients with LNM. In addition, low expression of *AP2α* and high expression of *MUC17* were confirmed as definitively independent factors associated with LNM in PDAC by multivariate analysis. Furthermore, low expression of *AP2α* and high expression of *MUC17* were shown to serve as prognostic factors for survival in patients with PDAC.

Activator protein 2 (*AP2*), which had low expression in PDAC of patients with LNM in this study, is a cell type-specific DNA-binding transcription factor family that has the ability to specifically regulate the expression of other genes in vertebrate organisms. The *AP2* family comprises five isoforms of 52 kDa protein: *AP2α*, *AP2β*, *AP2γ*, *AP2δ*, and *AP2ε*.[19] They share a common structure, possessing a proline/glutamine-rich transactivation domain in the N-terminal region and a helix-span-helix domain in the C-terminal region, which mediates dimerization and site-specific DNA binding.[19–21] Loss of *AP2α* expression has been associated with progression of melanoma, colorectal cancer, breast cancer, and pancreatic cancer, indicating that *AP2α* may have a tumor suppressive role.[20–23] We first found that the expression of *AP2α* was associated with not only LNM but also survival of PDAC patients.

In this study, we found that high expressions of three biomarkers (*MUC17*, *LI-cadherin*, and *XK*) were associated with LNM in PDAC, and these biomarkers were frequently expressed in metastatic lymph nodes in PDAC. Mucin 17 (*MUC17*), whose high expression was not only an independent factor associated with LNM in PDAC but also a prognostic factor in patients with PDAC, is a membrane-bound mucin identified recently and located in the mucin cluster at the chromosomal locus 7q22, along with *MUC3A/B*, *MUC11*, and *MUC12* mucins.[24] The full-length coding sequence of *MUC17* transcribes a 14.2 kb mRNA encompassing 13 exons.[24,25] Alternate splicing generates two variant codings, a membrane-anchored and a secreted form.[24,25] Moniaux *et al.*[25] reported that *MUC17* in pancreatic tumor cell lines and tumor tissues was overexpressed compared with the normal pancreas. Moreover, our data demonstrated that pancreatic cancer patients with LNM had higher expression of *MUC17*. Here, we show that *MUC17* is a new prognostic marker in PDAC patients through lymphatic metastasis, indicating that *MUC17* might be a molecular target for therapy of PDAC.

Previous studies showed that *LI-cadherin* was expressed only in the rat liver and intestine;[26] however, recent reports have revealed that various kinds of cancer in humans overexpressed *LI-cadherin*, including liver, stomach, colon, and pancreas cancers.[27,28] The structure of *LI-cadherin* is different from that of the classic type I cadherins such as *E-cadherin*, in which the cytoplasmic domain contains only 20 amino acids; therefore, *LI-cadherin* has no interaction with the catenin network or the actin cytoskelton.[26] However, the role of *LI-cadherin* in cancer is still not fully understood. *XK* is highly expressed in erythroid tissues, skeletal muscle, and the heart and brain.[29] Absence of *XK* expression at the surface of red blood cells and weakened Kell antigens define the McLeod syndrome phenotype through neurologic impairments.[29,30] No previous studies have reported the relationship between *XK* expression with carcinogenesis.

Although some genes that do not have available antibodies have likely been missed in the present study, *AP2α* and *MUC17* may be important in the metastasis of PDAC, suggesting that these genes may lead to improvements in making an early diagnosis and to the discovery of innovative therapeutic approaches for PDAC patients. The antibodies of *AP2α* and *MUC17* used in this study are also available for paraffin-embedded tissues; therefore, these antibodies may be useful for clinical markers. However, further molecular and cellular studies are needed to fully make use of this information.

Abbreviations

AP2	activating enhancer binding protein 2
C4BPB	complement component 4 binding protein, beta
DAB	3,3′-diaminobenzidine
DOK7	docking protein 7
FOXL1	forkhead box L1
LI-cadherin	liver–intestine cadherin
LNM	lymph node metastasis
LSD1	lysine-specific demethylase 1
MUC17	mucin 17
PDAC	pancreatic ductal adenocarcinoma
XK	X-linked Kx blood group

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