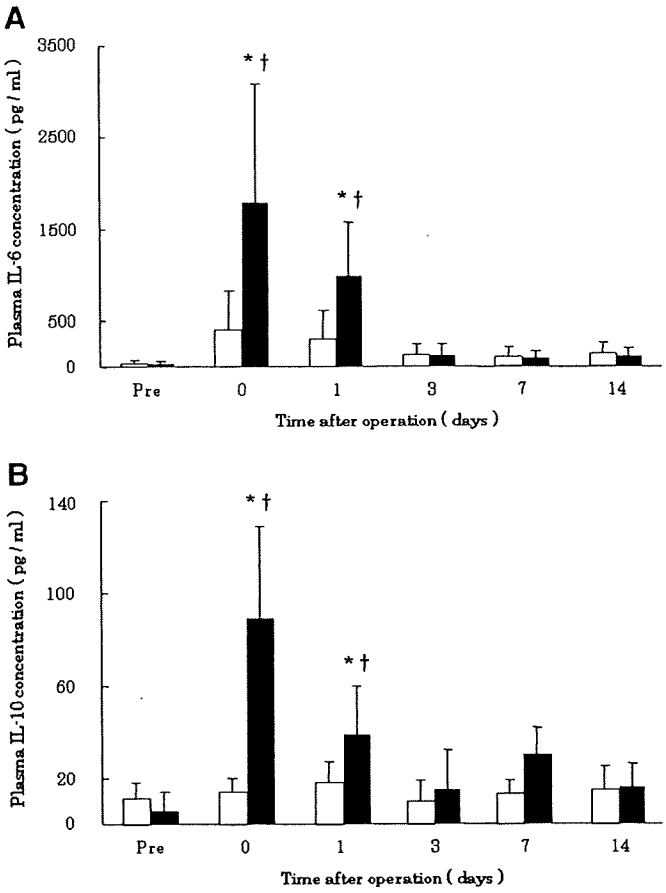

FIG. 1. Mean plasma concentrations of IL-6 (A) and IL-10 (B) following hepatobiliary pancreatic surgery. Open bars, patients without postoperative infection; filled bars, patients with postoperative infection. Error bars correspond to ± SD. *$P < 0.05$ *versus* patients without postoperative infection, †$P < 0.05$ *versus* preoperative value.

SSI. However, the incidence of postoperative infection was not affected by preoperative biliary drainage (Table 1). Eleven patients (37%) in the group with postoperative infection and 6 patients (23%) in the group without postoperative infection required blood transfusion ($P = 0.270$) (Table 1).

Plasma Concentrations of IL-6 and IL-10

Changes in plasma concentrations of IL-6 and IL-10 are presented in Fig. 1. Preoperative plasma concentrations of IL-6 and IL-10 were not significantly different between patients with postoperative infection and those without postoperative infection. Hepatobiliary pancreatic surgery resulted in a striking elevation of plasma IL-6 and IL-10 levels on postoperative d 0 and 1 compared with preoperative values ($P < 0.001$), followed by rapid declines ($P < 0.001$). Postoperative plasma IL-6 concentration was significantly higher in patients with postoperative infection than in those without postoperative infection on postoperative d 0 and 1 ($P < 0.01$) (Fig. 1A). Postoperative plasma IL-10 concentration was also significantly higher in patients with postoperative infection than in those without postoperative infection on postoperative d 0 and 1 ($P < 0.01$) (Fig. 2B). Plasma IL-6 level on postoperative d 0 correlated significantly with blood loss ($r = 0.489$, $P = 0.0004$) and operation time ($r = 0.454$, $P = 0.0012$). Plasma IL-10 level on postoperative d 0 correlated significantly with blood loss ($r = 0.577$, $P < 0.0001$) and operation time ($r = 0.310$, $P = 0.0358$).

Plasma Concentrations of sLAG-3 and sCD30

Perioperative plasma concentrations of sLAG-3 and sCD30 are presented in Fig. 2. Plasma concentration of sLAG-3 was not significantly different between patients with postoperative infection and those without postoperative infection throughout the study. Plasma concentration of sLAG-3 was not affected by surgery in both groups (Fig. 2A). Preoperative plasma concentration of sCD30 was significantly higher in patients with postoperative infection than in those without postop-

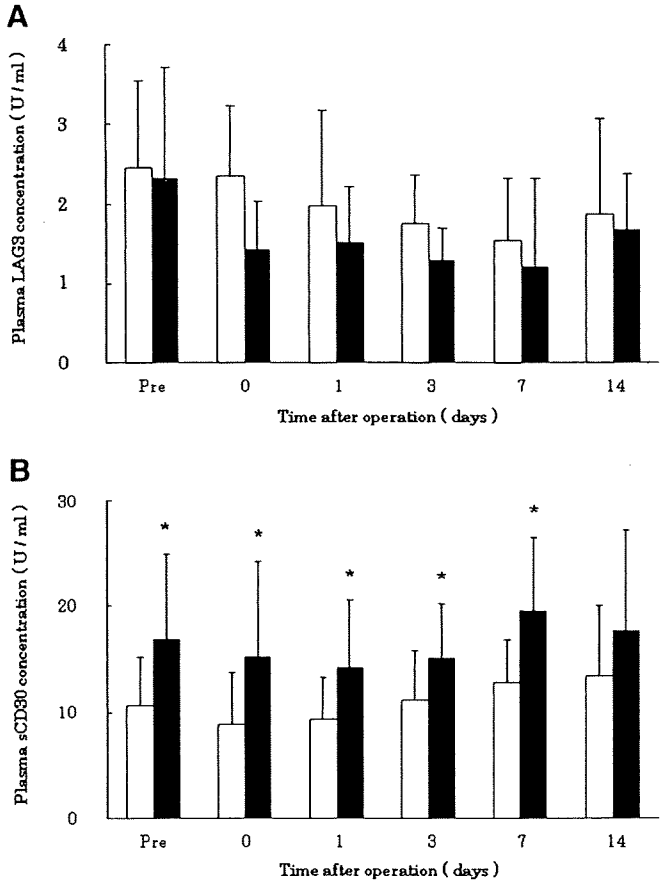

FIG. 2. Mean plasma concentrations of sLAG-3 (A) and sCD30 (B) following hepatobiliary pancreatic surgery. Open bars, patients without postoperative infection; filled bars, patients with postoperative infection. Error bars correspond to ± SD. *$P < 0.05$ *versus* patients without postoperative infection.

erative infection (16.8 ± 2.7 U/mL *versus* 10.6 ± 1.0 U/mL; $P = 0.0023$). Postoperative plasma concentration of sCD30 was also significantly higher in patients with postoperative infection than in those without postoperative infection on postoperative d 0, 1, 3, and 7 ($P < 0.001$). There were no significant changes in plasma sCD30 levels after surgery compared with preoperative value in both groups (Fig. 2B). Preoperative sCD30 level was not affected by cancer stage, and correlated significantly with length of postoperative hospital stay ($r = +0.45$, $P = 0.0011$). Preoperative sCD30 level did not correlate with depth of SSI.

Expressions Level of T-bet and GATA3 mRNA in the PBMCs

Expression level of the T-bet and GATA3 mRNA in PBMCs are presented in Fig. 3. Preoperative expression level of the T-bet mRNA in PBMCs was not significantly different between patients with postoperative infection and those without postoperative infection. Postoperative expression level of the T-bet mRNA in PBMCs was significantly higher in patients with postoperative infection than in those without postoperative infection on postoperative d 3 (0.086-fold ± 0.039-fold T-bet/GAPDH mRNA copy number *versus* 0.017-fold ± 0.003-fold T-bet/GAPDH mRNA copy number; $P = 0.0102$), d 7 ($P = 0.0401$), and d 14 ($P = 0.0087$). There were no significant changes in expression levels of T-bet mRNA after surgery compared with preoperative values in both groups (Fig. 3A). Preoperative expression level of the GATA3 mRNA in PBMCs was significantly higher in patients with postoperative infection than in those without postoperative infection (0.265-fold ± 0.041-fold GATA3/GAPDH mRNA copy number *versus* 0.171-fold ± 0.051-fold GATA3/GAPDH mRNA copy number; $P = 0.0106$). Postoperative expression level of the GATA3 mRNA in PBMCs was also significantly higher in patients with postoperative infection on postoperative d 0, 3, and 7 ($P < 0.05$). Postoperative expression level of the GATA3 mRNA in PBMCs was significantly lower on postoperative d 0 compared with preoperative value in patients with postoperative infection ($P = 0.001$) (Fig. 3B). Preoperative expression level of GATA-3 mRNA in PBMCs was not affected by cancer stage, and correlated significantly with length of postoperative hospital stay ($r = +0.33$, $P = 0.0446$). Preoperative expression level of GATA-3 mRNA in PBMCs did not correlate with depth of SSI.

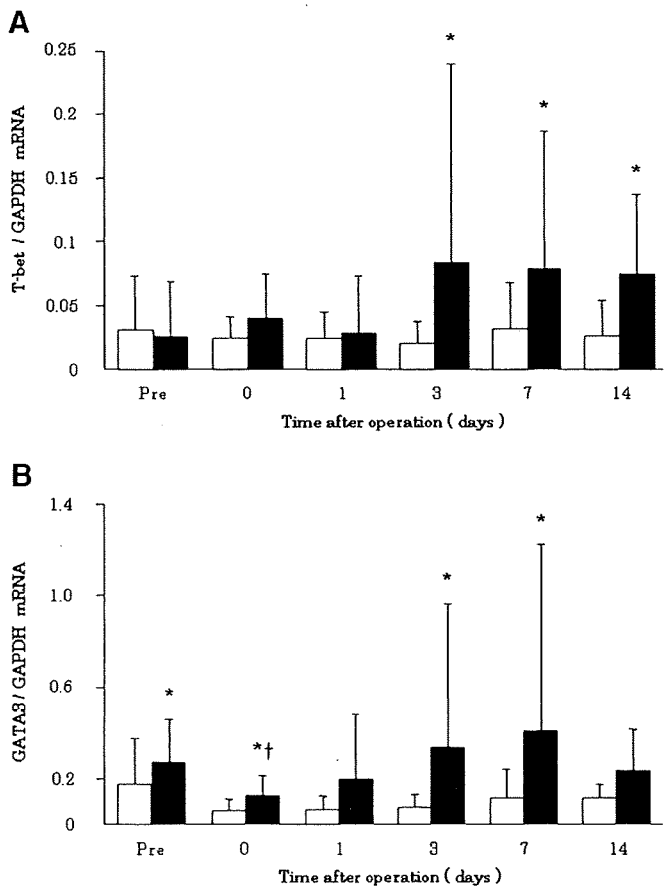

FIG. 3. Expression of T-bet (A) and GATA3 (B) mRNA in PBMCs from patients undergoing hepatobiliary pancreatic surgery. Open bars, patients without postoperative infection; filled bars, patients with postoperative infection. Error bars correspond to ± SD. *$P < 0.05$ *versus* patients without postoperative infection, †$P < 0.05$ *versus* preoperative value.

Expressions Level of TLR2 and TLR4 mRNA in PBMCs

Expression level of the TLR2 and TLR4 mRNA in PBMCs are presented in Fig. 4. Preoperative expression levels of the TLR2 and TLR4 mRNA in PBMCs were not significantly different between patients with postoperative infection and those without postoperative infection. There were no significant changes in expression levels of TLR2 and TLR4 mRNA after surgery compared with preoperative values in both groups (Fig. 4). Postoperative expression level of the TLR2 mRNA in PBMCs was significantly higher in patients with postoperative infection than in those without postoperative infection on postoperative d 3 (1.83-fold ± 0.52-fold TLR2/GAPDH mRNA copy number *versus* 0.536-fold ± 0.090-fold TLR2/GAPDH mRNA copy number; $P = 0.0042$) (Fig. 4A). Postoperative expression level of the TLR4 mRNA in PBMCs was significantly higher in patients with postoperative infection than in those without postoperative infection on postoperative d 3 (0.172-fold ± 0.079-fold TLR4/GAPDH mRNA copy number *versus* 0.047-fold ± 0.012-fold TLR4/GAPDH mRNA copy number; $P = 0.0455$) and d 14 ($P = 0.0440$) (Fig. 4B). Preoperative expression levels of TLR2 and TLR4 mRNA in PBMCs were not affected by liver cirrhosis or obstructive jaundice.

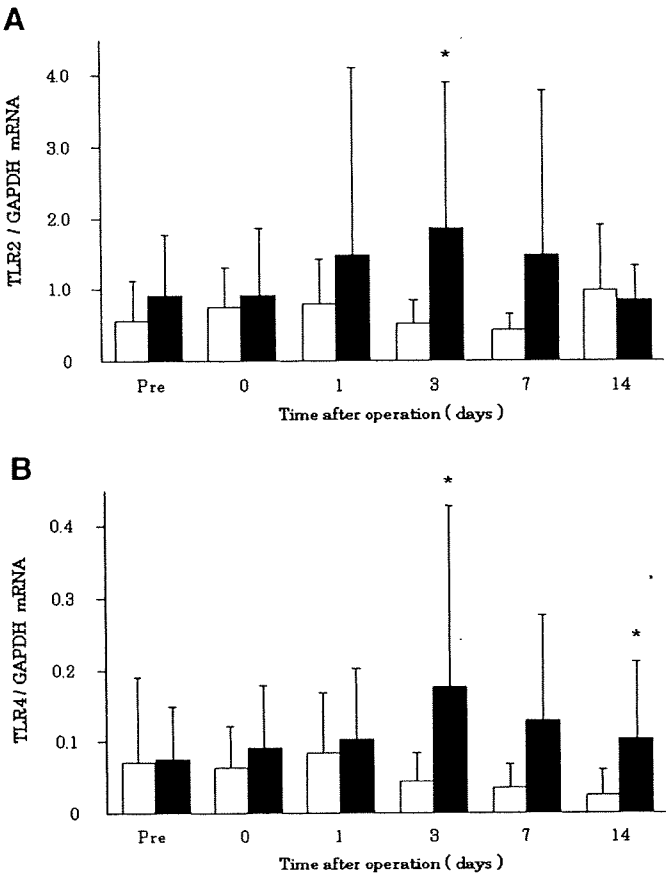

FIG. 4. Expression of TLR2 (A) and TLR4 (B) mRNA in PBMCs from patients undergoing hepatobiliary pancreatic surgery. Open bars, patients without postoperative infection; filled bars, patients with postoperative infection. Error bars correspond to ± SD. *$P <$ 0.05 *versus* patients without postoperative infection.

Correlation of T-bet and GATA-3 mRNA Expression with TLRs mRNA Expression

Preoperative expression levels of GATA-3 mRNA in PBMCs correlated significantly with those of TLR2 mRNA ($r = +0.45$, $P = 0.0042$) and TLR4 mRNA ($r = +0.34$, $P = 0.0380$) (Fig. 5). Preoperative expression levels of T-bet mRNA in PBMCs did not correlate significantly with those of TLR2 and TLR4 mRNA.

Predisposing Factors for Postoperative Infections

Logistic regression analysis was used to determine the most significant predisposing factors for postoperative infection. In univariate logistic regression analyses, postoperative infection was significantly associated with preoperative sCD30 > 9.69 U/mL (OR, 4.3; $P < 0.0170$), blood loss >691 g (OR = 4.3; $P = 0.0145$), operation time >402 min (OR, 2.6; $P = 0.0471$) (Table 2). These significant variables were included in a multivariate regression analysis. In this analysis, sCD30 on preoperative period >9.69 U/mL (OR, 5.4; $P =$ 0.0436), blood loss >691 g (OR, 8.0; $P = 0.0147$) were the significant factors connected to postoperative infection (Table 2).

DISCUSSION

In the present study, to evaluate Th1/Th2 balance, expression level of T-bet and GATA-3 mRNA in PBMCs, and circulating levels of sLAG-3 and sCD30 were determined. T-bet is essential for the development of Th1 cells, and GATA-3 performs an equivalent role in Th2 development [16]. These transcription factors are up-regulated in several cells that produce type 1 and type 2 cytokines and can be analyzed readily by RT-PCR using total RNA isolated from mixed cell populations, thereby providing a surrogate marker of Th1/Th2 cytokine balance under a variety of conditions [17, 18]. LAG-3 is a member of the immunoglobulin superfamily that is selectively transcribed in human activated

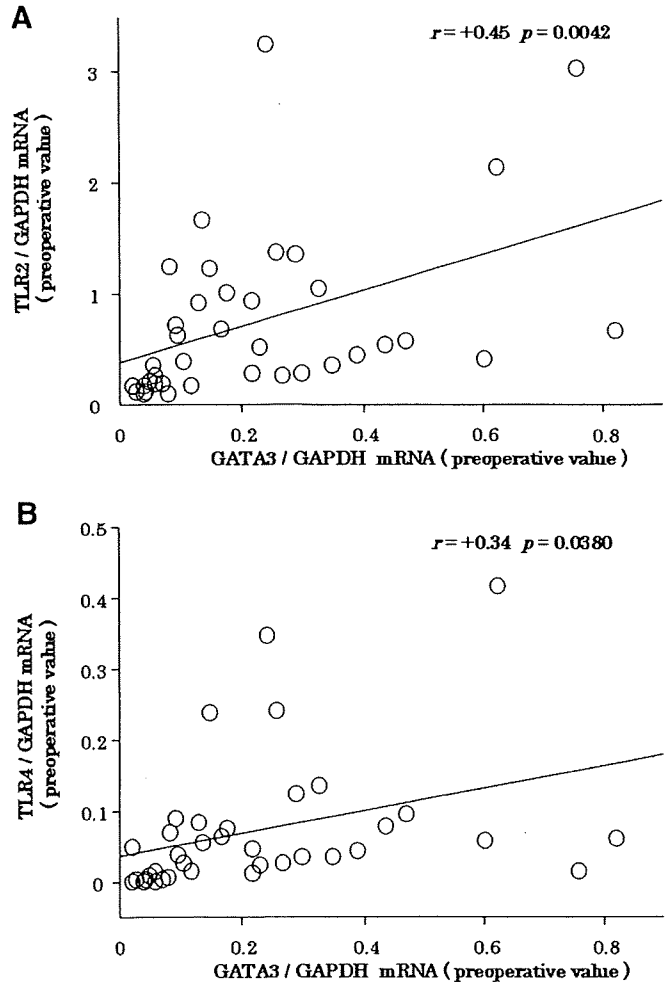

FIG. 5. Correlation of preoperative GATA3 mRNA expression with preoperative TLR2 (A) and TLR4 (B) mRNA expression; r is the coefficient of correlation and P is the statistical significance calculated according to the simple regression analysis.

TABLE 2

Results of Regression Analyses Examining Effects of Prognostic Factors on Postoperative Infection Following Hepatobiliary Pancreatic Surgery

	Univariate analyses		Multivariate analyses	
Variable	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Albumin* > 3.95 g/dL	0.5 (0.2–1.6)	0.2867		
sCD30* > 9.69 U/mLl	5.2 (1.5–17.4)	0.0082	19.1 (1.5–235)	0.0211
Operation time > 402 min	5.3 (1.6–16.8)	0.0069	2.7 (0.4–20.7)	0.3317
Blood loss > 691 g	5.1 (1.6–16.4)	0.0070	36.9 (2.7–511)	0.0071

OR = Odds ratio; CI = confidence interval.
* Preoperative values.

T and natural killer cells [19]. LAG-3 expression by activated CD4+ human T cells appears to be preferentially associated with the differentiation/activation pathway leading to the production of interferon-gamma [19]. CD30 is a 120 kD membrane glycoprotein that belongs to the tumor necrosis factor superfamily. Th1 clones expressed poor or no CD30 mRNA, and showed low or undetectable expression of both membrane and sCD30 protein, whereas Th2 clones showed both CD30 mRNA and membrane CD30 and released substantial amounts of sCD30 [20]. Thus, circulating sLAG-3 and sCD30 could be a potential serological marker of Th1/Th2 balance [21, 22].

In the present study, patients with postoperative infection showed up-regulation of GATA-3 mRNA expression before surgery and postoperative d 0, up-regulation of both GATA-3 and T-bet mRNA expression on postoperative d 3 and 7, and up-regulation of T-bet mRNA expression on postoperative d 14, compared with those without postoperative infection. These results suggest that Th1/Th2 balance of the patients with postoperative infection was shifted toward Th2 dominance before surgery and immediately after surgery, equilibrated by coexpression of Th1 and Th2 response on postoperative d 3 and 7, and finally shifted toward Th1 dominance 2 wk after surgery. Circulating sCD30 levels reflected the perioperative changes in expression level of GATA-3 mRNA, whereas T-bet failed to reflect expression level of T-bet mRNA.

It has been reported that in cancer patients the Th1/Th2 balance shifts toward Th2 dominance, which may promote cancer metastasis and the susceptibility to infection [23, 24]. However, in the present study, preoperative GATA-3 mRNA expression and circulating sCD30 levels were not affected by cancer stage. Malnutrition also polarizes Th0 cells toward a Th2 phenotype [25, 26]. In the present study, preoperative serum albumin level was significantly lower in patients with postoperative infection. Therefore, malnutrition might be one of the causes of preoperative Th2 dominance. Preoperative immunonutrition potentially corrects impaired Th1/Th2 balance and decreases postoperative morbidity rate [27]. TLRs may also play a pivotal role in regulation of Th1/Th2 balance [13]. In the present study, preoperative GATA-3 mRNA expression was correlated with TLR2 mRNA expression. This result suggests that TLR2 might take part in pathophysiology of preoperative Th2 dominance. It seems likely that subclinical infection might up-regulate TLR2 and GATA-3 mRNA expression [14]. However, precise mechanism remains obscure.

It has been reported that in patients undergoing thoracic esophagectomy for cancer, postoperative major infection is closely associated with preoperative suppression of cell-mediated immunity [28, 29]. Also in the present study, preoperative Th2 dominance might suppress the cell-mediated immunity, and result in postoperative infection. Correlations of GATA-3 mRNA expression and circulating sCD30 levels with duration of postoperative hospital stay support this hypothesis.

In the present study, expression of GATA-3 and T-bet mRNA, especially GATA-3 mRNA, seemed to be depressed on postoperative d 0 compared with preoperative values. It has been reported that in the early postoperative period, surgical stress induces the down-regulation of the Fas-mediated apoptotic response in neutrophils [30]. Contrarily, surgical trauma induces both up-regulation of death-signaling factors and down-regulation of survival-signaling factors, resulting in a high frequency of apoptosis of CD4(+) and CD8(+) cells [31]. Thus, in the early postoperative period, numbers and function of lymphocytes are depressed, while those of neutrophils increase [29, 32]. These phenomena should be taken into account to evaluate expression levels of T-bet and GATA-3 mRNA in the early postoperative period [33].

In humans, major injury shifts the Th1/Th2 balance toward Th2 dominance. Furthermore, there is a reciprocal relation between diminished Th1 function and increased Th2 function at approximately 1 wk after major injury [8–10]. However, in the present study, even in the patients with postoperative infection, postoperative Th2 dominance was not observed after surgery, excluding postoperative d 0, despite preoperative

Th2 dominance. It has been reported that Th2 cytokines are produced in response to surgical stress, and potentially modulate postoperative immune response, rendering patients susceptible to infection [34]. Also in the present study, postoperative IL-6 and IL-10 levels were correlated with surgical stress and postoperative infection. However, following the peak levels of these cytokines, T-bet mRNA expression was up-regulated on postoperative d 3, resulting in coexpression of Th1 and Th2 response followed by Th1 dominance 2 wk after surgery. These results suggest that increased levels of circulating Th2 cytokines do not play a major role in postoperative alteration of Th1/Th2 balance. Up-regulation of T-bet mRNA expression in patients with postoperative infection might be induced by organisms responsible for postoperative infection, including gram-positive and -negative bacteria and fungi [35, 36]. It has been reported that in patients with postoperative infection, a broad range of cytokines, chemokines, and stress hormones, including inflammatory and anti-inflammatory mediators, are detected in the circulation immediately after surgery [37]. These mediators might also take part in postoperative alteration of Th1/Th2 balance. Regardless of the mechanism, appearance of Th1 response in patients with postoperative infection seems to be a favorable immune response facilitating bacterial clearance [10, 38].

In the present study, expression levels of TLR2 and TRL4 mRNA in PBMCs were determined. It has been reported that although TLR1 and TLR6 are expressed in all cell types of immune cells, including monocytes, polymorphonuclear leukocytes, T and B cells, and natural killer cells, TLR2, TLR4, and TLR5 are expressed in myelomonocytic elements [39, 40]. Therefore, expression levels of TLR2 and TRL4 mRNA in PBMCs potentially reflect TLR2 and TRL4 expression of monocytes [40].

It has been reported that TLR2 expression of monocytes is significantly up-regulated in patients with cirrhosis [14, 41], while expression of TLR4 mRNA in PBMCs is down-regulated [41]. Moreover, tissue expression of TLR4 in the small intestine and liver is up-regulated in patients with obstructive jaundice [15]. These changes of TLR2 and TLR4 expression potentially modulate perioperative innate and adaptive immune response [13, 35, 36]. In the present study, however, preoperative expression of TLR2 and TLR4 mRNA in PBMCs was not affected by liver cirrhosis or obstructive jaundice. These results might depend on the fact that patients undergoing liver resection did not have severe cirrhosis, and all patients with obstructive jaundice underwent biliary drainage before surgery.

Effects of surgical stress on monocyte expression of TLR-2 and TLR-4 are controversial. It has been reported that in patients undergoing open heart surgery, monocyte expression of TLR-2 and TLR-4 is up-regulated on postoperative d 1 and d 2 [42]. By contrast, it has reported that TLR2 and TLR4 expression on monocytes are down-regulated by operation, and show the lowest values on postoperative d 3 and 1, respectively [43]. These contrasting results may depend upon the methodology of the flow cytometric gating technique used [44]. In the present study, expression of TLR2 and TLR4 mRNA was up-regulated on postoperative d 3 in patients with postoperative infection compared with patients without postoperative infection. It has been reported that monocytes from patients with sepsis exhibited higher TLR-2 and TLR-4 expression than cells from controls [45]. Furthermore, it has been reported that IL-6 up-regulates TLR-4 expression of isolated monocytes, tumor necrosis factor-alpha significantly down-regulates TLR-4 mRNA expression, whereas stimulation with IL-8 or IL-10 had no significant effects [46]. Therefore, it seems likely that increased IL-6 level and postoperative bacterial infection might up-regulate TLR-2 and TLR-4 mRNA expression in patients with postoperative infection. It has been reported that TLRs ligands specifically promote bacterial phagocytosis in human monocytes through induction of a phagocytic gene program [47]. Therefore, as well as up-regulation of Th1 response, up-regulation of TLR2 and TLR4 expression seems to be a favorable immune response facilitating bacterial clearance in patients with bacterial infection.

The present study revealed that intraoperative blood loss is also closely associated with postoperative infection independent of preoperative Th2 dominance. It has been reported that allogeneic blood transfusion potentially causes immunosuppression, which is associated with postoperative bacterial infection [48]. It has also been reported that intraoperative transfusion of packed red cells induces IL-6 and IL-8 release in patients [49, 50]. However, pathophysiology of blood transfusion induced immunosuppression remains obscure.

CONCLUSIONS

The conclusions are as follows: (1) that preoperative Th1/Th2 balance is shifted toward Th2 dominance in patients with postoperative infection compared with those without infection, (2) that hepatobiliary-pancreatic surgery increases Th1 response after surgery, resulting in coexpression of Th1 and Th2 response followed by Th1 dominance, (3) that expression of TLR2 and TLR4 mRNA in PBMCs is up-regulated after surgery in patients with postoperative infection compared with patients without infection, and (4) that hepatobiliary pancreatic cancer patients with preoperative high expression of GATA-3 mRNA and sCD30 can be identified as a higher risk population in the postoperative period.

ACKNOWLEDGMENTS

The authors acknowledge support for this work by a Grant-in-Aid for Scientific Research (C-2-14571178) from the Japan Society for the Promotion of Science.

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Cancer Science
The official journal of the Japanese Cancer Association

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

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(Received August 4, 2009/Revised September 3, 2009/Accepted September 5, 2009/Online publication October 11, 2009)

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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doi: 10.1111/j.1349-7006.2009.01359.x

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).

doi: 10.1111/j.1349-7006.2009.01359.x

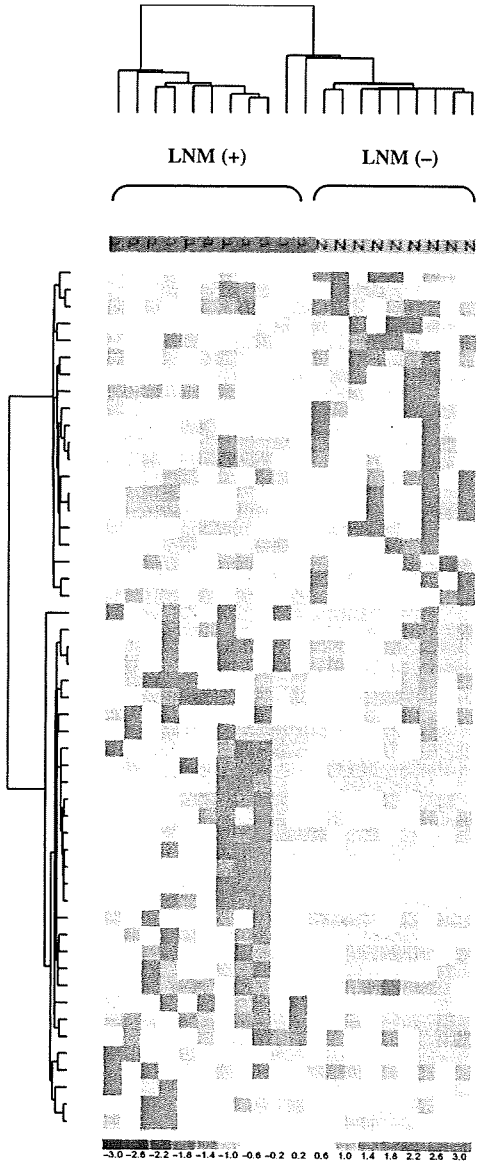

Fig. 1. Supervised hierarchical clustering of pancreatic ductal adenocarcinoma with and without lymph node metastasis using the selected 46 genes expressed differentially between two groups. Red, overexpressed genes; blue, underexpressed genes. LNM (+), positive lymph node metastasis; LNM (–), negative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

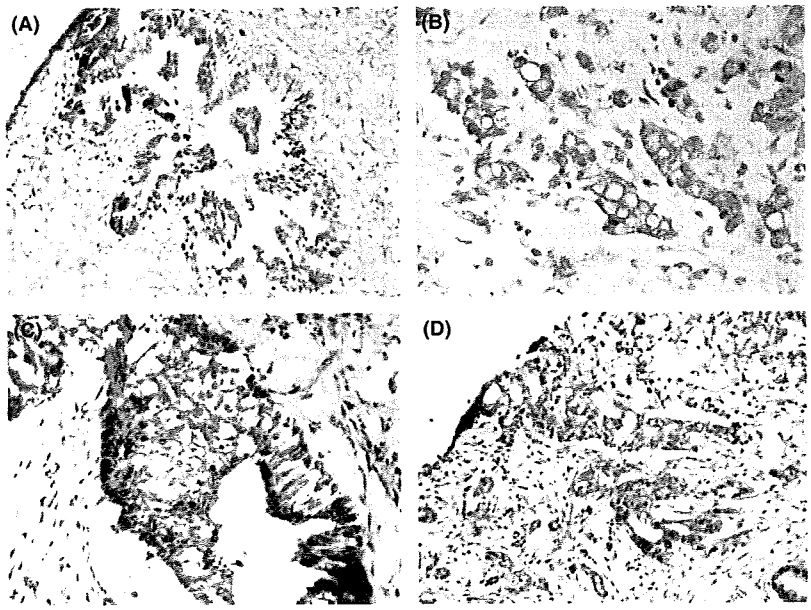

Fig. 2. Immunohistochemical staining of four genes associated with lymph node metastasis in pancreatic ductal adenocarcinoma patients (A–D). Activating enhancer binding protein 2 (*AP2α*) expressed in nucleus of pancreatic cancer cells (A). Liver–intestine cadherin (*LI-cadherin*) (B), mucin 17 (*MUC17*)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

doi: 10.1111/j.1349-7006.2009.01359.x

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

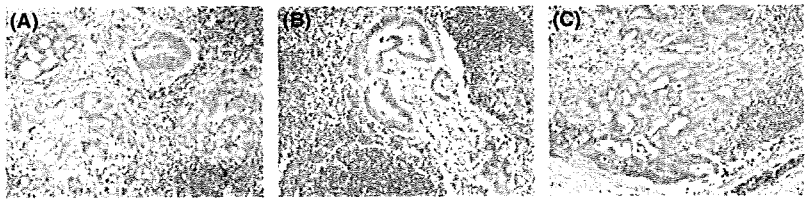

Fig. 3. Immunohistochemical staining in metastatic lymph node tissues from pancreatic ductal adenocarcinoma (A–C). High expression of liver–intestine cadherin (*LI-cadherin*) (A), mucin 17 (*MUC17*) (B), and X-linked Kx blood group (*XK*) (C) are shown in metastatic adenocarcinoma in lymph node.

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 *AP2*α-low expression and with *AP2*α-high expression.

Expression of molecular markers in metastatic lymph node tissues from PDAC. Protein expression of *AP2*α, *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 *AP2*α 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 *AP2*α-low expression was significantly worse than for those with *AP2*α-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 *AP2*α and *MUC17* expression, patients with *AP2*α-low and *MUC17*-high expression had a worse survival than those with *AP2*α-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, *AP2*α-low expression, *MUC17*-high expression, and *AP2*α-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

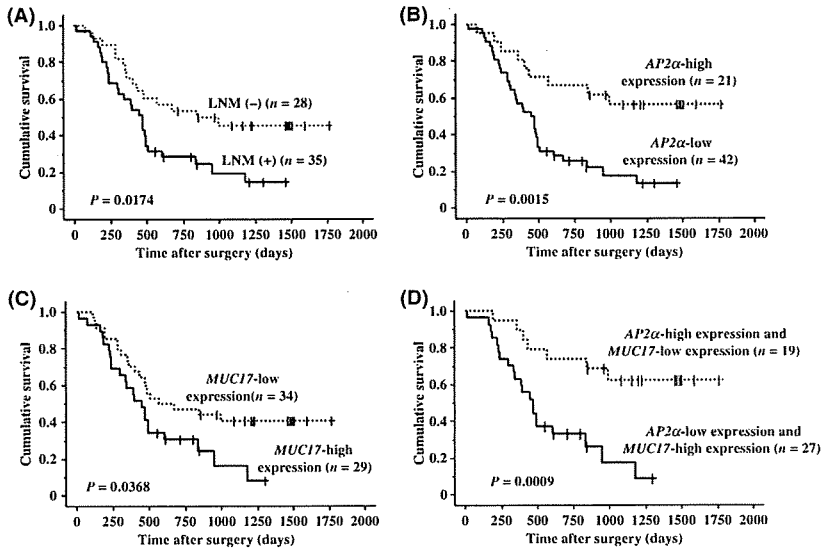

Fig. 4. (A) Overall survival (OS) without lymph node metastasis was better (median, 844 *vs* 470 days). (B) OS with *AP2*α-low expression was worse than that of high expression. (C) OS with *MUC17*-high expression was worse than that of low expression (median, 451 *vs* 567 days). (D) OS with *AP2*α-low and *MUC17*-high expression was worse than that of *AP2*α-high and *MUC17*-low expression. LNM (+), positive lymph node metastasis; LNM (–), negative lymph node metastasis.

doi: 10.1111/j.1349-7006.2009.01359.x

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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doi: 10.1111/j.1349-7006.2009.01359.x

Clamp-crushing Pancreas Transection in Pancreatoduodenectomy

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ABSTRACT

Background/Aims: Pancreatoduodenectomy is associated with high morbidity rates, resulting primarily from the occurrence of pancreatic fistula at pancreatojejunostomy. We transect the pancreas using a manual clamp-crushing technique to prevent postoperative pancreatic fistula (POPF) formation. The aim of this study was to clarify the usefulness of this new technique.

Methodology: Fifty patients with a normal soft pancreas who underwent pancreatoduodenectomy in the last 3 years were selected. During the last stage of the classic Whipple operation, the pancreas was transected using a clamp-crushing technique under blood-flow control. The pancreas parenchyma was crushed using forceps, and small pancreatic branch ducts were securely ligated and cut. The main pancreatic duct was identified, and pancreatojejunal reconstruction was done end-to-side with a duct-to-mucosa anastomosis, following approximation of the pancreatic stump to the jejunal wall using the one-layer suture technique.

Results: According to ISGPF (please use the first abbreviation for subtotal stomach - preserving pancreaticoduodenectomy) grading, POPF Grade B occurred in 10 (20%) patients. There were no Grade C patients, no postoperative hemorrhage and no POPF associated mortality.

Conclusion: The clamp-crushing technique appears to be a safe method for pancreatic transection that is feasible in cases with a normal soft pancreas.

KEY WORDS: Pancreatoduodenectomy, Pancreas transection, Clamp-crushing technique

ABBREVIATION: Endoscopic Retrograde Cholangiopancreatography (ERCP)

INTRODUCTION

Despite recent advances in surgical techniques and postoperative management, pancreatoduodenectomy (PD) is associated with high morbidity and mortality rates. A postoperative pancreatic fistula (POPF) is one of the most common complications following PD. Many surgeons have tried to prevent this complication through management of the pancreatic duct (1,2), pancreatoenterostomy (3,4), and appropriate postoperative management (5,6). However, these efforts have not given satisfactory results, particularly when the remnant pancreas was soft with a small main pancreatic duct. We hypothesized that transection of the pancreas is important to prevent POPF after PD and applied a clamp-crushing technique that is used in liver resection (7).

METHODOLOGY

Patients

Between 2005 Mar and 2007 Nov, 108 patients underwent PD in our hospital. Of these, 50 patients with a soft pancreas who underwent pancreatojejunal reconstruction were selected for this study. PD was performed for ampullary cancer in 14 patients, pancreatic cancer in 12, distal bile duct cancer in 9, duodenal cancer in 6, gallbladder cancer in 2, local recurrence of colon cancer in 2, and other causes in 5 (1 each of intraductal papillary mucinous neoplasm (IPMN), solid pseudopapillary tumor, metastasis from renal cell carcinoma, retroperitoneal liposarcoma, and pancreatitis). All patients had a soft pancreas.

Surgical techniques

After the head of the pancreas was dissected from the portal veins and the retroperitoneal cavity in the usual manner, the pancreas was transected along the line of the portal vein between the head and the body using the clamp-crushing technique. By cross-clamping the pancreas with intestinal forceps, the pancreatic parenchyma was crushed using a child Kelly clamp with strokes of a few millimeters (**Figure. 1a**). During transection, small branch pancreatic ducts and blood vessels were ligated with 4-0 absorbable sutures. Finally, the main pancreatic duct was identified and cut with a slight surplus for duct-to-mucosa anastomosis (**Figure. 1b**). The pancreatic cut surface was left without parenchymal suturing or oversewn. Pancreatic

Hepato-Gastroenterology 2009; 56:89-93

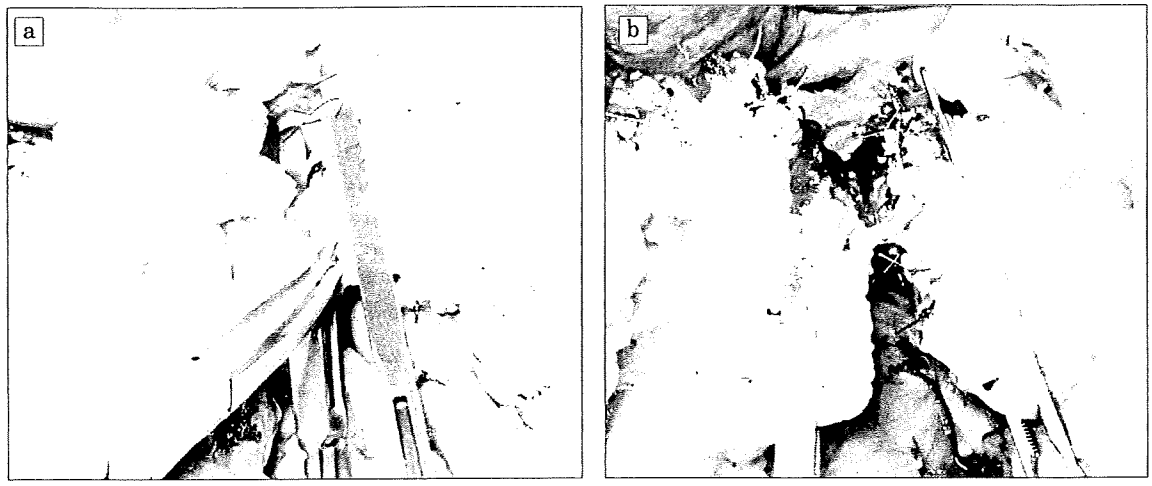

FIGURE 1 (a) Pancreas transection using a child Kelly clamp. **(b)** After pancreas transection, the main pancreatic duct is identified. Numerous absorbable suture knots remain on the cut surface of the pancreas

remnants were reconstructed in an end-to-side pancreatojejunostomy. Pancreatojejunostomy was performed by duct-to-mucosa anastomosis with interrupted sutures between the entire jejunal wall and the pancreatic duct, following approximation of the pancreatic stump to the jejunal wall, using the one-layer suture technique described by Kakita *et al.* (8). A pancreatic duct stent tube was inserted through the duct-to-mucosa anastomosis into the pancreatic duct for external drainage of pancreatic juice. A few drains were placed close to the pancreatojejunostomy and kept in place for at least 3 days, and the amylase content of the output was measured.

Diagnosis of pancreatic fistula

POPF was defined and graded according to the International Study Group on Pancreatic Fistula (ISGPF) criteria (9), as output via an operatively placed drain or a subsequently placed, percutaneous drain of any measurable volume of drain fluid on or after postoperative day 3, with an amylase content greater than 3 times the upper normal serum value (155 IU/l at our institute).

Histological analysis

Twenty-four specimens of the cut surface of the pancreas were fixed in 10% buffered formalin, embedded in paraffin by automatic tissue processing, and stained with hematoxylin and eosin (HE) and azan for collagen fiber to identify interlobular pancreatic branch ducts more easily (10); pancreatic ducts with more than 500 μm in diameter were counted microscopically.

Statistical analysis

Statistical analysis was performed using the *t*-test for continuous variables. A $p<0.05$ was considered significant.

RESULTS

A subtotal stomach - preserving pancreaticoduodenectomy (SSPPD), a modified Whipple operation, with four-fifths of the stomach preserved, was performed in 42 patients. For other patients, the classic Whipple operation was done because of co-existing gastric cancer or a previously performed gastrectomy for gastric disease in 5, duodenal cancer at the bulb portion in 1, and tumor invasion into the stomach wall in 2 patients. Combined partial colectomy was performed in 3 patients, and portal vein resection and reconstruction were performed in 3 patients.

The median operative time was 490 (range, 252 to 743) minutes, the median blood loss was 550 (range, 40 to 1400) ml, and blood transfusion was performed in 7 (14%) patients. The median time required for transection of the pancreas was 13 (range, 9 to 35) minutes, and during transection, pancreatic parenchyma was ligated 10 to 18 (median, 13) times. The main pancreatic ducts were identified in all patients, with a median size of 2 (range, 1-9) mm, and the duct to mucosa anastomosis was successfully done. Three patients with dilated main pancreatic duct more than 5 mm in diameter were included, two of whom suffered from ampullary cancer, one of whom IPMN. The median postoperative hospital stay was 30.5 (range, 19 to 83) days. Only one patient with autoimmune dermatitis died of multiple organ failure (MOF) due to sepsis on postoperative day 19, for a mortality rate of 2%. An autopsy was conducted based on the decreased patient's will, and the sepsis appeared to have been due to numerous subcutaneous abscesses and the patient's immunocompromised state, which resulted from frequent use of steroid ointment. Examination of the pancreatojejunostomy confirmed that it had healed in good condition without disturbance of the anastomosis or intra-abdominal infection.

POPF occurred in 24 (48%) patients and was classified according to ISGPF grading: more than a half of them, 14 patients (28%) were classified as Grade A, which required little change in management or deviation from the ordinary postoperative course; the remaining 10 patients (20%) were classified as Grade B, which were categorized into clinically relevant fistulas; Of whom 8 had prolonged drain placement due to

amylase-rich output without peripancreatic fluid collections on CT scan. One patient had peripancreatic fluid collection, which required repositioning of a drain at the bedside, but the patient was not septic and did not require intensive care. Another patient had no peripancreatic fluid collections, but disturbance of the anastomosis was proven on radiological examination. The patient was discharged with a drain on POD 52, and the drain was removed in the outpatient setting. None of these patients was life-threatened. None of the patients had Grade C POPF. Furthermore, none of the patients developed postoperative hemorrhage, required re-operation, or had POPF-associated mortality.

Figure 2 shows the postoperative amylase level of the drain output over time. Although the amylase level on POD 1 was significantly higher in patients with POPF than in patients without POPF (p=0.049), after POD 3, the amylase level decreased smoothly and there was no longer a significant difference between the two groups. The median drainage time was 19 (range, 5-54) days in the patients with POPF and 14 (range, 4-39) days in the patients without POPF (p=0.052). The median postoperative hospital stay was significantly longer in patients with Grade B POPF (39 days) than in patients with Grade A POPF (29 days, p=0.04) and in patients without POPF (29 days, p=0.01).

On histological examination (**Figure. 3**), the average number of interlobular pancreatic branch ducts more than 500 μm in diameter was 7 (range, 3-16), including thick pancreatic ducts with a similar thickness to the main pancreatic duct. While there were more pancreatic branch ducts, including smaller interlobular and intralobular ducts, in the cut surface, the distribution of the vessels was not similar to that of the pancreatic branch ducts.

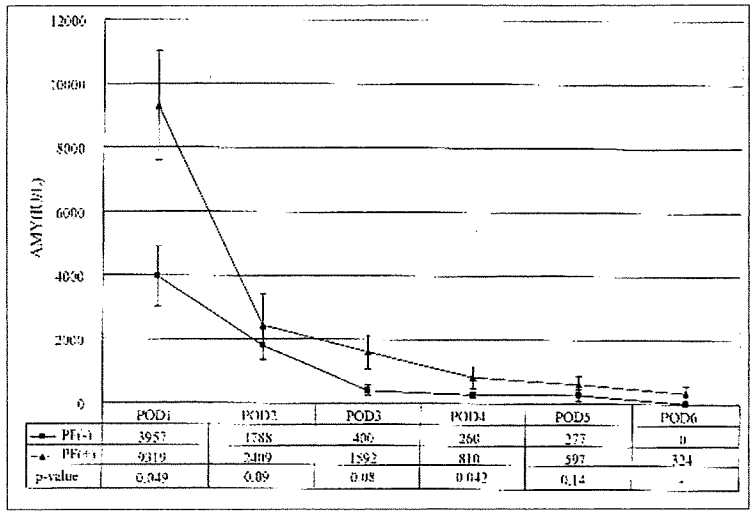

FIGURE 2 Postoperative amylase content of the drained fluid.

DISCUSSION

Reconstruction of continuity between the pancreas and the gastrointestinal tract during the Whipple operation is the most important and most unstable anastomosis, and there can be serious sequel. The rate of POPF after soft pancreas resection has been reported around 20% according to the ISGPF criteria (11,12). In particular, previous studies have shown that a soft pancreas is a significant risk factor for POPF (13-15). To prevent this complication, many surgeons have attempted various technical approaches, including pancreaticogastrostomy (3,4), management of the pancreatic cut surface (1,2), external pancreatic duct drainage(16), administration of somatostatin analogues (5,6), and approximation of other organs (8,17).

We use the end-to-side anastomosis of the pancreas and the jejunum, which is composed of interrupted suture of the main pancreatic duct and the jejunal mucosa, following the approximation of the jejunal wall to the pancreatic stump with the one-layer suture

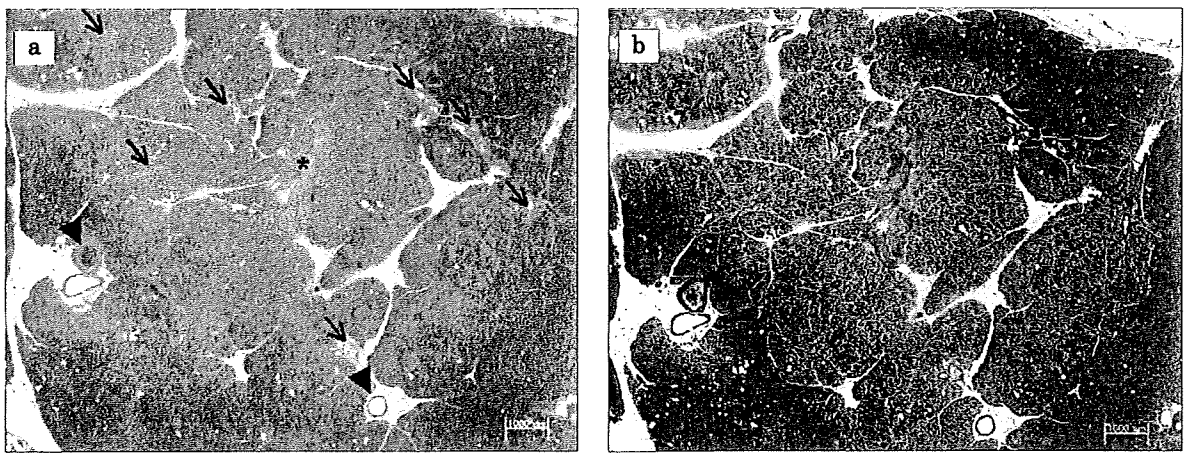

FIGURE 3 Microscopic view of the cut pancreatic surface. Original magnification, x10. **(a)** Hematoxylin and eosin staining. Several thick branch ducts (arrow) are around the main pancreatic duct (*). Vessels (arrow head) that do not accompany the pancreatic ducts are scattered. **(b)** Azan staining for collagen fibers enables easy identification of interlobular pancreatic branch ducts, which are lined with collagen tissue.

technique described by Kakita *et al.* (8). Pancreatic duct-to-mucosa anastomosis is one of the methods used to prevent POPF (18). Throughout this procedure, pancreatic juice in the main pancreatic duct drains well into the jejunum. Meanwhile, pancreatic juice naturally leaks from the branch ducts, which are exposed at the cut pancreatic stump, and collects around the anastomosis. Thus, reduction of such leakage from the unconnected ducts would be important in this procedure. Controlling minor leakage using the crush and ligation method appeared to be effective for stabilizing pancreatojejunostomy by preventing spillage of small amounts of pancreatic juice. In the present study, 50 cases with soft pancreas texture successfully underwent this procedure without mortality related to insufficiency of the pancreatojejunostomy.

Of the various studies dealing with reconstruction of continuity between the pancreas and the gastrointestinal tract, few have dealt with the management of the pancreatic stump. The pancreas is usually cut using a knife, electrocautery, or an ultrasonic-activated scalpel (19). Suzuki *et al.* (20) and Sugiyama *et al.* (1) transected the pancreas using an ultrasonic surgical aspirator and ligation of residual fibrous bundles. In the present study, the pancreatic parenchyma was crushed with a child Kelly clamp, and the remaining fibrous bundles were ligated and cut. The number of tied bundles was smaller in the clamp-crushing method than in the procedure using the ultrasonic aspirator (13 vs. 20-30) (1,20). Thus, the ultrasonic aspirator might permit surgeons to more delicately remove pancreatic tissue than the manual procedure. However, histological examination revealed that interlobular branch ducts that were 500 μm or more in diameter appeared to be fully covered with the manual method. While avoiding excessive skeletonizing of bridging tubules, and ligating the remaining fibrous bundles in a group as much as possible, not only interlobular pancreatic ducts but also intralobular pancreatic ducts could be closed securely.

In the previous studies, the rate of POPF (range, 14.8-25.1%) was considerably less than that of this study, but the incidence of Grade C POPF was comparatively frequent (range, 2.9-9%), in spite of including the cases with hard pancreas. It may have been suggested that there was Grade migration by insufficiency of the measurement of drain output, which led to the underestimaion of POPF and severe complications (21). Yamaguchi *et al.* (22) and Kamisawa *et al.* (23) reported the presence of an exceptional pancreatic duct, which takes a straight course through the body and tail to join the main pancreatic duct at the neck portion of the pancreas, as seen on ERCP or embryological study. Furthermore, since vessels and pancreatic branch ducts do not travel together in the pancreas, which is different from the hepatic portal tract, it may be difficult to close the branch ducts during the hemostatic procedure after a sharp cut of the pancreas. Manual pancreas transection by secure ligation that takes less than 15 minutes is practical and effective for stabilizing pancreatojejunostomy by reducing minor spillage from branch ducts, resulting in the smooth decrease of the level of amylase after POD 3 leading to no Grade C POPF, no postoperative hemorrhage, no re-operation, and no mortality due to POPF.

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