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Conflicts of interest

The authors disclose no conflicts.

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Akt/mTOR signaling pathway is crucial for gemcitabine resistance induced by Annexin II in pancreatic cancer cells

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ABSTRACT

Background: Although gemcitabine has been widely used as a first-line chemo reagent for patients with pancreatic cancer, the response rate remains low. We previously identified Annexin II as a factor involved in gemcitabine resistance against pancreatic cancer. The aims of this study were to elucidate the signaling mechanism by which Annexin II induces gemcitabine resistance and to develop a new therapy that overcomes the resistance against gemcitabine.

Methods: We compared the specific profiles of 12 targeted phosphorylated (*p*-) signaling proteins in gemcitabine-resistant (GEM-) and its wild-type pancreatic cancer cell lines (MIA PaCa-2) using the Bio-Plex assay system. We also evaluated the expression levels of Annexin II and two phosphoproteins, which showed different expressions in these two cell lines, by immunohistochemistry.

Results: Annexin II overexpression was significantly associated with rapid recurrence after gemcitabine-adjuvant chemotherapy in patients with resected pancreatic cancer ($P < 0.05$). Bio-Plex analysis showed up-regulation of *p*-Akt in GEM-MIA PaCa-2 cells in which Annexin II is highly expressed. The expression level of *p*-Akt was significantly correlated with that of the downstream protein, *p*-mTOR, in pancreatic cancer tissues. Inhibition of mTOR phosphorylation canceled gemcitabine resistance in GEM-MIA PaCa-2 cells.

Conclusions: The Akt/mTOR pathway is involved in mechanisms of gemcitabine resistance induced by Annexin II in pancreatic cancer cells. This indicates that combination therapy with the mTOR inhibitor may overcome gemcitabine resistance. Annexin II as an indicator for selection of gemcitabine resistance could thus be applied to the development of novel tailor-made approaches for pancreatic cancer treatment.

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1. Introduction

Recent advances in pancreatic cancer treatment have improved the prognosis of the disease. However, these improvements have yet to achieve satisfactory efficacy of patients with pancreatic cancer, in whom the 5-y survival rate is <10%. Although surgical resection is the only hope for a cure, the prognosis of the patient remains poor even after curative surgery because of the high recurrence rate [1,2].

Since Burris *et al* [3] showed the efficacy of gemcitabine as a chemotherapeutic reagent for pancreatic cancer, this drug has become first-line therapy for patients with unresectable pancreatic cancer. This new chemotherapy has also been applied to multidisciplinary treatments, combining surgery and chemotherapy. Oettle *et al* [4] indicated that adjuvant chemotherapy using gemcitabine improved disease-free survival in patients with resected pancreatic cancer.

Because the efficacy of gemcitabine is limited, with a response rate of about 5% [3], combinations with other cytotoxic agents or molecular targeting agents have been tested in systematic chemotherapy or the adjuvant therapy setting. To date, only minor or no additional effects have been reported [5,6]. Clarification of the molecular mechanisms of resistance against gemcitabine is needed for the development of a new therapy to overcome this resistance.

We recently reported Annexin II as a gemcitabine-resistant factor by comparing the protein profiling of gemcitabine-resistant (GEM-) and wild type (WT-) MIA PaCa-2 cell lines using two-dimensional gel electrophoresis [7,8]. Immunohistochemistry demonstrated Annexin II expression mainly at the surface of pancreatic cancer cells, as well as an association of its high expression with rapid recurrence after gemcitabine-adjuvant chemotherapy in postoperative patients with pancreatic cancer. We also found that inhibition of Annexin II expression in GEM-MIA PaCa-2 cells significantly increased the chemocytotoxic efficacy of gemcitabine [8]. Therefore, we pursued the role of Annexin II in gemcitabine resistance in pancreatic cancer cells.

Annexins comprise a family of calcium-dependent phospholipid-binding cell surface proteins that have diverse cellular functions, including membrane-cytoskeleton organization, vesicular trafficking, and regulation of ion channel activity [9]. Annexin II was also found to be a chemoresistant factor in human breast cancer [10–12]. Zhang *et al* [12] indicated that Annexin II has a critical role in the invasion and metastasis of Adriamycin-resistant breast cancer cells, and that this ability is associated with multidrug resistance. However, it is unclear how Annexin II induces chemoresistance in cancer cells.

Phosphorylation of multiple signal transduction pathways in cancer cells is crucial for cancer proliferation and survival. Signaling pathways such as extracellular signal-related kinase (ERK) signal and Akt/mammalian target of rapamycin (mTOR) pathways are well known to be involved in pancreatic cancer cell growth [13].

Herein, we tried to determine the signaling pathway involved in gemcitabine resistance by Annexin II using the Bio-Plex phosphorylation protein assay system, and found the Akt/mTOR signaling pathway to be involved in Annexin II-related gemcitabine resistance. We also found that application of both Annexin II and specific inhibition of the Akt/mTOR pathway may provide novel molecular targets for pancreatic cancer therapy.

2. Materials and methods

2.1. Tissue samples

We obtained formalin-fixed paraffin-embedded tissues from 107 patients with pancreatic cancer who had undergone pancreatectomy at Chiba University Hospital between 2001 and 2009. All patients had been diagnosed histologically with primary invasive pancreatic ductal adenocarcinoma for which surgery was curative. The ethics committee of our institute approved this protocol and we obtained written informed consent from each patient before surgery.

2.2. Immunohistochemistry

We performed immunohistochemical analysis as described previously [8]. We used the following primary antibodies and detected the epitopes using the LSAB+ kit (Dako, Tokyo, Japan): anti-Annexin II polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA; diluted 1:300), anti-human phospho(*p*)-Akt (Ser473) monoclonal antibody (Cell Signaling Technology, Beverly, MA; diluted 1:50), and anti-human *p*-mTOR (Ser2448) monoclonal antibody (Cell Signaling Technology; diluted 1:50). We scored staining patterns as described previously [8]: low expression = 0%–30% of tumor cells with positive staining, and high expression = >30% of tumor cells with positive staining.

2.3. Cell lines and reagents

We obtained the human pancreatic cancer cell line, MIA PaCa-2 (named WT-MIA PaCa-2 in this study), from American Type Culture Collection (Manassas, VA). We established the gemcitabine-resistant MIA PaCa-2 (GEM-MIA PaCa-2) cell line by exposing gemcitabine to WT-MIA PaCa-2 cells repeatedly, as previously described [7]. We maintained WT- and GEM-MIA PaCa-2 cells as previously described [7], and purchased gemcitabine and rapamycin from Toronto Research Chemicals (Toronto, Canada) and Calbiochem (Darmstadt, Germany), respectively.

2.4. Bio-Plex phosphorylation protein assay system

Phosphoproteins were analyzed using Bio-Plex phosphoprotein array (Bio-Rad, Hercules, CA) based on multiplex sandwich bead immunoassays. We used phosphoprotein determination kits (Bio-Rad) according to the manufacturer's recommendations. We prepared protein extracts using a cell lysis kit (Bio-Rad).

Briefly, we incubated protein extracts overnight in 96-well plates with fluorescent capturing beads coupled to antibodies directed against 12 phosphoproteins (*p*-Akt, *p*-NF-κBp65, *p*-ERK1/2, *p*-JNK, *p*-MEK1, *p*-p38MAPK, *p*-CREB, *p*-p70S6kinase, *p*-HSP27, *p*-PDGF receptor-β, *p*-Stat3, and *p*-Stat6) and 8 total

proteins (t-Akt, t-IκB-α, t-ERK1/2, t-JNK, t-MEK1, t-p38MAPK, t-CREB, and t-HSP27). We washed plates and incubated them with biotinylated antibodies fixing each target protein. We then added streptavidin-phycoerythrin solution. We recorded results as mean fluorescence intensities and compared them with negative controls. We performed all experiments in duplicate, two independent times.

2.5. *Western blotting*

We purchased anti-t-Akt, anti-*p*-Akt (Ser473), anti-t-mTOR, and anti-*p*-mTOR (Ser2448) monoclonal antibodies from Cell Signaling Technology, and anti-Annexin II polyclonal antibody and anti-β-actin antibody from Santa Cruz Biotechnology. We washed subconfluent WT- and GEM-MIA PaCa-2 cells in six-well plates twice with phosphate-buffered saline, lysed them with appropriate lysis buffer, and incubated them after centrifugation, as previously described [8]. We separated the supernatant proteins (20 μg) by electrophoresis on 10%–20% gradient gels (PerfectNT Gel; DRC, Tokyo, Japan) for t-Akt and *p*-Akt, and 7.5% gels for t-mTOR and *p*-mTOR. We transferred these proteins to polyvinylidene fluoride membranes (Millipore, Bedford, MA), and reacted the membranes with anti-t-Akt (diluted 1:1000), anti-*p*-Akt (diluted 1:2000), anti-t-mTOR (diluted 1:1000), and anti-*p*-mTOR (diluted 1:1000) for 2 h at room temperature. We used goat anti-rabbit immunoglobulin G horseradish peroxidase (diluted 1:2000) and rabbit anti-goat immunoglobulin G horseradish peroxidase (Cappel, West Chester, PA; diluted 1:500) in blocking buffer as secondary antibodies. We detected antigens on the membrane using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). We measured the intensity of each band using NIH image (NIH, Bethesda, MD) and calculated the relative protein levels normalized to that of β-actin.

2.6. *Inhibition of Annexin II expression using short interfering RNA (siRNA) in vitro*

We performed the specific inhibition of Annexin II expression as described previously [8]. We used siRNA that specifically targeted Annexin II mRNA (Anx2 siRNA) to reduce Annexin II expression. The target sequences for Annexin II RNA interference were composed of a duplex of siRNA: 5′-GUUACAGCCC UUAUGACAUTT-3′ and 5′-AUGUCAUAAGGGCUGUAACTT-3′. We purchased double-stranded synthetic Anx2 siRNA and luciferase (GL2) siRNA, as a negative control, from Sigma Aldrich (Tokyo, Japan) and Qiagen (Tokyo, Japan), respectively. We performed *in vitro* transfection using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

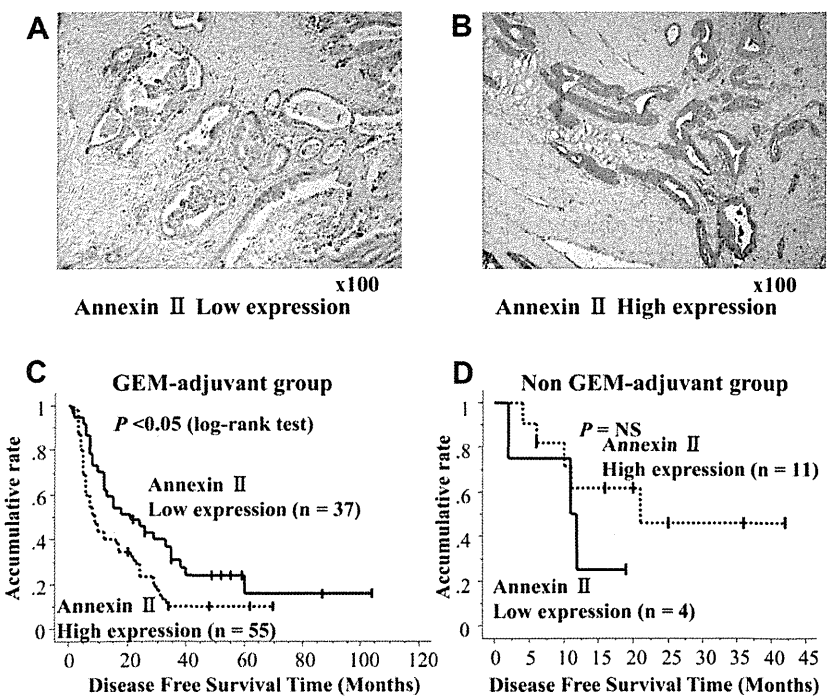


Fig. 1 – Annexin II expression level in pancreatic cancer tissue correlated with disease-free survival after gemcitabine adjuvant chemotherapy. (A, B) Representative findings of Annexin II immunohistochemistry in pancreatic cancer tissues. Microscopic magnification at ×100. Low Annexin II expression (A). High Annexin II expression (B). (C, D) Disease-free survival of patients administered gemcitabine as the adjuvant therapy (GEM-adjuvant group) and those without adjuvant therapy (non GEM-adjuvant group). Disease-free survival was shorter in patients showing a high expression of Annexin II than in those showing a low expression ($P < 0.05$, log-rank test) in the GEM-adjuvant group (C) but not in the non GEM-adjuvant group (D). We estimated disease-free survival using the Kaplan-Meier method and determined the difference between curves using the log-rank test. (Color version of figure is available online.)

Table 1 – Characteristics of 92 pancreatic cancer patients with gemcitabine treatment in IHC analysis.

	Total (92)	Annexin II-IHC staining		P
		Low expression (37)	High expression (55)	
Age (y)	64.8 ± 9.3	64.3 ± 7.7	65.1 ± 10.3	NS
Sex (M/F)	50/42	21/16	29/26	NS
UICC stage				NS
IA	1	0	1	
IB	2	2	0	
IIA	20	10	10	
IIB	64	25	39	
III	2	0	2	
IV	3	0	3	
Resection status				NS
R0	71	27	44	
R1	21	10	11	
Histology				NS
Tubular adenocarcinoma				
Well-differentiated	12	5	7	
Moderately differentiated	56	23	33	
Poorly differentiated	15	6	9	
Papillary	2	1	1	
IPMC (invasive)	4	1	3	
Anaplastic carcinoma	2	1	1	
Adenosquamous carcinoma	1	0	1	

UICC = International Union Against Cancer; NS = not significant; IPMC = intraductal papillary-mutinous carcinoma; R0 = microscopic margin free; R1 = macroscopic margin free.

2.7. Cell cytotoxicity assay

We plated WT- and GEM-MIA PaCa-2 cells at 5×10^3 cells/well in 96-well plates in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and incubated them for 48 h. For cytotoxicity assay, after we changed the medium with gemcitabine (50 ng/mL) and/or rapamycin (50 nmol/L), we incubated the cells for 72 h. We quantified the number of viable cells by colorimetric cell proliferation assay using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. We performed all experiments in triplicate, four independent times.

2.8. Statistical analysis

We applied Student's t-test for statistical analysis of comparative data. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Patients with Annexin II overexpression in pancreatic cancer are significantly associated with rapid recurrence

We first examined Annexin II staining of pancreatic cancer tissues by immunohistochemistry to confirm our previous result showing that Annexin II expression level is correlated with gemcitabine efficacy [8]. We performed immunohistochemical analysis on 107 cancer tissues from patients with curatively resected pancreatic cancer. We treated most of these patients (n = 92) with gemcitabine as the adjuvant chemotherapy after operation (GEM-adjuvant); the other 15 patients, including eight who treated with S-1 as the adjuvant therapy, did not receive gemcitabine adjuvant therapy (non

Table 2 – Uni- and Multivariate disease free survival analysis in patients with gemcitabine treatment.

Variables	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% confidence interval	P	Hazard ratio	95% confidence interval	P
Age (>64 y/≤ 63 y)	1.201	0.762–1.894	NS			
Sex (F/M)	1.200	0.766–1.879	NS			
Tumor location (Head/body-tail)	0.561	0.328–0.959	<0.05	0.586	0.343–1.001	NS
UICC stage (IA, IB, IIA/IIB, III, IV)	0.434	0.246–0.766	<0.005	0.045	0.006–0.351	<0.005
N (–/+)	0.492	0.282–0.857	<0.05	9.658	1.323–70.482	<0.05
Tumor differentiation (Poorly/well-moderately)	1.595	0.857–2.967	NS			
Annexin II-IHC staining (high/low)	1.787	1.117–2.860	<0.05	1.802	1.121–2.896	<0.05
Resection status (R0/1)	0.775	0.456–1.317	NS			

N = lymph node status; R0 = histologically tumor-free surgical margins; R1 = macroscopically tumor-free surgical margins; NS = not significant.

A **Three cases of repeat pancreatectomy after gemcitabine adjuvant chemotherapy**

Case	Age	Sex	DFS after initial operation (months)	Histology (initial / second)	GEM total amount (g)	Annexin II expression (initial / second)
1	68	F	38	IPMC (invasive) / tubular (mod)	91.2	+ / ++
2	76	F	40	tubular (well-mod) / tubular (mod)	13.9	- / +++
3	62	F	29	tubular (mod) / tubular (mod)	79.0	- / ++

DFS, disease free survival; IPMC, intraductal papillary-mucinous carcinoma; tubular, tubular adenocarcinoma; well, well differentiated type; mod, moderately differentiated type; -, no expression; +, weak expression; ++, strong expression; +++, very strong expression.

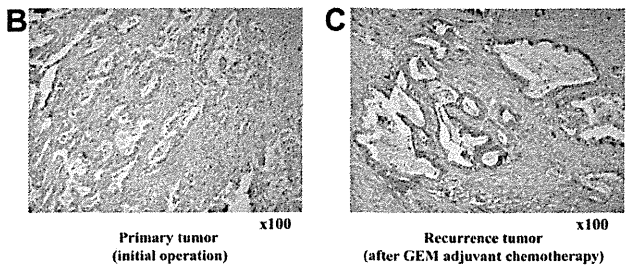


Fig. 2 – Annexin II expression is increased in recurrent pancreatic cancer after gemcitabine adjuvant chemotherapy. (A) The backgrounds of three patients who underwent repeated pancreatectomy for recurrent cancer in remnant pancreas. Annexin II expression is increased in recurrent cancer tissues compared with that in primary tumor in all three patients. (B, C) Representative images of Annexin II immunohistochemistry in primary (B) and recurrent (C) pancreatic cancer tissues after gemcitabine adjuvant chemotherapy (Case 3 in [A]). (Color version of figure is available online.)

GEM-adjuvant). There were no statistical differences in disease-free and overall survival between patients in GEM-adjuvant and non GEM-adjuvant group (data not shown); this result may have been because of adjuvant therapy with S-1, which showed the same level of anti-tumor effect with gemcitabine [14]. We analyzed Annexin II expression and evaluated it as low or high (Fig. 1A and B). We observe a high level of expression of Annexin II in 55 of 92 patients (59.8%) in the GEM-adjuvant group and 11 of 15 patients (73.3%) in the non GEM-adjuvant group; the Annexin II expression pattern was not significantly different between groups (data not shown).

We next focused on patients treated with gemcitabine as the adjuvant therapy, to analyze whether Annexin II expression level is correlated with the efficacy of this drug. Because many patients in GEM-adjuvant group received other chemotherapy after recurrence, we focused on the disease-free survival of these patients to analyze the efficacy of gemcitabine. Kaplan-Meier analysis showed that a high expression of Annexin II was significantly associated with rapid recurrence ($P < 0.05$, log-rank test) (Fig. 1C). Median disease-free survival was 9 mo for patients characterized with a high expression and 21 mo for those characterized with a low expression of Annexin II. Table 1 lists the characteristics of patients with pancreatic cancer in the GEM-adjuvant group; we found no significant differences in patient background between those in the low and high Annexin II expression groups. Univariate and multivariate analysis showed that the Annexin II expression level was an independent prognostic factor for disease-free survival in these patients (Table 2). In contrast, Kaplan-Meier analysis of patients in the non GEM-adjuvant group revealed no correlation between Annexin II expression level and disease-free survival time (Fig. 1D).

3.2. *Annexin II expression is increased in recurrent cancer tissues of patients administered GEM as adjuvant chemotherapy*

Of the clinical cases, three patients in the GEM-adjuvant group underwent a second operation for recurrent pancreatic cancer in remnant pancreas after gemcitabine adjuvant chemotherapy. Fig. 2A shows the characteristics and clinical data of these three patients. We analyzed changes in the expression level of Annexin II *in situ* after long-term use of gemcitabine in these patients. Fig. 2B and C (case 3) shows representative results of Annexin II immunohistochemistry. In the primary tumor resected at the initial operation, we observed weak and faint Annexin II expression, which was increased in the recurrent tumor compared with that in the primary tumor (Fig. 2C). We saw this increase in Annexin II expression in the recurrent tumor in all three patients (Fig. 2A). Thus, Annexin II expression may gradually increase in recurrent cancer tissue under exposure to gemcitabine.

3.3. *Akt signal is highly activated in gemcitabine-resistant pancreatic cancer cells*

We next investigated the molecular mechanisms of resistance against gemcitabine induced by Annexin II overexpression. We analyzed the signaling pathways up-regulated in the gemcitabine-resistant cell line, which overexpresses Annexin II, compared with the wild-type cell line. Using the Bio-Plex phosphorylation protein assay system, we compared the expression levels of 12 phosphorylated and eight total proteins (see details in Materials and Methods) in WT- and GEM-MIA PaCa-2 cells. Among these proteins, the Akt signal

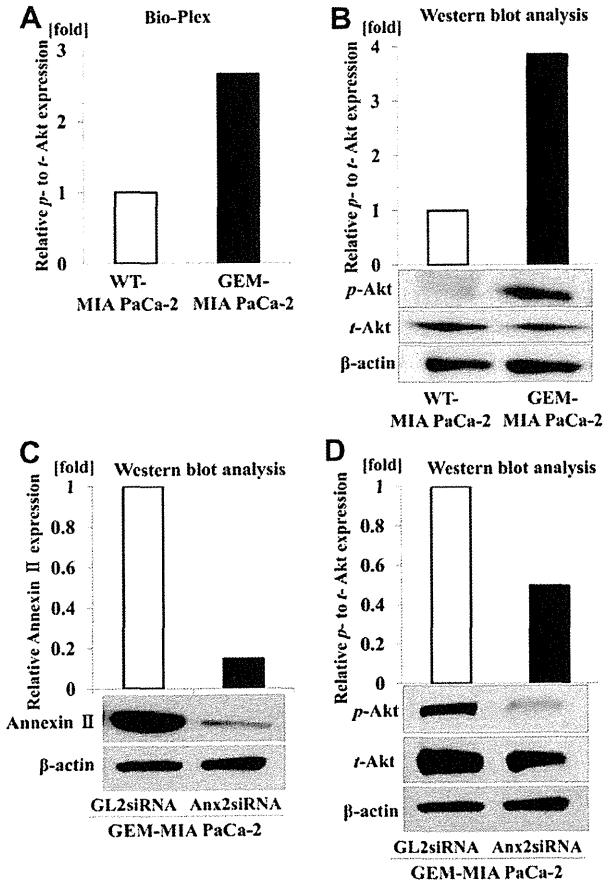


Fig. 3 – Phosphorylation of the Akt signal is up-regulated in gemcitabine-resistant pancreatic cancer cells expressing Annexin II. (A) The relative phosphorylated (*p*)– and total (*t*)-Akt expression is 2.5-fold higher in GEM-MIA PaCa-2 cells than in WT-MIA PaCa-2 cells, as detected by Bio-Plex phosphorylation protein assay analysis. (B) Western blot analysis confirms up-regulation of Akt phosphorylation in GEM-MIA PaCa-2 cells. The relative *p*- and t-Akt expression in GEM-MIA PaCa-2 cells is approximately 3.9-fold higher than in WT-MIA PaCa-2 cells. (C) Anx2 siRNA inhibits Annexin II expression in GEM-MIA PaCa-2 cells. Western blot analysis of Annexin II and β-actin in GEM-MIA PaCa-2 cells treated with GL2 siRNA as the negative control or Anx2 siRNA. (D) The relative *p*- and t-Akt expression is suppressed in Annexin II-inhibited GEM-MIA PaCa-2 cells. Western blot analysis of *p*- and t-Akt, and β-actin as the control, in GEM-MIA PaCa-2 cells treated with GL2 siRNA or Anx2 siRNA. The intensity of each band is measured and the relative protein levels normalized to that of β-actin are calculated (B–D).

was highly activated in GEM-MIA PaCa-2 cells. The relative *p*- to *t*-Akt expression in GEM-MIA PaCa-2 cells was 2.7-fold higher than that in WT-MIA PaCa-2 cells (Fig. 3A). We confirmed this increase in Akt phosphorylation in GEM-MIA PaCa-2 cells by Western blot analysis (Fig. 3B).

Based on these results, we examined whether inhibition of Annexin II expression by siRNA affects Akt signal activation. Western blot analysis confirmed that Annexin II expression was effectively inhibited by transfection of Anx2 siRNA (Fig. 3C). Gene knockdown by the Anx2 siRNA resulted in the suppression of Akt signal activation (down-regulation of relative *p*- to t-Akt expression) in GEM-MIA PaCa-2 cells (Fig. 3D). These results suggest that Annexin II overexpression in gemcitabine-resistant cells is associated with cellular signaling activation of the Akt pathway *in vitro*.

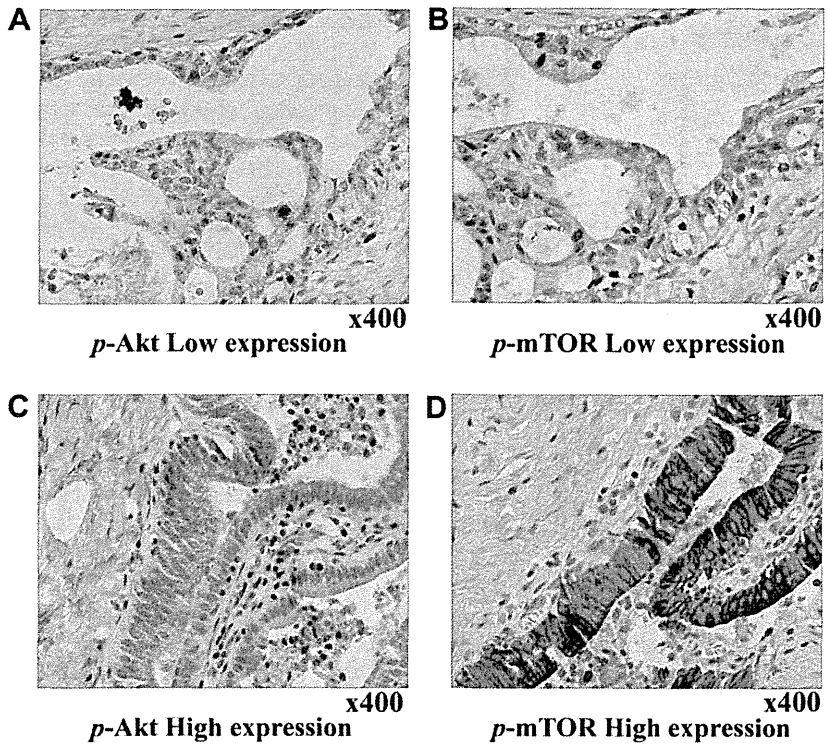
3.4. *Phosphorylation levels of Akt and mTOR are significantly correlated in pancreatic cancer tissues*

In all 92 patients in the GEM-adjuvant group, we analyzed expression levels of activated Akt and mTOR (*p*-Akt and *p*-mTOR) by immunohistochemistry. We mainly observed expression of both *p*-Akt and *p*-mTOR in the cytoplasm of cancer cells (Fig. 4A–D). We observed strong staining (i.e., high expression) of *p*-Akt and *p*-mTOR in 38 (41.3%) and 43 (46.7%) cases, respectively. Notably, high expression of *p*-Akt was significantly associated with that of *p*-mTOR in serial sections of the same specimen ($P = 0.0005$) (Fig. 4E).

3.5. *Inhibition of mTOR activation recovers sensitivity to gemcitabine in GEM-MIA PaCa-2 cells*

To confirm whether mTOR activation is correlated with gemcitabine resistance, we treated GEM-MIA PaCa-2 cells with the mTOR inhibitor, rapamycin. We first confirmed gemcitabine resistance in GEM-MIA PaCa-2 cells, which showed less toxicity against gemcitabine compared with WT-MIA PaCa-2 cells. We maximized the difference of this cytotoxicity between GEM- and WT-MIA PaCa-2 cells with 50 ng/mL gemcitabine (Fig. 5A). According to this result, we used gemcitabine with 50 ng/mL concentration in the following examination. We also confirmed the inhibition of m-TOR activation by rapamycin. Western blot analysis of *p*- and t-mTOR in WT- and GEM-MIA PaCa-2 cells exposed to rapamycin showed that rapamycin effectively inhibits the activation of mTOR (relative *p*- to t-mTOR expression) in both WT- and GEM-MIA PaCa-2 cells (Fig. 5B).

We next examined whether the inhibition of mTOR phosphorylation affects sensitivity to gemcitabine. As shown in Fig. 5A, gemcitabine treatment showed more cytotoxicity in WT- than in GEM-MIA PaCa-2 cells (Fig. 5C, second and sixth columns). Moreover, WT-MIA PaCa-2 cells showed limited cytotoxicity to treatment of rapamycin alone (Fig. 5C, third column) and no additional cytotoxicity to the combined treatment of gemcitabine and rapamycin, compared with cells treated with gemcitabine alone (Fig. 5C, second and fourth columns). Treatment of rapamycin alone showed the same limited cytotoxicity to GEM-MIA PaCa-2 cells as that to WT-MIA PaCa-2 cells (Fig. 5C, third and seventh columns). Interestingly, the combination of rapamycin and gemcitabine showed increased cytotoxicity compared with gemcitabine treatment in GEM-MIA PaCa-2 cells (Fig. 5C, sixth and eighth columns). This additional effect of rapamycin in gemcitabine cytotoxicity reached a maximum at only the 25-nmol/L concentration (Fig. 5D). This cytotoxicity of the combination of rapamycin and gemcitabine in GEM-MIA PaCa-2 cells was almost at same level as that in gemcitabine-treated WT-MIA



E Relationship between *p*-Akt and *p*-mTOR expression.

		p-mTOR		
		High (n = 43)	Low (n = 49)	*P*
p-Akt	High (n = 38)	26	12	0.0005
	Low (n = 54)	17	37	

Fig. 4 – *p*-Akt and *p*-mTOR expression in pancreatic cancer are well correlated. (A–D) Representative immunohistochemical staining of *p*-Akt and *p*-mTOR in pancreatic cancer tissues. Low expression of *p*-Akt (A) and *p*-mTOR (B). High expression of *p*-Akt (C) and *p*-mTOR (D). Microscopic magnification at ×400. (E) There is a significant correlation between the expression levels of *p*-Akt and *p*-mTOR in pancreatic cancer tissue (P = 0.0005). (Color version of figure is available online.)

PaCa-2 cells (Fig. 5C, second and eighth columns), which suggests cancellation of gemcitabine resistance in GEM-MIA PaCa-2 cells by rapamycin.

4. Discussion

Although gemcitabine has led to important progress in pancreatic cancer treatment in the past decade, the efficacy of this reagent is still limited. Thus, elucidation of the mechanisms underlying gemcitabine resistance is important.

Using a proteomic approach, we previously demonstrated that Annexin II is the important factor of gemcitabine resistance. Chuthapisith *et al* [10] showed that Annexin II is involved in chemoresistance to breast cancer. They reported 20 proteins including Annexin II with altered expression in both Adriamycin- and paclitaxel-resistant cells using two-dimensional gel electrophoresis and Matrix Assisted Laser Desorption and Ionization time-of (MALDI-TOF) peptide mass fingerprinting. They also reported that Annexin II positivity correlated with a poor pathological response of neoadjuvant chemotherapy in large or locally advanced breast cancer [11]. Annexin II was thus thought to induce chemoresistance through interruption of the apoptotic pathway, including p53 [15].

Vishwanatha *et al* [16] first indicated that Annexin II mRNA and protein expression are up-regulated in pancreatic cancer tissues and cell lines. Immunocytochemical analysis of co-localization of Annexin II and proliferating cell nuclear antigen further suggested that Annexin II has a role in pancreatic cancer cell proliferation. In this study, we confirmed through Annexin II immunohistochemical staining of pancreatic cancer tissues that high Annexin II expression is correlated with short disease-free survival for patients who receive gemcitabine adjuvant chemotherapy after surgery. On the other hand, for patients who do not receive gemcitabine adjuvant therapy, we found no correlation between Annexin II expression in cancer cells and disease-free survival. Together with the fact that more than half of patients with non

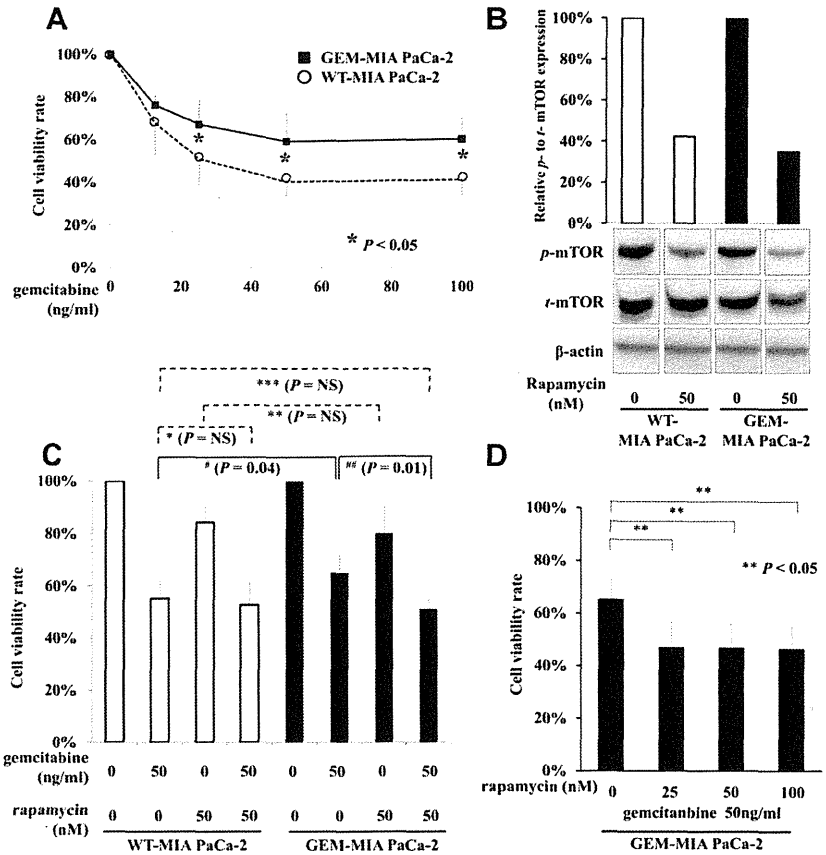


Fig. 5 – Gemcitabine and rapamycin combined therapy recovers gemcitabine sensitivity in GEM-MIA PaCa-2 cells. (A) Gemcitabine is less cytotoxic in GEM-MIA PaCa-2 cells than in WT-MIA PaCa-2 cells. Each point shows the relative number of viable cells treated with gemcitabine in indicated concentrations compared with the number of untreated cells. The asterisk indicates the difference between GEM- and WT-MIA PaCa-2 cells in each gemcitabine concentration. (B) mTOR phosphorylation is inhibited by rapamycin in both WT- and GEM-MIA PaCa-2 cells. Western blot analysis of p- and t-mTOR, and β-actin as the control. The intensity of each band is measured and the relative protein levels normalized to that of β-actin are calculated. (C) The relative number of viable cells compared with the number of untreated cells. More GEM-MIA PaCa-2 cells survived than WT-MIA PaCa-2 cells treated with 50 ng/mL gemcitabine. Rapamycin treatment cancelled the gemcitabine-resistant state of GEM-MIA PaCa-2 cells, returning to the same levels of WT-MIA PaCa-2 cells. Each column and bar shows the mean ± SD. NS = not significant. (D) Cell toxicity of combination treatment with 50 ng/mL gemcitabine and rapamycin of indicated concentrations in GEM-MIA PaCa-2 cells. Cell viability rate is calculated as relative cell number of each treatement compared with cell number without gemcitabine and rapamycin treatment.

GEM-adjuvant group received S-1 adjuvant therapy, this finding confirms the specific role of Annexin II in gemcitabine resistance to pancreatic cancer.

Ortiz-Zapater *et al* [17] identified that t-PA-related signaling pathways in pancreatic cancer are mediated by epidermal growth factor receptor and Annexin II. This signaling pathway activates the downstream proteins of ERK1/2, which suggests the possibility that Annexin II-related gemcitabine resistance involves proliferative signaling transduction. To elucidate the molecular mechanisms of gemcitabine resistance in pancreatic cancer cells, we investigated the signaling pathways that correlate with Annexin II. We found more activation of the Akt signal in gemcitabine-resistant cells, which strongly express Annexin II, than in wild-type cells. The PI3K/Akt pathway is a major signaling pathway involved in oncogenesis in many types of malignancies. Ng *et al* [18] reported that the combination of a PI3K inhibitor with gemcitabine may have therapeutic potential in pancreatic cancer cells, and that the PI3K/Akt pathway is constitutively activated in most human pancreatic cancer cell lines [19]. Further evidence suggests that this pathway is activated in pancreatic cancer [20–22].

We revealed that both Akt and its downstream protein, mTOR, are simultaneously activated in pancreatic cancer tissues. mTOR is one of the effectors regulated via the PI3K/Akt pathway and has a central role in cell survival and proliferation [23]. The mTOR inhibitor rapamycin and its structurally related compounds CCI-779, RAD001, and AP23573 are currently in clinical development for use as molecular targeting cancer therapies [24]. Our *in vitro* study showed that the gemcitabine sensitivity of a gemcitabine-resistant cell line, expressing a high level of Annexin II, recovered to the same level as that of the wild-type cell line

using rapamycin, which indicates that the Akt/mTOR pathway is involved in the mechanism of gemcitabine resistance by Annexin II.

This finding is supported by several similar reports that show anti-tumor effects of mTOR inhibitor and gemcitabine combination therapy in pancreatic cancer [25,26]. On the other hand, Wolpin *et al* [27] reported no clinical benefits of everolimus, another mTOR inhibitor, for patients with advanced, gemcitabine-refractory pancreatic cancer. They considered the insufficient activity of everolimus to result from the presence of a negative feedback loop. That is, inhibition of mTOR leads to an increase in Akt phosphorylation and activation of other Akt target proteins that promote cell survival [28,29]. Our *in vitro* results also suggest insufficient activity of the mTOR inhibitor on pancreatic cancer, because rapamycin alone showed only limited toxicity to both wild-type and gemcitabine-resistant cells. Taken together, our *in vitro* study shows that both gemcitabine and rapamycin in combined therapy may be effective for patients with gemcitabine-resistant pancreatic cancer.

Annexin II could be a good clinical indicator for early recurrence of pancreatic cancer treated with gemcitabine as adjuvant chemotherapy. The Akt-mTOR pathway is involved in the mechanisms of gemcitabine resistance by Annexin II, and mTOR inhibition recovers the sensitivity of resistance. Thus, analysis of the Annexin II expression level in pancreatic cancer tissue may be useful for selecting the chemotherapeutic reagent and may contribute to the establishment of a novel tailor-made therapy for patients with pancreatic cancer. Moreover, combination therapy with the mTOR inhibitor may increase the cell toxic effect of gemcitabine on pancreatic cancer. To confirm this, further clinical prospective studies are needed.

Acknowledgments

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ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

Surgical Resection after Downsizing Chemotherapy for Initially Unresectable Locally Advanced Biliary Tract Cancer: A Retrospective Single-center Study

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ABSTRACT

Background. Surgical resection is the only method for curative treatment of biliary tract cancer (BTC). Recently, an improved efficacy has been revealed in patients with initially unresectable locally advanced BTC to improve the prognosis by the advent of useful cancer chemotherapy. The aim of this study was to evaluate the effect of downsizing chemotherapy in patients with initially unresectable locally advanced BTC.

Methods. Initially unresectable locally advanced cases were defined as those in which therapeutic resection could not be achieved even by proactive surgical resection. Gemcitabine was administered intravenously once a week for 3 weeks followed by 1 week's respite. Patients whose disease responded to chemotherapy were reevaluated to determine whether their tumor was resectable.

Results. Chemotherapy with gemcitabine was provided to 22 patients with initially unresectable locally advanced BTC. Tumor was significantly downsized in nine patients, and surgical resection was performed in 8 (36.4%) of 22 patients. Surgical resection resulted in R0 resection in four patients and R1 resection in four patients. Patients who underwent surgical resection had a significantly longer survival compared with those unable to undergo surgery.

Conclusions. Preoperative chemotherapy enables the downsizing of initially unresectable locally advanced BTC, with radical resection made possible in a certain proportion of patients. Downsizing chemotherapy should be proactively carried out as a multidisciplinary treatment strategy for patients with initially unresectable locally advanced BTC with the aim of expanding the surgical indication.

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Surgical resection is the only method for curative treatment of biliary tract cancer (BTC). This condition has few subjective symptoms in its initial stage, and early diagnosis is difficult despite current advances in diagnostic imaging technology. This means that most cases are discovered at an advanced stage and curative resection is possible in only a limited number of cases, making the prognosis for this disorder extremely bleak.[1–4] Although cancer chemotherapy has been generally considered as the first-choice treatment for unresectable BTC, BTC has been reported to be highly resistant to antineoplastic treatment until the recent advent of effective chemotherapeutic agents. However, at the start of the 21st century, numerous reports have shown effectiveness of gemcitabine for BTC, and this has become accepted as the standard therapeutic agent for advanced unresectable BTC.[5–7]

Downsizing chemotherapy and subsequent surgical resection has been already reported as an effective new approach in multidisciplinary therapy for colorectal cancer hepatic metastases and pancreatic cancer.[8–10] Since the arrival of gemcitabine, an improved efficacy has been revealed in patients with initially unresectable locally advanced BTC to improve the prognosis. Therefore, downsizing chemotherapy and subsequent surgical resection in cases of initially unresectable localized BTC with no distal metastases may be a considerable possible therapeutic new strategy.

To the best of our knowledge, there have been no previous reports of the utility of preoperative downsizing

chemotherapy for BTC, with the present study representing the first. We here report on the feasibility of surgical resection in patients who have undergone downsizing chemotherapy with gemcitabine for initially unresectable locally advanced BTC.

PATIENTS AND METHODS

Determination of Patient Eligibility

This study comprised a retrospective chart review of treatment performed at Chiba University Hospital. All patients underwent pulmonary computed tomography (CT), bone scintigraphy, and positron emission tomography to confirm the absence of distal metastases. Thus, patients with Union for International Cancer Control stage IVB disease was excluded. Each of the cases was discussed in a multidisciplinary setting. Initially unresectable locally advanced cases were defined as those in which surgical resection could not be achieved even by aggressive surgical procedure, including combined vascular resection. In practice, this referred to (1) local invasion of the hepatic artery to be unable to reconstruct; (2) local invasion of the portal vein to be unable to reconstruct; (3) local invasion of the hepatic vein to be unable to reconstruct; (4) extensive infiltration of the bile duct to be unable to achieve a curative resection; and (5) extensive hepatic invasion to be unable to excise due to insufficient remnant liver volume even after portal vein embolization (Table 1). Subjects had Eastern Cooperative Oncology Group performance status values of 0 or 1, no active infection, no major cardiac or cerebrovascular disorders, and no active malignant tumors other than BTC. Patients who had previously undergone chemotherapy and/or radiotherapy to treat BTC were excluded.

In terms of blood biochemistry findings, chemotherapy administration criteria comprised leukocytes $\geq$3,000/mm^3, hemoglobin $\geq$9.0 g/l, platelets $\geq 10 \times 10^4$, creatinine $\leq$1.5 g/dl, and total bilirubin, AST, and ALT less than three times the upper limit of normal. Preoperative biliary drainage was performed for patients with obstructive jaundice, and surgery was carried out after serum total bilirubin levels had dropped to $\leq$3.0 mg/dl. When hepatic resection was required in order to perform curative resection in patients with obstructive jaundice, portal vein embolization was carried out in cases in which the remnant liver volume ratio was <40%, and surgical resection including hepatectomy was performed after the remnant liver volume ratio had reached $\geq$40%.[11] In patients with normal liver function, portal vein embolization was indicated when the anticipated future remnant liver volume ratio was <35%.

TABLE 1 Definition of initially unresectable locally advanced biliary tract cancer

- Local invasion of the hepatic artery to be unable to reconstruct.
- Local invasion of the portal vein to be unable to reconstruct.
- Local invasion of the hepatic vein to be unable to reconstruct.
- Extensive infiltration of the bile duct to be unable to achieved a curative resection.
- Extensive hepatic invasion to be unable to excise due to insufficient remnant liver volume even after portal vein embolization.

Chemotherapy Schedule and Evaluation of Response

Gemcitabine (1,000 mg/m^3) was administered intravenously once a week for 3 weeks followed by 1 week's respite, with a single course comprising 4 weeks. Toxicity was evaluated according to the National Cancer Institute Common Terminology Criteria for adverse events version 3.0. If toxicity of grade 3 or above was observed, administration was immediately discontinued and resumed after recovery to normal values with the gemcitabine dosage reduced to 800 mg/m^3. If toxicity was still observed, the dose was further reduced to 600 mg/m^3.[12]

At the end of every two courses of administration, the response was evaluated by enhanced CT on the basis of Response Evaluation Criteria in Solid Tumors (RECIST) group criteria and also evaluated whether tumor was downsized to be sufficient for surgical resection. Patients who were not indicated for surgical resection at that point and who were evaluated as having no change in their tumor continued with gemcitabine administration, whereas those with progressive disease were switched to S-1 (tegafur–gimeracil–oteracil) chemotherapy agents.

Data Analysis

The survival curve was calculated according to the Kaplan–Meier method, and a log rank test was used to test for significant differences. A P value of less than 0.05 was regarded as statistically significant.

RESULTS

From January 2004 to December 2010, total 391 patients with BTC were referred to our hospital (Fig. 1). Of these 391 patients, 266 patients underwent laparotomy. Among these 266 patients, 17 patients judged unresectable on the basis of distant metastases in 16 (peritoneal dissemination in ten and distant lymph node metastases in six) and locally advanced disease in one. Thus, 249 patients judged resectable and underwent surgical resection. The other 125 patients were considered unresectable according to preoperative examination (distant metastases in 96 and locally advanced disease in 29). Of these 29 patients with locally

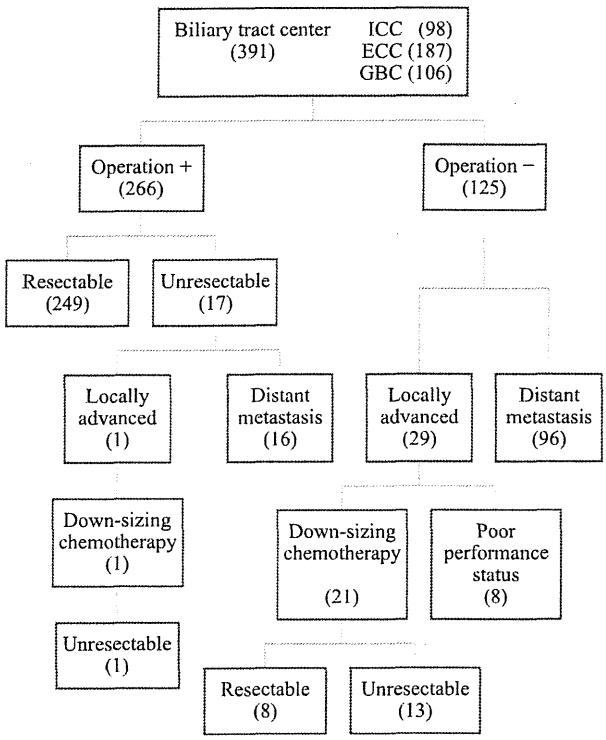

FIG. 1 Treatment schema and outcome for the patients with biliary tract cancer. Numbers in *parentheses* are numbers of patients. *ICC* intrahepatic cholangiocarcinoma, *ECC* extrahepatic cholangiocarcinoma, *GBC* gallbladder carcinoma

advanced disease, eight could not undergo downsizing chemotherapy as a result of poor performance status. Finally, 22 patients represent the study population with downsizing chemotherapy. Their ages ranged from 57 to 85 years, with an average of 65.3 years, and there were 12 men and 10 women. The final diagnosis was intrahepatic cholangiocarcinoma (ICC) in seven patients, extrahepatic cholangiocarcinoma (ECC) in eight, and gallbladder carcinoma (GBC) in seven (Table 2). Preoperative biliary drainage was performed in 14 (63.6%) of 22 patients with obstructive jaundice, with percutaneous transhepatic biliary drainage in nine cases and endoscopic retrograde biliary drainage in five. Biliary drainage was performed in one of seven patients with ICC, all eight patients with ECC, and five of seven patients with GBC. Chemotherapy was initiated in these patients with obstructive jaundice after serum total bilirubin levels had decreased to $\leq$3.0 mg/dl.

According to our definition of initially unresectable locally advanced BTC, the reason for unresectability was local invasion of the hepatic artery in 18 patients, local invasion of the portal vein in seven, local invasion of the hepatic vein in two, extensive infiltration of the bile duct in three, and insufficient remnant liver volume in two. Ten patients had more than one reason for unresectability. Three patients (patients 5, 6, and 21) with insufficient remnant liver volume underwent portal vein embolization and started chemotherapy 2 weeks later. Among three patients who underwent portal vein embolization, two patients could undergo surgical resection because the remnant liver volume was sufficient. One patient could not undergo surgical resection because of insufficient remnant liver volume even after portal vein embolization. One patient with insufficient remnant liver volume did not undergo portal vein embolization because of multiple bilobular hepatic metastases in ICC (patient four).

Response was evaluated every two courses, tumor was significantly downsizing in nine patients. On the basis of the RECIST criteria, three patients had a partial response, 11 had stable disease, and eight had progressive disease. Obvious reduction of tumor size, including disappearance of invasion of the hepatic vein and hepatic artery, was seen in eight patients with downsizing tumor, and surgical resection was judged to be feasible in these patients.

Surgical resection was performed in 8 (36.7%) of 22 patients after obtaining tumor downsizing by chemotherapy. Preoperative biliary drainage was performed in 3 (37.5%) of eight patients who underwent surgical resection. The period from the start of chemotherapy to surgery in these eight patients was 5.4 $\pm$ 2.3 months (mean $\pm$ standard deviation), and the dose of gemcitabine administered was 21,400 $\pm$ 12,000 mg (mean $\pm$ standard deviation). Hepatectomy procedures were selected left trisectionectomy in two, left hemihepatectomy with caudate lobectomy in two, right hemihepatectomy with caudate lobectomy in two, and central inferior hepatectomy in two patients. Extrahepatic bile duct resection was performed in seven patients. Combined portal vein resection and inferior vena cava resections were performed in three patients each. After surgery, cholangiojejunal anastomotic failure occurred in one and intraperitoneal abscess in two patients. However, all patients who underwent surgery were discharged from hospital, with a surgical mortality rate of zero (Table 3).

No macroscopic residual tumor (R2) was observed in any of the patients who underwent surgical resection, with four patients classified as R0 and four as R1. Gemcitabine was administered to all patients who underwent surgical resection as postoperative adjuvant chemotherapy. Two of eight patients who underwent surgical resection have now survived for 42 and 13 months, respectively, without recurrence. Although four patients with recurrence died of cancer, two patients with recurrence are still alive after switching to chemotherapy with S-1. The 2 years overall survival rate in patients with surgical resection after downsizing chemotherapy and chemotherapy alone without surgical resection were 45.0 and 19.0%, respectively. Patients who underwent surgery survived significantly longer after the induction of chemotherapy compared with

TABLE 2 Patient characteristics of initially unresectable locally advanced biliary tract cancer

Patient no.	Age (years)	Sex	Diagnosis	Reasons for unresectability	Biliary drainage	Chemotherapy	Downsizing (RECIST)	Operation	Status	Survival (months)
1	60	F	ICC	Hepatic vein invasion	No	GEM	Yes (SD)	Yes	Alive	66
2	60	M	ICC	Hepatic vein invasion	No	GEM	Yes (PR)	Yes	Alive	44
3	67	M	ICC	Arterial invasion	Yes	GEM	Yes (SD)	Yes	Dead	10
4	72	M	ICC	Insufficient remnant liver volume	No	GEM	Yes (PR)	Yes	Alive	13
5	57	F	GBC	Arterial invasion	Yes	GEM	Yes (SD)	Yes	Alive	42
6	57	F	GBC	Arterial invasion	Yes	GEM	Yes (PR)	Yes	Dead	18
7	57	F	GBC	Arterial invasion Portal vein invasion	No	GEM	Yes (SD)	Yes	Dead	19
8	61	M	GBC	Arterial invasion	No	GEM	Yes (SD)	Yes	Dead	8
9	57	M	ICC	Arterial invasion	No	GEM	No (PD)	No	Dead	11
10	77	F	ICC	Arterial invasion Portal vein invasion	No	GEM → S-1	No (SD)	No	Alive	13
11	84	F	ICC	Arterial invasion Portal vein invasion	No	GEM	No (SD)	No	Dead	6
12	75	M	ECC	Arterial invasion Broad biliary infiltration	Yes	GEM → S-1	Yes (SD)	No	Dead	30
13	69	F	ECC	Arterial invasion Portal vein invasion	Yes	GEM	No (SD)	No	Alive	27
14	65	M	ECC	Arterial invasion	Yes	GEM	No (PD)	No	Dead	5
15	66	M	ECC	Arterial invasion Portal vein invasion	Yes	GEM → S-1	No (PD)	No	Dead	6
16	81	M	ECC	Arterial invasion	Yes	GEM → S-1	No (SD)	No	Dead	11
17	59	F	ECC	Arterial invasion Portal vein invasion	Yes	GEM	No (PD)	No	Dead	4
18	63	M	ECC	Arterial invasion	Yes	GEM	No (PD)	No	Dead	8
19	65	F	ECC	Arterial invasion	Yes	GEM → S-1	No (SD)	No	Dead	19
20	49	M	GBC	Arterial invasion Broad biliary infiltration	Yes	GEM	No (PD)	No	Dead	9
21	71	M	GBC	Broad biliary infiltration Insufficient remnant liver volume	Yes	GEM	No (PD)	No	Dead	4
22	64	F	GBC	Arterial invasion Portal vein invasion	Yes	GEM	No (PD)	No	Dead	5

ICC intrahepatic cholangiocarcinoma, *GBC* gallbladder carcinoma, *ECC* extrahepatic cholangiocarcinoma, *GEM* gemcitabine, *S-1* tegafur–gimeracil–oteracil, *PR* partial response, *SD* stable disease, *PD* progressive disease

those unable to undergo surgery ($P = 0.032$) (Fig. 2a). The survival rate at 5 years is 40.8% in 249 patients with initially resectable BTC. Similar survival rate was obtained in patients with initially unresectable locally advanced BTC (survival rate at 5 years of 45.0%) after downsizing chemotherapy and subsequent surgical resection (Fig. 2b)

DISCUSSION

Since 1999, when no effective chemotherapy existed for BTC, there have been numerous clinical trials of gemcitabine for unresectable advanced BTC, with comparatively good results reported.[5–7] According to these reports, the response rate ranges 10–60%, with a median survival of 5–14 months. Such dramatic advances in chemotherapy in recent years mean that improved response rates and longer survival periods can be anticipated even in cancers formerly regarded as untreatable in patients with BTC.

In 2004, Adam et al. administered chemotherapy for initially unresectable colorectal cancer liver metastases and performed hepatectomy on patients in whom major response was obtained, reporting good results, with a 5 years survival rate of 33%.[13] Numerous reports of the results of hepatectomy after downsizing for colorectal

TABLE 3 Surgical procedure and histopathologic features of resected patients with biliary tract cancer after downsizing chemotherapy

Patient no.	Diagnosis	Operation methods	Vascular resection	Operation time (min)	Blood loss (g)	T	N	Stage	R
1	ICC	Left trisectionectomy, BDR	IVC	580	1,285	4	1	IVA	0
2	ICC	Left trisectionectomy, BDR	IVC	519	8,900	4	0	IVA	0
3	ICC	Left hemihepatectomy with caudate lobectomy, BDR	IVC, PV	637	1,150	4	1	IVA	1
4	ICC	Left hemihepatectomy with caudate lobectomy	–	590	3,115	4	0	IVA	0
5	GBC	Right hemihepatectomy with caudate lobectomy, BDR	–	400	490	4	1	IVA	0
6	GBC	Right hemihepatectomy with caudate lobectomy, BDR	PV	439	1,080	4	1	IVA	1
7	GBC	Central inferior hepatectomy (S4a + S5), BDR	PV, RHA	404	240	4	1	IVA	1
8	GBC	Central inferior hepatectomy (S4a + S5), BDR	–	499	2,080	4	1	IVA	1

BDR bile duct resection, *IVC* inferior vena cava, *PV* portal vein, *RHA* right hepatic artery

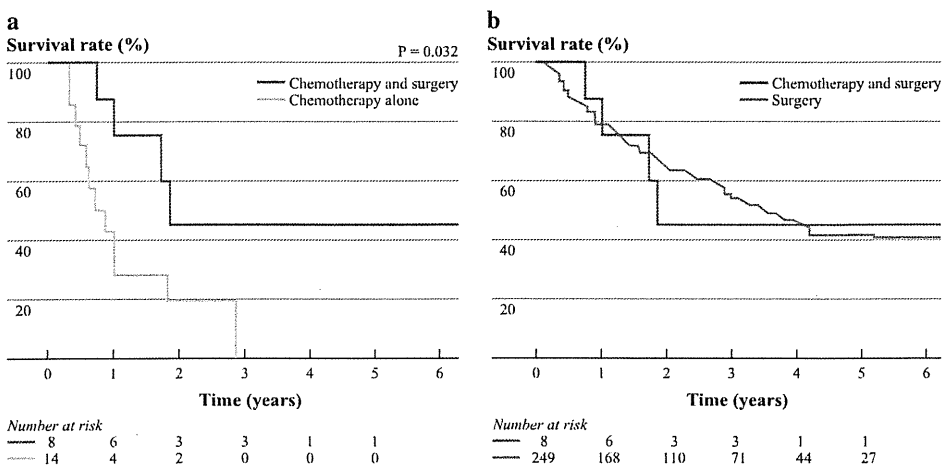

FIG. 2 Survival curves after the induction of chemotherapy for initially unresectable locally advanced BTC. **a** The *black line* represents patients in whom surgical resection was possible after downsizing. The *gray line* represents patients in whom resection was not possible. Median survival time (MST) in patients with surgical resection after downsizing chemotherapy and chemotherapy alone without surgical resection was 19.3 and 7.5 months, respectively; $P = 0.032$ (log rank test). **b** The *solid line* represents patients in whom surgical resection was possible after downsizing. The *dotted* line represents the patients with initially resectable BTC. The survival rate at 5 years did not differ between the patients with initially resectable BTC and initially unresectable locally advanced BTC after surgical resection after downsizing chemotherapy

cancer metastases subsequently appeared from many institutions. According to these reports, the downsized rate at which initially unresectable tumors became resectable was 11–36%.[14–18]

Reported resection rates for initially unresectable pancreatic cancer after downsizing chemotherapy range 8.3–64.2%.[19] The postulated reason for this wide range of resection rates might be due to the different definitions of unresectability at different institutions. The National Comprehensive Cancer Network (NCCN) has produced guidelines for pancreatic cancer with definitions of unresectability and borderline resectability. Although the current NCCN guidelines indicate clear criteria for resectability, there is still scope for debate on the indications of borderline resectability and unresectability.[20] In addition, even when radical resection is performed, surgical resection of pancreatic cancer at high-volume centers tends to comprise active surgical resection including combined vascular resection, and postoperative rates of complications and mortality are also low, with reports accordingly stating that surgical treatment should be carried out at such specialist facilities.[21,22] There are currently

no definitions for unresectability or borderline resectability in BTC, with completely different indications for resection used by different institutions and practitioners. Proactive expansion of surgical indications in high-volume centers lead to curative resection for advanced BTC with vascular invasions and extensive biliary infiltration. Several authors have described combined vascular resection for advanced BTC and have advocated an aggressive surgical strategy. Thus, the reason for unresectability in this paper was defined as local vascular invasion to be unable to reconstruct, extensive infiltration of the bile duct to be unable to achieve a curative resection, and extensive hepatic invasion to be unable to excise due to insufficient liver volume even after portal vein embolization.

In general, securing a clear margin after surgical resection is regarded as an important prognostic factor. Curative resection (R0) is based on the concept of our surgical treatment. We have previously reported that the aggressive combined vascular resection to achieve a curative resection bring about the beneficial effects on the prognosis in the patients with advanced BTC.[23] On the other hands, a number of recent reports have stated that because of advances in multidisciplinary treatment, even resection with microscopic residual tumor at the resected margin (R1) now does not always have the affect on the prognosis or risk of recurrence compared with R0 surgery in colorectal liver metastases and pancreas cancer.[12,13,24] In present study, all patients who underwent R0 surgical resection are currently alive with a mean follow-up of 41 months. Thus, R0 surgical resection should be performed in patients with initially unresectable locally advance BTC even after downsizing chemotherapy. We have compared the survival time after surgical resection between initially unresectable locally advanced BTC and initially resectable BTC. Similar survival rate was obtained between two groups. This result indicated that surgical resection after downsizing chemotherapy might be beneficial to the initially unresectable locally advanced BTC.

Recently, the possibilities of gemcitabine-based multidrug regimens have been investigated, with good results reported for the coadministration of gemcitabine with cisplatin.[25,26] In the United Kingdom, good results were obtained in a randomized phase III trial (ABC-02) of gemcitabine with cisplatin, with a 81.4% tumor control rate in the group given both drugs compared with 71.8% for the single drug.[27] Also in Japan, a randomized, controlled trial of combined therapy with gemcitabine and cisplatin for unresectable BTC compared with gemcitabine alone is underway as a joint study with other institutions. This study was also demonstrating similarly favorable results.[28] The establishment of gemcitabine and cisplatin combined therapy as a standard therapy for unresectable BTC is therefore anticipated. In future the introduction of gemcitabine and cisplatin combination therapy showed evaluated for the effect on preoperation neoadjuvant chemotherapy.

In this series, there was no patient with ECC judged to be resectable after chemotherapy. All patients with ECC underwent biliary drainage, and the tube provoked inflammation in the bile duct and surrounding tissues, making it difficult to reach an accurate diagnosis of the extent of tumor progression by means of CT. This meant that vascular invasion and bile duct invasion may have been overestimated on CT performed after tube insertion, making it difficult to select resectable patients. A new modality of assessment should be required to allow the use of downstaging chemotherapy response in BTC especially in ECC after biliary stent insertion. BTC also encompasses various diseases of ICC, ECC, and GBC with different biological characteristics, and these may also differ in terms of the effectiveness of chemotherapy and prognosis. For this reason, separate study and analyzes might be required for ICC, ECC, and GBC. However, although individual facilities treat limited numbers of BTC patients, large-scale clinical trials, such as multicenter studies, should be done for obtaining the appropriate results.

There are no clear criteria concerning the period for which chemotherapy should be administered preoperatively or the appropriate time for surgery in patients whose disease becomes resectable after chemotherapy. It has been reported that few patients who have developed resistance to a first-line regimen for colorectal cancer liver metastases can undergo resection after switching to a second-line regimen. Although there are few second-line chemotherapy regimens available for BTC, surgical resection might have to be performed immediately when it is determined to be possible.[29] Furthermore, attention must also be paid to chemotherapy-induced liver toxicity because major hepatic resection is usually required for radical surgical resection for advanced BTC. There have been no reports, however, of increased liver toxicity or postoperative complications when gemcitabine is used as preoperative chemotherapy. In our series, there were no cases of liver damage caused by long-term administration of gemcitabine. However, further care should be required when gemcitabine is provided as part of multidrug therapies with agents such as cisplatin.

In conclusion, in this study, preoperative chemotherapy enabled the downsizing of initially unresectable locally advanced BTC, with surgical resection made possible in a certain proportion of patients. Proactive resection can also be anticipated in these patients to bring about an improved survival. Downsizing chemotherapy should be proactively carried out as a new multidisciplinary treatment strategy for the treatment of initially unresectable locally advanced BTC for the aim of expanding the surgical indication.

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Geminin Expression in Pancreatic Neuroendocrine Tumors

Possible New Marker of Malignancy

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Objectives: We evaluated geminin labeling index (LI) as a new prognostic indicator of pancreatic neuroendocrine tumor.

Methods: Twenty-seven patients who underwent surgery were retrospectively referred. Labeling indices for geminin and Ki-67 were calculated and compared with clinicopathologic factors. Then, the concordance of positivity between 2 LIs was evaluated using the color difference quotation.

Results: The median (range) of LIs for geminin and Ki-67 was 1.0% (0.05%–14.9%) and 1.5% (0.02%–8.8%), respectively. When the high LI was defined as more than 2.0% according to the receiver operating characteristic curves determining the metastasis, both geminin LI (hazard ratio [HR], 31.382; 95% confidence interval [CI], 3.177–309.99; $P = 0.003$) and Ki-67 LI (HR, 6.182; 95% CI, 1.221–31.298; $P = 0.028$) were significant risk factors of recurrence in the univariate analysis. The Kaplan-Meier curves consistently exhibited the superiority of geminin LI (log rank, $P < 0.001$) to Ki-67 LI (log rank, $P = 0.041$) in predicting the disease-free survival. In the color difference quotation, the median ΔE of geminin stain (16.12; range, 5.8–41.9) was significantly larger than that of Ki-67 stain (13.17; range, 3.4–37.9).

Conclusions: The geminin LI was suggested to be more closely correlated with outcome and had more consistent positivity than the Ki-67 LI.

Key Words: geminin, neuroendocrine tumor, color difference quotation, prognostic factor, Ki-67

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The annual incidence of pancreatic neuroendocrine tumor (PNET) is about 0.32 cases per population of 100,000 in the United States and 2.23 cases per population of 100,000 in Japan. Pancreatic neuroendocrine tumors are thought to represent 1%–2% of all pancreatic neoplasms. The apparent incidence and prevalence of PNET have increased substantially during the last 30 years, probably because of the rapid progress of innovative diagnostic techniques.[1] The best treatment for PNET is curative surgical resection, which has a disease-free survival rate of 82% after surgery.[2] Pancreatic neuroendocrine tumors have a wide spectrum of clinical presentations. Therefore, multiple studies have attempted to develop staging and grading systems to better define prognosis.[2–5] The 2000 World Health Organization (WHO) classification system used both stage-related criteria (size and presence of metastases) and grade-related criteria (mitotic rate, perineural invasion, angioinvasion, and Ki-67 proliferative index) to predict outcome. Though this approach included most well-accepted pathologic prognostic factors, the multiple grading parameters made it difficult to reproduce grades reliably among pathologists and institutions, and this grading system has since been replaced by the current WHO classification.[6] Immunohistochemistry for Ki-67 protein is commonly used to evaluate the proliferative activity of tumor cells, and numerous studies have shown that the labeling index (LI) of the Ki-67 protein is correlated with the clinical outcome of patients with a variety of malignant tumors, including PNET.[2,3,7–10] The Ki-67 protein is detected during all active cell cycle phases (G_1, synthesis, G_2, and mitosis and cytokinesis) but not in resting (G_0) cells, although its function remains uncertain.[11,12] Although histologic grade-based estimations of prognosis are extremely useful for interpreting biopsy samples, additional reliable markers are needed.

Geminin, a negative regulator of DNA replication, has recently been described as a novel marker of malignant potential.[13] During the G_1 phase, DNA replication is initiated by the recruitment of the origin recognition complex, composed of cell division cycle-6 and Cdt1, to specific points of replication origin in the genome; this recruitment, in turn, loads the minichromosome maintenance (Mcm) complex, which is composed of Mcm-2 to Mcm-7.[14] Geminin is specifically expressed during the S, G_2, and early M phases and interacts with Cdt1 to prevent the loading of the Mcm complex to points of origin that have already been initiated, thus ensuring a single replication per 1 cell cycle.[15,16] Geminin expression has been widely observed in various malignant neoplasms, and the number of geminin-positive cells is reportedly proportional to the cell proliferation index, as measured using Ki-67 expression.[13] High levels of geminin expression are reportedly correlated with a poorer clinical outcome in breast cancer,[17] renal cell carcinoma,[18] prostatic adenocarcinoma,[19] salivary gland carcinomas,[20] lung cancer,[21] and gastric hyperplasia.[22] However, the prognostic significance of geminin expression in PNET remains unknown.

The purpose of this study was to determine whether geminin expression defines the aggressiveness of PNET and to compare the clinical and diagnostic use of the geminin LI with that of the Ki-67 LI.

MATERIALS AND METHODS

Patients

Between 1994 and 2010, a total of 27 consecutive patients underwent primary surgical treatment for PNET at our institution. The medical records and surgical specimens of these patients were retrospectively examined in the present study.

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Patients with recurrent tumors were excluded. Follow-up clinical information was obtained from the patients' medical records. The follow-up time was measured from the date of surgery until disease-caused death or the end of the follow-up period.

Clinicopathologic Parameters

The grading and staging of each tumor were performed according to the WHO classification, the cancer staging manual of the American Joint Committee on Cancer (AJCC), and the classification proposed by the European Neuroendocrine Tumor Society (ENETS).[23,24]

The prognostic values of the following clinicopathologic parameters were examined in the present study: tumor diameter (<2 vs ≥2 cm), lymphatic or blood vessel infiltration (absent vs present), perineural invasion (absent vs present), serosal or retroperitoneal invasion (absent vs present), tumor extension beyond the pancreas (absent vs present), mitotic index per 10 high-power fields (10 HPFs) (<2 vs ≥2), regional and distant metastasis (absent vs present), and pathological stage (AJCC stage ≥ IIA vs AJCC stage ≤ I, ENETS stage ≥ IIb vs ENETS stage ≤ IIa).

Histologic Examination and Immunohistochemistry

Surgical specimens were fixed in 10% formalin and embedded in paraffin. Two pathologists (M.A. and M.K.), who were unaware of the clinical data, reviewed all the hematoxylin-and-eosin–stained sections and reclassified and graded the specimens according to the histologic parameters.

Serial 4-μm sections were used for immunohistochemical staining. Deparaffinized and rehydrated sections were immersed in 0.3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. Heat-induced antigen retrieval was performed for 20 minutes at 95°C with a 10-mM citrate buffer (pH 6.0). After the slides had cooled at room temperature for 1 hour, they were exposed to 2% normal swine serum in phosphate-buffered saline for 30 minutes, then allowed to react overnight at 4°C with the following mouse monoclonal antibodies: antihuman geminin (diluted 1:40, clone EM6; Novocastra, Newcastle, United Kingdom) or antihuman Ki-67 (diluted 1:100, clone MIB-1; Dako, Glostrup, Denmark). After washing with phosphate-buffered saline 3 times, the sections were reacted with EnVision plus (Dako) for 30 minutes at room temperature. The peroxidase reaction products were developed with 3,3′-diaminobenzidine, and the sections were counterstained with hematoxylin.

The LI of each marker was calculated by manually counting the number of brown-stained tumor cell nuclei among the total number of tumor cells in the most highly immunoreactive area at a magnification of 400-fold, with the aid of an eyepiece grid (5 × 5 squares). Indices were expressed as a percentage value corresponding to the number of positive cells among approximately 2000 tumor cells.

Evaluation of Color Difference Quotation

Immunohistochemically stained full-face sections from each case with geminin and Ki-67 overexpression were digitized using the Slide Path and the NanoZoomer Digital Pathology System (Hamamatsu, Welwyn Garden City, United Kingdom). Approximately 7 minutes was required to scan a slide at a resolution of 40×. Subsequently, 400-fold magnified images from highly immunoreactive areas were exported for analysis.

The image analysis was performed using Photoshop (version 7; Adobe Systems, San Jose, CA). First, the image mode was converted from RGB to Lab color mode. Two hundred fifty representative positive cells were selected in each of 4 cases with high geminin expression levels (LI ≥ 2.0%) and 7 cases with high Ki-67 expression levels (LI ≥ 2.0%). All the cells with a recognizable brown stain were measured. The L*a*b* value of the nuclear areas was outputted as a range from 0 to 255. The values represented a Lab color space composed of 3 axes in a spherical form: L^*, a^*, and b^*. The L^* axis was associated with the lightness of the color, whereas the a^* and b^* axes were associated with the red-green scale and the yellow-blue scale, respectively. After the conversion of the scale to the CIE LAB color system, the L^* value was described as a decimal scale from 0 to 100, and the a^* and b^* values ranged from −128 to 127. The difference between the average values for a positive cell and an adjacent negative cell was calculated as ΔL^*, Δa^*, and Δb^*. Then, the color difference ΔE was estimated using the formula $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. ΔE was then compared between the geminin-stained and Ki-67–stained sections.

Statistical Analysis

The Spearman rank correlation test was used to determine associations between continuous variables. Receiver operating characteristic curves were plotted to calculate the sensitivity, specificity, positive predictive value, and negative predictive value for the presence of metastasis. The cutoff values for the geminin LI and the Ki-67 LI were chosen so as to obtain the best combination of predictive values. A univariate analysis using the Cox proportional hazards model was applied to estimate the associations of clinicopathologic factors, including the immunohistochemical results, with the disease-free survival period. Survival curves were drawn using the Kaplan-Meier method, and the differences were analyzed using a log-rank test. Differences in nonparametric data were estimated using the Mann-Whitney U test. All P values < 0.05 were considered statistically significant. The statistical analyses were performed using Dr. SPSS II for Windows (SPSS Japan, Tokyo, Japan).

RESULTS

Demographic Characteristics and Tumor-Related Factors

Among the 27 patients, the median age at the time of diagnosis was 56 years; 14 patients (51.9%) were men, and 13 patients (48.1%) were women. In all the patients, a curative resection was performed, followed by a histologic assessment of the tumor grading. The tumor-related factors are summarized in Table 1. The tumor was located in the pancreatic head in 11 cases (40.7%), in the body in 12 cases (44.4%), and in the tail in 4 cases (14.8%). Only 1 case of functional PNET (insulinoma) was included. The median and range of the maximum tumor diameter were 26 mm and 8 to 92 mm, respectively. The diameter was 2 cm or larger in 11 cases (40.7%). Local invasion was observed in 2 cases (7.4%). One of these cases exhibited an obstruction of the inferior common bile duct, and another case presented with invasion to both the splenic artery and vein. Lymph node metastasis was encountered in 10 cases (37.0%), and a solitary liver metastasis was resected in 1 case (3.7%). With respect to mitosis, fewer than 2 mitoses per 10 HPFs were present in 19 cases (70.4%), from 2 to 10 mitoses per 10 HPFs were encountered in 8 cases (29.6%), and none of the cases had more than 20 mitoses per 10 HPFs.

The tumor grade classifications according to the WHO system and tumor staging according to the AJCC or ENETS criteria are shown in Table 1. Nineteen cases (70.4%) were classified as grade 1 neuroendocrine tumor (NET), and 8 cases