all markers. The baseline levels of CEC ($P = 0.03$), IL-6 ($P < 0.01$), and IL-10 ($P = 0.03$) were found to be significantly higher among patients with PD than among those with PR or SD. The blood concentrations of HGF ($P < 0.001$), IL-6 ($P < 0.01$), and IL-8 ($P < 0.001$) were also significantly higher among patients with clinical stage IV disease and recurrence than among those with stage III disease. When the association between CEC number and the expression of other angiogenic factors was examined, the number of CECs was found to correlate positively with the levels of VEGF ($r = 0.34$, $P = 0.04$), HGF ($r = 0.37$, $P = 0.02$), IL-8 ($r = 0.38$, $P = 0.02$), and IL-10 ($r = 0.45$, $P = 0.006$), suggesting that the number of CECs is related to the expression of these markers (Table 3).

Discussions

In most cases, CECs are apoptotic or necrotic cells that are released into circulation as a byproduct of vascular turnover. In some cancer patients, the level of CECs is significantly higher than that of healthy individuals, and this increased level has been identified as a surrogate marker of angiogenesis and anti-angiogenic drug activity [10,11]. The present study has shown that baseline CEC levels are markedly higher among pancreatic carcinoma patients than in healthy individuals. Our results also support the hypothesis that CEC levels are associated with clinical outcome in pancreatic carcinoma patients undergoing gemcitabine chemotherapy, and may be a prognostic factor for this disease. A previous study found that the baseline level of CECs, identified as $CD45^{-}CD31^{+}CD34^{+}$ by flow cytometry, was inversely associated with OS in patients who had gemcitabine-refractory metastatic pancreatic carcinoma and were treated with bevacizumab plus erlotinib [12]. CEC ($CD45^{-}CD31^{+}CD146^{+}$) detection by flow cytometry requires careful discrimination between blood cell populations with overlapping phenotypes showing hallmarks of T cells ($CD45^{-}CD31^{-}CD146^{+}$) and platelets ($CD45^{-}$ $CD31^{high}CD146^{-}$). These cells populations show distinct regulation during cancer therapy, and their concomitant analysis may offer extended prognostic and predictive information [13].

Table 2 Univariate and multivariate Cox analyses of prognosis

Univariate analysis	HR	95% CI	*P*
Age: Over 70 vs. Below 70	0.52	0.25–1.13	0.1
Sex: Male vs. Female	1.00	0.48–2.08	0.99
Stage: IV + Recurrence vs. III	2.21	1.03–4.71	0.04
ECOG PS: 2 + 1 vs. 0	2.72	1.29–5.70	0.008
Pancreatic tumor location: Head vs. Others	0.94	0.46–1.90	0.86
CA19-9 (cut-off: 10,000 U/mL): CA19-9^{high} vs. CA19-9^{low}	1.77	0.75–4.15	0.19
CRP level (cut-off: 1.0 mg/dL): CRP^{high} vs. CRP^{low}	2.49	1.14–5.42	0.02
Histology: Poorly differentiated vs. Others	1.09	0.52–2.27	0.82
Second line therapy: Yes vs. No	0.61	0.30–1.24	0.17
CEC level (cut-off: 166 cells/4 mL): CEC^{high} vs. CEC^{low}	5.18	2.23–12.03	<0.001
IL-6 (cut-off: 19.3 pg/mL): IL-6^{high} vs. IL-6^{low}	2.52	0.73–8.64	0.14
IL-8 (cut-off: 11.3 pg/mL): IL-8^{high} vs. IL-8^{low}	1.74	0.82–3.67	0.15
IL-10 (cut-off: 7.82 pg/mL): IL-10^{high} vs. IL-10^{low}	5.05	1.55–16.39	0.007
VEGF (cut-off: 44.1 pg/mL): VEGF^{high} vs. VEGF^{low}	1.22	0.60–2.47	0.59
PDGF-BB (cut-off: 1127.5 pg/mL): PDGF-BB^{high} vs. PDGF-BB^{low}	0.93	0.43–2.04	0.86
HGF (cut-off: 471.3 pg/mL): HGF^{high} vs. HGF^{low}	2.52	1.22–5.21	0.01
SDF-1 alpha (cut-off: 110.6 pg/mL): SDF-1 alpha^{high} vs. SDF-1 alpha^{low}	1.23	0.60–2.53	0.56
Multivariate analysis	**HR**	**95% CI**	***P***
Stage: IV + Recurrence vs. III	2.04	0.78–5.35	0.15
ECOG PS: 2 + 1 vs. 0	2.58	0.98–6.76	>0.05
CRP level (cut-off: 1.0 mg/dL): CRP^{high} vs. CRP^{low}	2.04	0.62–6.76	0.24
CEC level (cut-off: 166 cells/4 mL): CEC^{high} vs. CEC^{low}	5.14	1.83–14.45	0.002
IL-10 (cut-off: 7.82 pg/mL): IL-10^{high} vs. IL-10^{low}	5.26	1.26–22.22	0.02
HGF (cut-off: 471.3 pg/mL): HGF^{high} vs. HGF^{low}	1.34	0.46–3.91	0.59

Abbreviations: HR = hazard ratio; CI = confidence interval; ECOG PS = Eastern Cooperative Oncology Group performance status; CEC = circulating endothelial cells; IL = interleukin; PDGF-BB = platelet-derived growth factor-B chain; VEGF = vascular endothelial growth factor; HGF = hepatocyte growth factor; CA19-9 = carbohydrate antigen 19–9; CRP = C-reactive protein; CEA = carcinoembryonic antigen.

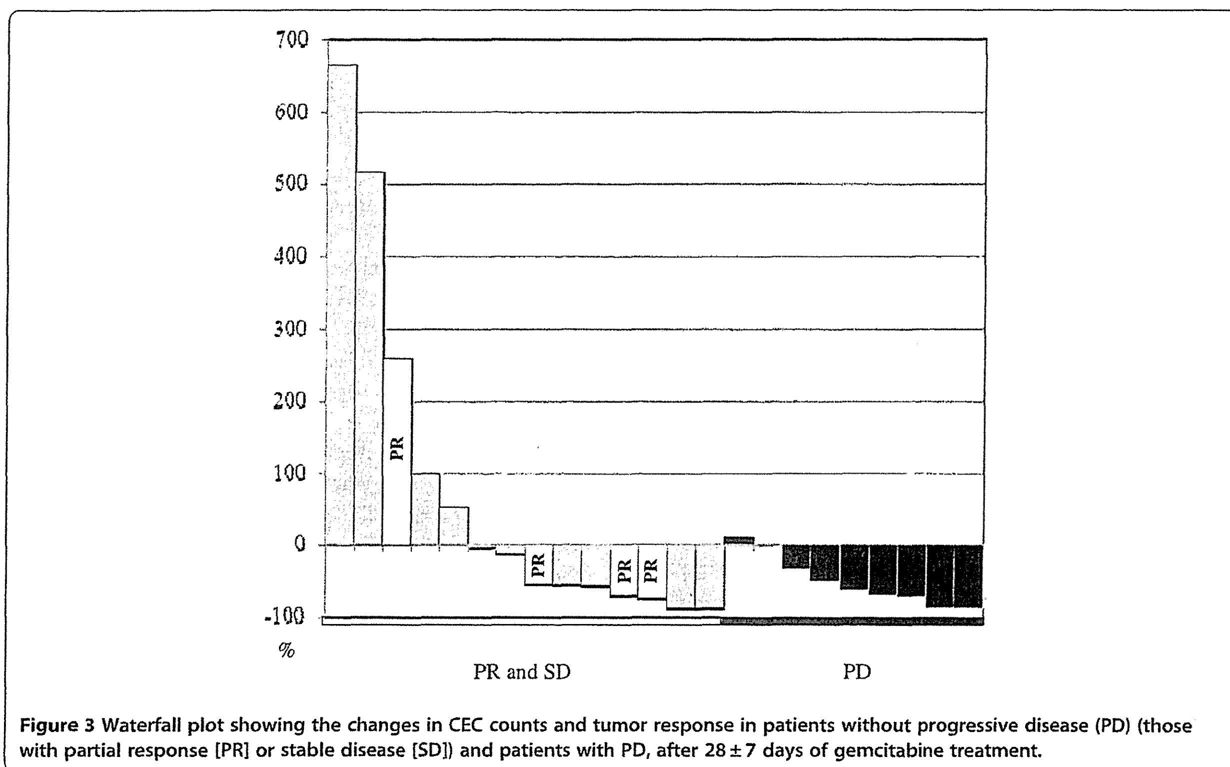

Figure 3 Waterfall plot showing the changes in CEC counts and tumor response in patients without progressive disease (PD) (those with partial response [PR] or stable disease [SD]) and patients with PD, after 28 ± 7 days of gemcitabine treatment.

Our study also found the baseline level of CECs, as well as the levels of HGF, IL-6, and IL-10, which are associated with gemcitabine resistance or stemness, to be significantly higher among PD patients. Univariate Cox model analysis further demonstrated that PS, clinical stage, CRP levels, and CEC levels are all associated with the survival of pancreatic carcinoma patients, while multivariate Cox analysis showed that CEC and IL-10 levels are strongly associated with survival.

The number of CECs detectable in individuals has previously been found to be associated with the plasma levels of VCAM-1 and VEGF in cancer patients [14] [15]. Our findings further show that, in addition to VEGF, CEC levels are strongly associated with the expression levels of IL-8, IL-10, and HGF in pancreatic carcinoma patients. These molecules, among others, play important roles in tumor biology and have been implicated in several cellular phenotypes. Chemokines,

Table 3 Association between CECs and other factors

	Mean ± SD	Spearman's rank correlation coefficient	*P*
CEC (cells/4 mL)	166.2 ± 228.9	1	-
IL-6 (pg/mL)	19.3 ± 52.4	0.17	0.30
IL-8 (pg/mL)	11.3 ± 10.1	0.38	0.02
IL-10 (pg/mL)	7.82 ± 26.9	0.45	0.006
VEGF (pg/mL)	44.1 ± 38.8	0.34	0.04
PDGF-BB (pg/mL)	1,127.5 ± 941.5	0.24	0.16
HGF (pg/mL)	471.3 ± 249.0	0.37	0.02
SDF-1alpha (pg/mL)	110.6 ± 43.7	0.15	0.37
CRP (mg/dL)	1.9 ± 3.9	0.31	0.06
CA19-9 (U/mL)	18,229.1 ± 55,377.8	0.11	0.50
CEA (ng/mL)	18.3 ± 51.0	0.03	0.88

Abbreviations: CEC = Circulating endothelial cell; IL = interleukin; PDGF-BB = platelet-derived growth factor-B chain; VEGF = vascular endothelial growth factor; HGF = hepatocyte growth factor; CA19-9 = carbohydrate antigen 19–9; CRP = C-reactive protein; CEA = carcinoembryonic antigen.

including IL-8 and IL-10, are small peptides involved in controlling cell migration, particularly in leukocytes, during inflammation and the immune response. Chemokines are also important in tumor biology as they influence tumor growth, invasion, metastasis, and angiogenesis. For instance, VEGF, HGF and IL-8 significantly stimulate the proliferation, migration, and invasion of cancer cells. CEC are shed from vessels and this process may be amplified by an aberrant vascular turnover/remodeling associated with high local levels of VEGF required for CEC survival [16]. The chemokine SDF-1 has likewise been found to enhance the production of IL-8 by pancreatic cells in a paracrine manner [17]. Although our results did not indicate that SDF-1 levels were associated with CEC or IL-8 levels in the pancreatic cancer patients examined, it is likely that several of the proangiogenic factors examined in this study interact with each other to promote vascular turnover and remodeling, thereby leading to a higher number of CECs in the peripheral blood of cancer patients.

Drugs targeting angiogenesis, such as those that inhibit the VEGF pathway, have had a major impact in the treatment of many types of cancer. The VEGF pathway is also an independent prognostic factor for patient survival in pancreatic carcinoma. Although preclinical models have suggested that VEGF-VEGF receptor inhibitors would be effective in the treatment of pancreatic carcinoma, patients who received bevacizumab and axitinib therapy in addition to gemcitabine have not shown a survival advantage when compared to those treated with gemcitabine alone [6,18]. These results add to the increasing evidence that suggests that targeting VEGF signaling is an ineffective strategy in the treatment of pancreatic carcinoma. However, many antiangiogenic therapies modulate the expression levels of proangiogenic factors [19], and many factors are associated with tumor angiogenesis. Therefore, there are a variety of potential therapeutic targets that may be exploited in order to target angiogenesis, potentially including those examined in this study.

In advanced non-small cell lung cancer (NSCLC), patients with higher baseline CEC counts have PR/SD and longer PFS. It has also previously been reported that the elevated CEC numbers exhibited in NSCLC patients decrease following treatment with carboplatin in combination with paclitaxel [9]. Paclitaxel and docetaxel are categorized as mitotic spindle agents with potent antiangiogenic properties [20-22]. Therefore, it seems that the baseline CEC count is a promising predictor of clinical response to the carboplatin plus paclitaxel regimen, as well as of survival. However, although several other clinical studies that have examined CECs have also found chemotherapy to be associated with either an increase or decrease in CEC number [23,24], no association was detected between gemcitabine treatment and CEC number in the pancreatic carcinoma patients in our study. Although gemcitabine has anti-angiogenic properties, higher baseline CEC levels were associated with PD in pancreatic carcinoma patients receiving gemcitabine therapy, and patients with high CEC counts exhibited poor clinical condition. It is therefore likely that the tumor type, anti-cancer drugs being administered, and the amount of time between the start of treatment and the time when CEC counts are obtained influence the number of CECs detected in cancer patients after treatment. In this study, we measured CEC levels before starting chemotherapy and at 28 ± 7 days after starting chemotherapy, the time of sampling might influence the changes of CEC level. Moreover, the diversity in literature regarding CEC up-or down-regulation during cancer therapy and the associated prognostic and predictive evidence might in part be explained by a differential focus on or by the lack of discrimination between these cell populations [13].

Conclusions

Although the number of patients examined in this study was small, and patients were recruited prospectively, this study, along with others, has shown the clinical importance of CEC number as a prognostic factor in advanced pancreatic carcinoma treated with gemcitabine chemotherapy, whereby high CEC counts are associated with poor prognosis. This study also found that elevated CEC counts are associated with the high expression levels of several chemokines and proangiogenic factors involved in the regulation of tumor immunological and angiogenic factors. Although this correlation between blood parameters is not proof of a causal relationship, these factors may provide viable therapeutic targets for the treatment of pancreatic carcinoma in the future. Further studies in a larger population will be required to confirm our findings.

Abbreviations

CEC: circulating endothelial cell; ECOG: Eastern Cooperative Oncology Group; CA19-9: Carbohydrate antigen 19–9; CRP: C-reactive protein; IL: Interleukin; PDGF-BB: Platelet-derived growth factor-B chain; VEGF: Vascular endothelial growth factor; HGF: Hepatocyte growth factor; PD: Progressive disease; PR: Partial response; HR: Hazard ratio; CI: confidence interval; SD: Stable disease.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SK and KT designed and participated in all stages of the study. SK and JH performed most of the experiments. FK and CM participated in CEC analysis, as well as the statistical analyses and discussion of the results. HU and TO recruited the patients, collected the tumor biopsy samples, and helped to draft the manuscript. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Effect of biliary drainage on chemotherapy in patients with biliary tract cancer: an exploratory analysis of the BT22 study

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Abstract

Background/purpose: Complications from biliary drainage in biliary tract cancer (BTC) may influence the relative dose intensity of chemotherapy or increase adverse events during chemotherapy. BT22 was a randomized phase II trial, the results of which were consistent with those of a phase III trial in non-Japanese that demonstrated the effectiveness of gemcitabine plus cisplatin combination therapy (GC) in BTC. The purpose of this exploratory analysis of the BT22 study was to identify the possible effects of biliary drainage on the efficacy and safety of GC or gemcitabine monotherapy (G).

Patients and Methods: The 83 BTC patients who received GC or G in BT22 were retrospectively analysed in two subgroups dependent upon whether biliary drainage was performed before study entry. Efficacy and safety of treatment (GC vs. G) were compared in these two groups.

Results: The GC arm had a higher 1-year survival rate and longer median survival time (MST) than the G arm independent of prior biliary drainage. Patients in the drainage subgroup developed cholangitis more frequently, however, the frequency of grade 3/4 adverse events did not differ between the treatment regimens with/without drainage.

Conclusions: Biliary drainage before chemotherapy did not affect the therapeutic efficacy or tolerability of chemotherapy using G or GC.

Keywords

biliary drainage, chemotherapy, gemcitabine, cholangitis, cisplatin, biliary tract cancer

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Introduction

Biliary tract cancer (BTC), while relatively rare in Western countries, is more common in Japan where it is the sixth leading cause of cancer death with approximately 17 000 deaths every year.[1] The mortality caused by BTC in Japan is higher than any other country, and far exceeds all Western countries.[2]

Developing an effective BTC treatment has become a high priority for Japan. At present the only curative treatment is surgical resection, and although an increasing number of patients undergo surgery each year, outcomes have met with only a varying degree of success. Patients with unresectable disease can only be managed with chemotherapy and supportive care for palliation of disease including biliary decompression. However, prognosis remains extremely poor in these patients.

No standard chemotherapy for BTC has been established. Many clinical trials of systemic chemotherapy have been conducted in BTC patients, but most of these were phase II trials that had small sample sizes and lacked a control group because of the rarity and heterogeneity of BTC. In 2009, the results of a phase III study of gemcitabine plus cisplatin (GC) vs. gemcitabine monotherapy (G) conducted in the United Kingdom (ABC-02 Study) were reported.[3] This previous study included 410 patients and is the largest clinical trial to be conducted in this field. The GC arm had

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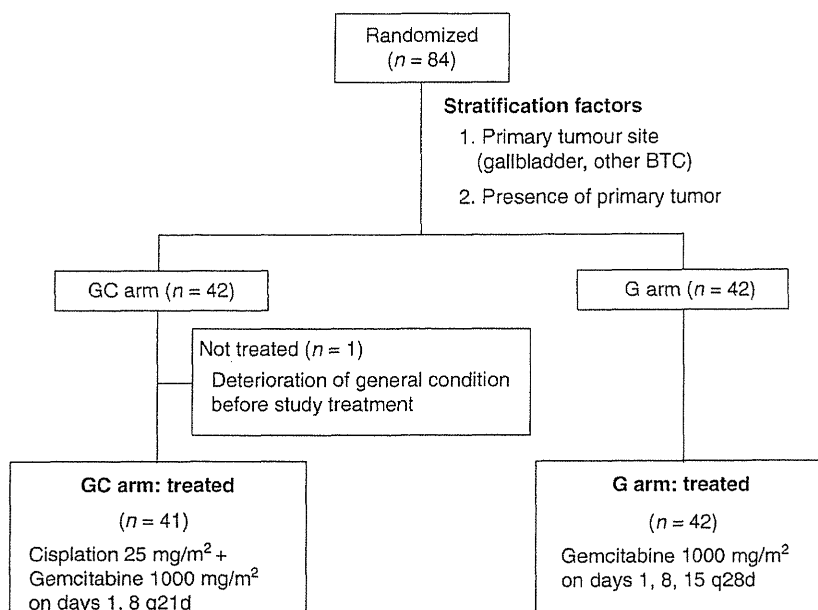

Figure 1 Patient disposition of the BT22 Study. BTC, biliary tract cancer; G arm, gemcitabine monotherapy; GC arm, combination therapy with gemcitabine and cisplatin

a significantly better median survival time (MST) [11.7 months (95% confidence interval (CI) 9.5 to 14.3) vs. 8.1 months (95% CI 7.1 to 8.7); $P < 0.001$] and progression-free survival (PFS) [8.0 months (95% CI 6.6 to 8.6) vs. 5.0 months (95% CI 4.0 to 5.9); $P < 0.001$] than the G arm. A comparison of grade 3 and 4 toxicities showed that the GC combination added little toxicity. From the results of the ABC-02 study, GC was recognized as the standard of care for the treatment of advanced BTC. A randomized phase II study comparing GC and G was also conducted in Japan (BT22 study; Clinical Trial.gov Identifier NCT00380588). Median survival time [11.2 vs. 7.7 months; hazard ratio (HR) 0.69 (95% CI 0.42 to 1.13)] and PFS [5.8 vs. 3.7 months; HR 0.66 (95% CI 0.41 to 1.05)] were similar to the results seen in the ABC-02 study confirming the status of GC as the worldwide standard.[4]

For patients with unresectable disease, biliary decompression is often required if chemotherapy is contemplated.[5] Usually, biliary obstruction will be managed by percutaneous or endoscopic drainage rather than a surgical approach because of the presence of incurable disease and high operative risk. However, complications resulting from insufficient biliary drainage, morbidities such as obstructive jaundice, cholangitis, and sepsis, often require that chemotherapy be interrupted or discontinued.[6] Obstructive jaundice may impact on prognosis by necessitating dose modification of chemotherapy or by complications as a consequence of biliary obstruction.

In the present study, we analysed the data from the BT22 study conducted in Japan to determine the impact of biliary drainage on the efficacy and adverse events associated with gemcitabine-based chemotherapy.

Patients and methods

Patients

This analysis included all patients who received at least one dose of study treatment in the BT22 study, a multicentre study conducted at nine medical institutions in Japan. From September 2006 to October 2008, 84 BTC patients were enrolled. The patients were randomized to either the GC arm [a weekly intravenous (i.v.) infusion of cisplatin 25 mg/m^2 followed by gemcitabine 1000 mg/m^2 for 2 weeks, followed by dose suspension at the third week, repeated as one course] or the G arm (weekly i.v. infusion of gemcitabine 1000 mg/m^2 for 3 weeks followed by dose suspension at the fourth week, repeated as one course). Randomization was stratified by primary tumour site (gallbladder cancer or other BTC) and the presence or absence of a primary tumour. One patient in the GC arm was discontinued before the start of the study treatment for deterioration of a general condition caused by another complication, so the analysis was conducted with 41 GC arm patients and 42 G arm patients (Fig. 1).

The specific criteria for study eligibility have been reported previously[4] and are only summarized here:

- patients with unresectable locally advanced or metastatic intrahepatic bile duct cancer, extrahepatic bile duct cancer, gallbladder cancer, or ampullary carcinoma that is histologically or cytologically confirmed adenocarcinoma (including adenosquamous carcinoma);
- patients with at least one measurable lesion;
- patients with no prior chemotherapy;
- patients with a performance status of 0 or 1;

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Table 1 Patient characteristics at baseline

Characteristics	BD (n = 34)		Non-BD (n = 49)	
	n (%)		n (%)	
	GC arm (n = 16)	G arm (n = 18)	GC arm (n = 25)	G arm (n = 24)
Gender				
Male	7 (43.8)	9 (50.0)	11 (44.0)	12 (50.0)
Female	9 (56.3)	9 (50.0)	14 (56.0)	12 (50.0)
Age				
Median (years)	64.5	65.5	65.0	68.5
PS				
0	13 (81.3)	12 (66.7)	21 (84.0)	16 (66.7)
1	3 (18.8)	6 (33.3)	4 (16.0)	8 (33.3)
Primary tumour site				
Gallbladder	6 (37.5)	9 (50.0)	9 (36.0)	8 (33.3)
Non-gallbladder	10 (62.5)	9 (50.0)	16 (64.0)	16 (66.7)
Presence of a primary tumour				
Present	14 (87.5)	18 (100.0)	16 (64.0)	13 (54.2)
Absent	2 (12.5)	0 (0.0)	9 (36.0)	11 (45.8)
Extent of disease				
Locally advanced	5 (31.3)	7 (38.9)	0 (0.0)	1 (4.2)
Metastatic	11 (68.8)	11 (61.1)	25 (100.0)	23 (95.8)

BD, biliary drainage; G arm, gemcitabine monotherapy; GC arm, combination therapy with gemcitabine and cisplatin; PS, performance status.

- patients with an estimated life expectancy of more than 3 months; and
- patients with adequate organ function (e.g. bone marrow, liver and kidney).

Methods

The BT22 study was a randomized study that compared patients from two arms: GC vs. G.

Patients with obstructive jaundice had to achieve a certain degree of jaundice reduction with biliary drainage before study entry (i.e. total bilirubin was three times the upper limit of normal or less). The protocol contained no specific provisions about biliary drainage. The approach (endoscopic or percutaneous transhepatic), drainage type (internal biliary drainage or external biliary drainage) and stent material (plastic stent or metallic stent) could be decided by the investigator. The primary endpoint was 1-year survival rate. Sample size was calculated by the method proposed by Simon *et al.*[7] The 83 treated patients were retrospectively analysed and classified into subgroups of patients who had undergone biliary drainage before the start of the study (BD subgroup) and patients who had not (non-BD subgroup) to compare the efficacy and safety of the treatment regimens (GC vs. G arms). Progression-free survival and overall survival (OS) curves were constructed using the Kaplan–Meier method, and estimates of median OS and the respective 95% CIs were calculated from the Kaplan–Meier estimates. Cox's proportional hazard model was used to estimate the HR. Adverse events were graded according to the Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE v. 3.0). A multivariate Cox proportional hazard model was used to identify prognostic factors.

Results

Of the 83 patients treated in the BT22 study, 34 were in the BD sugroup (16 in the GC arm and 18 in the G arm) and 49 were in the non-BD subgroup (25 in the GC arm and 24 in the G arm). Table 1 shows patient baseline characteristics. More of the patients in the BD subgroup (n = 34) had a primary tumour [GC: (14/16) 87.5%, G: (18/18) 100%], whereas the percentages of patients in the non-BD subgroup (n = 49) without a primary tumour were relatively higher [GC: (9/25) 36.0%, G: (11/24) 45.8%]. However, no substantial imbalances were noted between the two subgroups in gender, age or primary tumour site.

Efficacy

Efficacy data for the subgroups are shown in Table 2 and Fig. 2. In the BD subgroup, a comparison of OS in the GC and G arms showed 1-year survival of 40.9% vs. 27.8% and MST of 11.3 vs. 8.1 months [HR of 0.59 (95% CI 0.27 to 1.30)], respectively. In the non-BD subgroup, a comparison between the GC and C arms showed 1-year survival rate of 37.8% vs. 33.3% and MST of 9.6 vs. 7.5 months [HR of 0.76 (95% CI 0.40 to 1.45)], respectively.

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Table 2 Overall survival and progression-free survival with or without biliary drainage by treatment arm

	BD (*n* = 34)		Non-BD (*n* = 49)	
	GC arm (*n* = 16)	G arm (*n* = 18)	GC arm (*n* = 25)	G arm (*n* = 24)
Overall survival				
1-year survival rate	40.9%	27.8%	37.8%	33.3%
Median survival time (months)	11.3	8.1	9.6	7.5
Hazard ratio (95% confidence interval)	0.588 (0.266–1.301)		0.758 (0.397–1.447)	
Progression-free survival				
6-month progression-free survival	53.3%	27.8%	43.7%	27.5%
Median progression-free survival (months)	7.1	3.9	4.5	3.3
Hazard ratio (95% confidence interval)	0.479 (0.222–1.032)		0.748 (0.407–1.374)	

BD, biliary drainage; G arm, gemcitabine monotherapy; GC arm, combination therapy with gemcitabine and cisplatin.

Subgroup analysis results based on HRs for OS by biliary drainage, performance status (PS), primary tumour site, presence of primary tumour and extent of disease are shown in Fig. 3. Hazard ratios (GC vs. G) for OS were less than one in every subgroup.

Safety

Adverse events observed in the GC and G arms with a frequency of at least 30% in the BT22 study have been reported.[4] In this analysis, the frequency of grade 3 and 4 events for the most common adverse events (frequency ≥30%) in the BD subgroup was compared with that in the non-BD subgroup (Table 3). Events in the BD subgroup that were more common in the GC arm were haemoglobin decrease (43.8% vs. 5.6%), thrombocytopenia (37.5% vs. 5.6%) and red blood cell decrease (37.5% vs. 5.6%). Events in the non-BD subgroup that were more common in the GC arm were leukopaenia (32.0% vs. 12.5%), neutoropenia (64.0% vs. 33.3%), and thrombocytopaenia (40.0% vs. 8.3%). There were no significant differences in the incidence of non-haematological events between the GC and G arms in either the BD or non-BD subgroup.

Although the incidence of cholangitis was higher in the BD subgroup than in the non-BD subgroup, the ratio of cholangitis in the GC arm to that in the G arm was not appreciably different in each of the BD and non-BD subgroups. For the BD subgroup, the incidence of Grade 3 and 4 cholangitis in the G arm was relatively higher than that in the GC arm (Table 4).

Prognostic factors

A multivariate Cox's proportional hazard model was used with the following six factors: biliary drainage, PS (0 vs. 1), primary tumour site (gallbladder vs. non-gallbladder), the presence of a primary tumour (present vs. absent), extent of disease (locally advanced vs. metastatic) and chemotherapy regimen (GC arm vs. G arm) (Table 5). The HR of the GC arm to the G arm was 0.72 [95% CI 0.44 to 1.20] after multivariate adjustment for several variables. The primary tumour site of non-gallbladder [HR of gallbladder vs. non-gallbladder 1.72 (95% CI 1.01 to 2.93)] and the absence of a primary tumour [HR of presence vs. absence 2.79 (95% CI 1.40 to 5.56)] were significantly related to a longer OS. Biliary drainage was suggested to have favourable clinical relevance [HR 0.72 (95% CI 0.39 to 1.32)], as well as PS 0 and locally advanced disease.

Discussion

Many studies on unresectable BTC retrospectively investigated whether biliary drainage should be performed endoscopically or with a percutaneous transhepatic approach,[8,9] and whether a plastic or metallic stent should be used.[10–15] Several of the few prospective studies conducted have stent patency or complication-free survival as a primary endpoint. However, only a few of those have considered the impact of drainage on chemotherapy efficacy or adverse events. This analysis represents an important exploratory investigation of the impact of biliary drainage on chemotherapy efficacy in the BT22 study which was a prospectively controlled study.

Insufficient biliary drainage leads to problems during chemotherapy, such as recurrent obstructive jaundice with or without cholangitis, which in turn often results in suspension or discontinuation of chemotherapy. In the BT22 study, the incidence of cholangitis during initial chemotherapy in the GC and G arms in the BD subgroup was higher than that in the respective arms of the non-BD subgroup. Although the profiles of grade 3 and 4 non-haematological adverse events in the GC and G arms did not differ, haematological toxicities were slightly more severe in the GC arm. GC therapy is expected to lead to an increased incidence of cholangitis or progression to severe cholangitis in patients undergoing biliary drainage who are at high risk of cholangitis mainly because the regimen has a more severe haematological toxicity profile than G. These events could undermine the efficacy of GC therapy.

In the BD subgroup, however, MST in the GC and G arms were 11.3 vs. 8.1 months, respectively, with an HR of 0.59 (95% CI 0.27 to 1.30). Median survival time in the GC arm was longer than MST in the G arm. As with adverse events overall, grade 3 and 4 adverse events in the BD subgroup were slightly more severe in the

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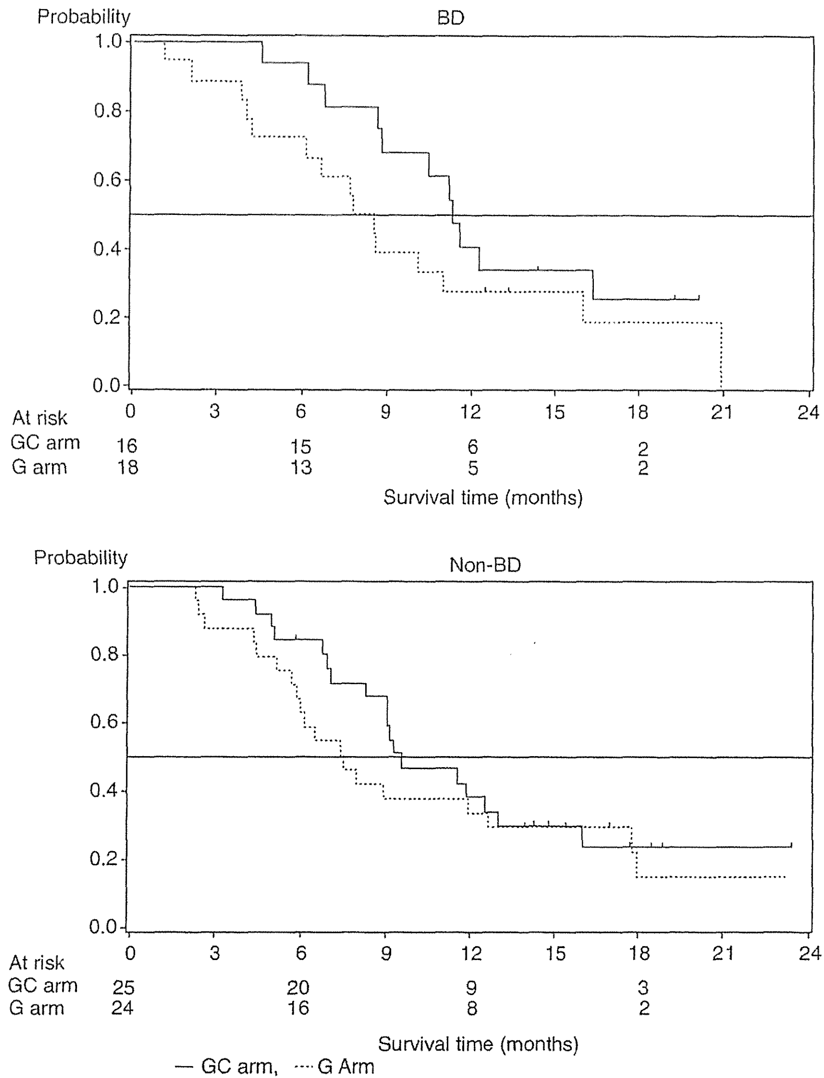

Figure 2 Survival curves in the BD subgroup (a) and non-BD subgroup (b) by the treatment arm. Solid line (—) indicates the GC arm and the broken line (---) the G arm. BD, biliary drainage; G arm, gemcitabine monotherapy; GC arm, combination therapy with gemcitabine and cisplatin

Subgroup		HR*	*P*-value	hazard ratio with 95% confidence interval
Biliary drainage	BD	0.588	0.1902	
	Non-BD	0.758	0.4006	
PS	0	0.759	0.3520	
	1	0.592	0.3344	
Primary tumour site	Gallbladder	0.852	0.6746	
	Non-gallbladder	0.578	0.1097	
Presence of primary tumour	Present	0.722	0.2525	
	Absent	0.602	0.3891	
Extent of disease	Locally advanced	0.591	0.4757	
	Metastatic	0.715	0.2181	
				0 0.5 1 1.5 2 2.5 3

Figure 3 Hazard ratios for overall survival by patient baseline characteristics. HR*, hazard ratio of the combination therapy with gemcitabine and cisplatin (GC) arm to the gemcitabine monotherapy (G) arm

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Table 3 Incidence of grade 3 or 4 events among most common adverse events[a]

Most common adverse events	BD (n = 34)		Non-BD (n = 49)	
	n (%)		n (%)	
	GC arm (n = 16)	G arm (n = 18)	GC arm (n = 25)	G arm (n = 24)
Hematologic				
WBC count decreased	4 (25.0)	5 (27.8)	8 (32.0)	3 (12.5)
Neutrophil count decreased	7 (43.8)	8 (44.4)	16 (64.0)	8 (33.3)
RBC decreased	6 (37.5)	1 (5.6)	8 (32.0)	5 (20.8)
Haemoglobin decreased	7 (43.8)	1 (5.6)	8 (32.0)	6 (25.0)
Haematocrit decreased	1 (6.3)	0 (0.0)	1 (4.0)	0 (0.0)
Platelet count decreased	6 (37.5)	1 (5.6)	10 (40.0)	2 (8.3)
Non-haematological				
Anorexia	0 (0.0)	0 (0.0)	0 (0.0)	2 (8.3)
Nausea	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Vomiting	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Constipation	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Diarrhoea	1 (6.3)	0 (0.0)	0 (0.0)	0 (0.0)
Fatigue	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.2)
Pyrexia	0 (0.0)	2 (11.1)	0 (0.0)	0 (0.0)
Weight decreased	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
AST increased	3 (18.8)	5 (27.8)	4 (16.0)	2 (8.3)
ALT increased	4 (25.0)	5 (27.8)	6 (24.0)	2 (8.3)
GGT increased	6 (37.5)	7 (38.9)	6 (24.0)	8 (33.3)
LDH increased	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
ALP increased	1 (6.3)	3 (16.7)	2 (8.0)	4 (16.7)
Blood sodium decreased	4 (25.0)	2 (11.1)	3 (12.0)	2 (8.3)
C-reactive protein increased	0 (0.0)	2 (11.1)	0 (0.0)	1 (4.2)

[a]Most common, incidence ≥30% of all grades; events were graded according to CTCAE v3.0.
AST, aspartate aminotransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; BD, biliary drainage; G arm, gemcitabine monotherapy; GC arm, combination therapy with gemcitabine and cisplatin.

Table 4 Incidence of cholangitis with or without biliary drainage by treatment arm

	BD (n = 34)		Non-BD (n = 49)	
	n (%)		n (%)	
	GC arm (n = 16)	G arm (n = 18)	GC arm (n = 25)	G arm (n = 24)
Cholangitis (all grades)	5 (31.3)	7 (38.9)	2 (8.0)	3 (12.5)
Cholangitis (≥ grade 3)	2 (12.5)	5 (27.8)	0 (0.0)	1 (4.2)

BD, biliary drainage; G arm, gemcitabine monotherapy; GC arm, combination therapy with gemcitabine and cisplatin.

GC arm than in the G arm, but no particularly frequent events were encountered. In addition, patients with biliary drainage in the G arm had a relatively increased incidence of grade 3 and 4 cholangitis, possibly as a result of a lack of efficacy and inferior biliary drainage. Although careful monitoring of cholangitis is still needed, the above findings indicate GC to be an appropriate standard chemotherapy for unresectable BTC in both patients with and without biliary drainage.

Because this analysis was a retrospective investigation of the BT22 study, several biases could arise after this retrospective approach. Although the biases cannot be completely circumvented using a multivariate Cox's proportional hazard model, the results adjusted with other prognostic factors suggested that biliary drainage would not have a negative impact on the anticancer effect of chemotherapy. The results suggested that baseline biliary drainage did not adversely impact patient prognosis. The presence of a primary tumour (present vs. absent) and primary tumour site (gallbladder vs. non-gallbladder) had the greatest impact on the prognosis, which suggests that the stratification factors of the present study were appropriately selected.

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Table 5 Multivariate analysis of prognostic factors using Cox's proportional hazard model

Covariate	*P*-value*	Hazard ratio (95% CI)
Biliary drainage (BD vs. non-BD)	0.2875	0.717 (0.389–1.323)
PS (1 vs. 0)	0.1620	1.532 (0.843–2.785)
Primary tumour site (gallbladder vs. non-gallbladder)	0.0454	1.722 (1.011–2.934)
Presence of primary tumour (present vs. absent)	0.0036	2.789 (1.398–5.564)
Extent of disease (metastatic vs. locally advanced)	0.4333	1.391 (0.609–3.176)
Treatment arm (GC arm vs. G arm)	0.2093	0.724 (0.437–1.199)

*Chi-square test.
BD, biliary drainage; CI, confidence interval; G arm, emcitabine monotherapy; GC arm, combination therapy with gemcitabine and cisplatin; PS, performance status.

As the BT22 study was conducted to compare chemotherapy regimens, the data available for investigating biliary drainage, e.g. the site of bile duct obstruction, the approach (endoscopic or percutaneous transhepatic) and the stent material (plastic stent or metallic stent), were limited. Thus, patient baseline characteristics varied substantially. A detailed subgroup analysis on biliary drainage was unfortunately infeasible with the limited sample size of the study. Moreover, this analysis was conducted to investigate the impact of whether or not biliary drainage was performed before starting chemotherapy, and patients requiring biliary drainage during chemotherapy were consequently included in the non-BD subgroup. Data on adverse events occurring during the primary treatment period are available, but adverse events occurring with more advanced cancer in secondary and subsequent treatments were not investigated. No definite conclusions about the relationship of biliary drainage to chemotherapy may therefore be drawn based on the findings of this analysis alone.

Conclusion

In this analysis, GC combination therapy was safely administered and the therapeutic efficacy of the GC arm was maintained in patients with or without biliary drainage. The presence or absence of biliary drainage was not found to impact the efficacy or adverse events in each treatment arm for unresectable BTC. Based on these results, it appears that adequate efficacy with gemcitabine-based chemotherapy can be expected in patients with BTC even with biliary drainage.

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Conflicts of interest

Masanori Taketsuna and Minori Koshiji are employees of Eli Lilly Japan K.K. Takuji Okusaka has received funding to research and attend/present at conference from Eli Lilly Japan K.K. Junji Furuse has received funding to attend/present at conference from Eli Lilly Japan K.K. The authors report no other conflicts of interest.

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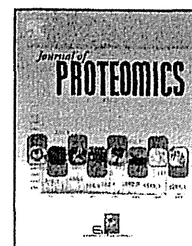

Macrophage-capping protein as a tissue biomarker for prediction of response to gemcitabine treatment and prognosis in cholangiocarcinoma

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ABSTRACT

Cholangiocarcinoma is one of the deadliest malignancies worldwide. Recent studies reported that treatment with gemcitabine was effective in prolonging survival. However, as the treatment only benefited a limited subset of patients, selection of patients before treatment is required. To discover biomarkers predictive of the response to gemcitabine treatment in cholangiocarcinoma, we examined the proteome of three types of material resource; ten cell lines, nine xenografts and nine surgically resected primary tumors from patients who exhibited different response to gemcitabine treatment. Two-dimensional difference gel electrophoresis generated quantitative protein expression profiles including 3571 protein spots. We detected 172 protein spots with significant correlation with response to gemcitabine treatment. All proteins corresponding to these 172 protein spots were identified by mass spectrometry. We found that the macrophage-capping protein (CapG) was associated with response to gemcitabin treatment in all three types of material source. Immunohistochemical validation in an additional set of 196 cholangiocarcinoma cases revealed that CapG expression was associated with lymphatic invasion status and overall survival. Multivariate analysis showed that CapG protein expression was an independent prognostic factor for overall survival. In conclusion, CapG was identified as a novel candidate biomarker to predict response to gemcitabine treatment and survival in cholangiocarcinoma.

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1. Introduction

Cholangiocarcinoma is one of the leading causes of cancer death, the incidence of which is rising worldwide [1–3]. Cholangiocarcinoma is classified into the intra- and extrahepatic types, both having poor clinical outcome; the 5-year survival rate after resection is 8–47% and 20–54% for intra- and extrahepatic cholangiocarcinoma (IHCC and EHCC) respectively [4]. Previous studies have reported that surgical resection is the only curative treatment [5–8], and no standard chemotherapy regimen has been established for inoperable or recurrent cases after surgical resection.

Gemcitabine (GEM, 2′-deoxy-2′-difluorodeoxycytidine: Gemzar, Eli Lilly, Indianapolis, IL), a deoxycytidine analog with structural and metabolic similarities to cytarabine, has been reported to benefit patients with unresectable, locally advanced or metastatic adenocarcinoma, and has been considered as a first-line chemotherapy for cholangiocarcinoma [1]. However, in cholangiocarcinoma, response rates for gemcitabine treatment range from 8 to 36%, and the median survival period of the patients subjected to gemcitabine treatment ranges from 6.3 to 16 months [1]. These observations suggest that certain molecular variables may exist that explain the different response to GEM treatment in cholangiocarcinoma. The identification of biomarkers predictive of the patients' response to GEM treatment will allow selective use of gemcitabine and is urgently needed in practice. To date, however, there has been no attempt to clarify the molecular mechanisms of the varying response to GEM treatment in cholangiocarcinoma.

The proteome is a functional translation of the genome directly regulating cancer phenotypes, and cancer proteomics has revealed the molecular background of carcinogenesis and cancer progression of a range of tumor types. Proteomic studies have identified biomarker candidates and possible therapeutic targets in hepatocellular carcinoma [9,10], cholangiocarcinoma [11,12], and pancreatic cancer [13,14]. However, proteomic tools have not yet been employed to develop biomarkers predictive of the efficacy of GEM treatment in any type of malignancy, probably because of the difficulty in obtaining suitable clinical material.

In this report, we investigated the proteomic features corresponding to the response to GEM treatment in cholangiocarcinoma in three types of material resource; the proteome of cell lines, tumor xenografts and primary tumors from cholangiocarcinoma patients who exhibited different response to GEM treatment were examined by two-dimensional difference gel electrophoresis (2D-DIGE) [15]. As a result, macrophage-capping protein (CapG) was identified as a biomarker candidate predictive of the efficacy of GEM treatment. The prognostic performance of CapG was confirmed by immunohistochemistry in an additional set of 196 cholangiocarcinoma cases. This is the first report concerning the predictive and prognostic value of CapG expression in cholangiocarcinoma. By measuring CapG expression in primary tumors, we will be able to optimize current therapeutic strategies for patients with cholangiocarcinoma.

2. Materials and methods

We examined the protein expression profiles of ten cholangiocarcinoma cell lines, nine xenografts and nine surgically resected tissues from patients who exhibited different response to gemcitabine treatment.

2.1. *Cell lines*

Ten human cholangiocarcinoma cell lines were included in this study (Table 1). Six cell lines, NCC-CC1, NCC-CC3-1, NCC-CC3-2, NCC-CC4-1, NCC-BD1 and NCC-BD2, were established in the National Cancer Center Research Institute [16]. TKKK and TGBC24TKB were purchased from RIKEN Bio Resource Center (Tsukuba, Japan), and OZ and HuCCT1 from the Japanese Collection of Research Bioresources (Osaka, Japan). The TKKK, NCC-CC1, NCC-CC3-1, NCC-CC3-2, and NCC-CC4-1 cell lines were derived from IHCC, while the OZ, TGBC24TKB, HuCCT1, NCC-BD1 and NCC-BD2 cell lines from EHCC [16]. These cell lines were classified into the sensitive and resistant group based on their 50% inhibition (IC50) value for GEM according to our previous report [16]. The protein expression profiles of these cell lines were generated. Cell pellets were embedded in paraffin blocks for immunohistochemistry.

2.2. *Xenografts*

Cells from all cell lines were subcutaneously implanted in 5–7 weeks old congenital athymic female C.B17/Icr-scid (scid/scid) mice (CLEA Japan, Tokyo). The mice were sacrificed when tumor size reached 1–2 cm in diameter. As implantation of NCC-BD2 cells did not result in the development of tumors, samples from nine xenografts were used in the proteomic study (Table 1). The xenografts were grouped in two groups according to the characteristics of the cell lines from which they derived; xenografts from the GEM-sensitive (GEM-sensitive xenografts) and GEM-resistant (GEM-resistant xenograft) cell lines. The resected xenografts were cut into 2–4 mm^3 pieces, snap-frozen in liquid nitrogen, and stored at −80 °C until use. The recovered specimens were histologically examined by a certified pathologist (H.O.) [16].

Mice were kept at the Animal Care and Use Facilities of the National Cancer Center (Tokyo, Japan) under specific pathogen-free conditions. All experiments were approved by the Animal Care and Ethical Review Board of the National Cancer Center.

2.3. *Case selection*

Among the 100 patients who underwent surgery for cholangiocarcinoma between September 2003 and October 2007, 34 patients had recurrence and received chemotherapy, and were followed up for at least six months. Among these 34 patients, the 24 patients who were treated with GEM were initially selected for the study. The median follow-up period in these 24 patients was 498 days. A further 15 of these cases were excluded because: (1) the drug administration period was less than one month (three cases), (2) there was disagreement on the diagnosis of tumor recurrence offered between the oncologist (T.O.) and radiologist (H.O.) (three cases), (3) the efficacy of GEM treatment was not evaluated adequately (five cases), (4) the histological diagnosis was that of an uncommon type of carcinoma (bile duct cystadenocarcinoma, solid adenocarcinoma and combined carcinoma) in three

Table 1 – Characteristics of samples.

Cell lines

Sample number [a]	Cell line name [b]	Origin of cell lines [b]	Drug sensitivity [c]	Xenograft [d]
1	TGBC24TKB	RIKEN Bio Resouce Center, EHCC	Sensitive	26
2	Hucct1	Japanese Collection of Research Bioresources, EHCC	Sensitive	25
3	TKKK	RIKEN Bio Resouce Center, IHCC	Resistant	31
4	OZ	Japanese Collection of Research Bioresources, EHCC	Resistant	27
5	NCC-CC1	National Cancer Center, IHCC	Resistant	32
6	NCC-BD1	National Cancer Center, EHCC	Sensitive	28
7	NCC-CC3-2	National Cancer Center, IHCC	Sensitive	30
8	NCC-CC4-1	National Cancer Center, IHCC	Sensitive	33
9	NCC-BD2	National Cancer Center, EHCC	Resistant	–
10	NCC-CC3-1	National Cancer Center, IHCC	Sensitive	29

Xenografts

Sample number [a]	Name of cell line [e]	Drug sensitivity [f]
25	Hucct1	Sensitive
26	TGBC24TKB	Sensitive
27	OZ	Resistant
28	NCC-BD1	Sensitive
29	NCC-CC3-1	Sensitive
30	NCC-CC3-2	Sensitive
31	TKKK	Resistant
32	NCC-CC1	Resistant
33	NCC-CC4-1	Sensitive

Primary tumors with recurrence that underwent gemcitabine therapy after surgery

Sample number [a]	Patient age	Patient gender	Pathological diagnosis [g]	Histologic type [h]	TNM stage [i]		Duration of SD (month) [j]
34	72	Female	Upper BDCa	Adeno/mod	pT4N1M0	III	0.20
35	48	Male	Hilar BDCa	Adeno/well	pT3N1M0	IIB	3.67
36	60	Male	Entire BDCa	Pap	pT2N0M0	II	1.43
38	61	Male	Middle BDCa	Adeno/mod	pT3N1M0	IIB	6.83
39	67	Male	Lower BDCa	Adeno/mod	pT3N1M0	IIB	8.33
40	75	Male	CCC	Adeno/mod	pT3N0M0	IIIA	10.30
41	68	Male	CCC	Adeno/mod	pT4N0M0	IIIB	7.60
43	50	Male	CCC	Adeno/mod	pT1N0M0	I	6.23
44	72	Male	Lower BDCa	Adeno/mod	pT2N0M0	IB	3.67

[a] Sample numbers correspond to those in GeMDBJ Proteomics (https://gemdbj.nibio.go.jp/dgdb/DigeTop.do).
[b] The characteristics of cell lines were described in our previous paper (16). IHCC, intrahepatic cholangiocarcinoma; EHCC, extrahepatic cholangiocarcinoma.
[c] The *in vitro* assay for determining the sensitivity of the treatment with gemcitabine was detailed in our previous paper (16).
[d] These xenografts were made following transplantation of cell line material. Sample numbers correspond to those in GeMDBJ Proteomics (https://gemdbj.nibio.go.jp/dgdb/DigeTop.do).
[e] These cell lines provided the material used for transplantation.
[f] Determining the sensitivity was based on the drug sensitivity of the original cell lines.
[g] CCC, Cholangiocellular carcinoma; Upper BDCa, Upper third of extrahepatic bile duct carcinoma; Hilar BDCa, Hilar Bile duct carcinoma; Entire BDCa, Entire extrahepatic bile duct carcinoma; Middle BDCa, Middle third of extrahepatic bile duct carcinoma; Lower BDCa, Lower third of extrahepatic bile duct carcinoma.
[h] Adeno, Adenocarcinoma; mod, moderately differentiated; well, well differentiated; Pap, Papillary adenocarcinoma; Muc, Mucinous adenocarcinoma.
[i] TNM Classification of Malignant Tumours, Sobin LH, Wittekind Ch (eds): International Union Against Cancer (UICC): "TNM classification of malignant tumors". 6th ed. New York: Wiley; 2002 (18).
[j] The duration of the period in which the disease was stable (SD) after treatment with gemcitabine.

cases, (5) preoperative radiation therapy had been performed in one patient. Finally, nine cases met all the criteria and were subjected to the proteomic study (Table 1). The effects of chemotherapy were assessed by an oncologist (T.O.) and a radiologist (H.O.) in accordance with the chemotherapy response evaluation criteria in solid tumors (RECIST) guidelines [17]. None of the nine patients was judged as showing a complete response (CR) or a partial response (PR). The patients were grouped into the effective or non-effective group. The "effective" group included five patients whose disease state was judged as stable (SD) for more than six months during chemotherapy. The "non-effective" group included four patients whose disease state was judged as stable (SD) for less than six months, or as progressive (PD) during chemotherapy.

The expression of CapG was evaluated in an additional set of 196 patients with cholangiocarcinoma who underwent initial surgical resection between February 1990 and June 2004 at the National Cancer Center Hospital. No patients in this study received any preoperative therapy. The clinicopathological features of the patients are listed in Supplementary Table 1. Tumors were classified according to the International Union against Cancer tumor-node-metastasis (TNM) classification [18]. This study was approved by the Ethical Review Board of the National Cancer Center, and informed consent was obtained from all patients in this study.

2.4. *Laser microdissection*

As cholangiocarcinoma tissues are rich in stroma cells, laser microdissection was performed to recover pure populations of tumor cells [15,19] (Fig. 1A). In brief, 10 μm thick frozen sections were prepared from tumor tissues and stained with hematoxylin and eosin (HE) or hematoxylin alone. The cells were recovered under microscopic observation with the use of ultraviolet laser (MMI CellCut; Molecular Machines & Industries, Glattburg, Switzerland). One mm^2 area of tumor tissue (~3000 cells) was recovered for each 2D-DIGE gel image. The proteins were extracted from the recovered tissues using a urea lysis buffer containing 6 M urea, 2 M thiourea, 3%{3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate}, and 1% Triton X-100, and stored at −80 °C until use.

2.5. *2D-DIGE and image analysis*

2D-DIGE was performed as previously described [15,19]. In brief, a common internal control sample was created by mixing a small portion of all protein samples used in this study, and labeled with Cy3 fluorescent dye (CyDye DIGE Fluor saturation dye, GE Healthcare Biosciences, Uppsala, Sweden). Individual samples were labeled with Cy5 fluorescent dye (CyDye DIGE Fluor saturation dye, GE Healthcare Biosciences). These differently labeled protein samples were mixed together, and separated by two-dimensional gel electrophoresis (2D-PAGE), according to the isoelectric point and molecular weight of the proteins (Fig. 1B). The first dimension separation was achieved using a 24 cm-length immobiline gel (IPG, pI 4–7, GE Healthcare Biosciences), while the second separation using a home-made gradient gel and GiantGelRunner (Biocraft, Tokyo, Japan), with

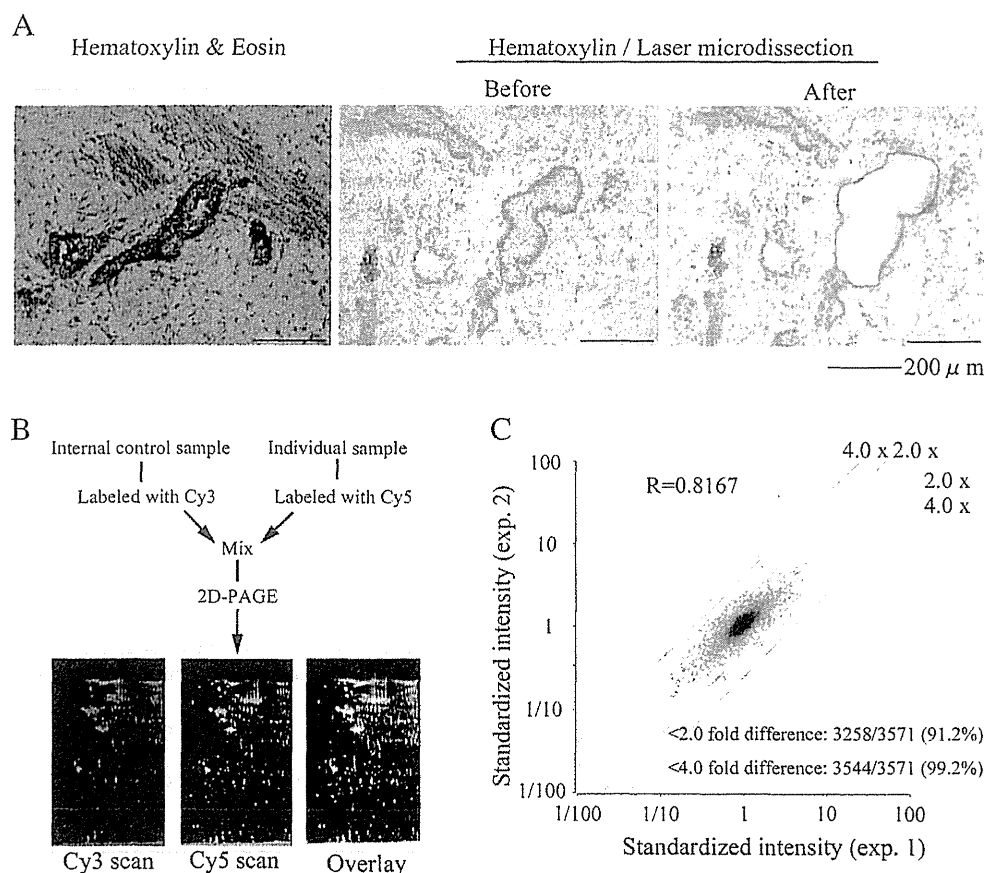


Fig. 1 – (A) Pure populations of tumor cells were recovered using laser microdissection to achieve accurate and cell-specific expression profiles. Tissue sections were stained with standard hematoxylin and eosin, and neighboring sections were stained with hematoxylin alone for laser microdissection. (B) The proteins extracted from laser microdissection tissues were labeled with fluorescent dyes. After mixing the internal control sample with the individual one, the protein samples were separated by 2D-PAGE. After gel electrophoresis, gel images were obtained by laser scanning. (C) Scatter graph demonstrating the high reproducibility of 2D-DIGE. The reproducibility was examined by running the identical sample, Sample 3 (Table 1), twice, and the intensities of all spot pairs were compared.

a separation distance of 36 cm. The gels were scanned using a laser scanner (Typhoon Trio, GE Healthcare Biosciences) at the appropriate wavelength for Cy3 or Cy5. For all protein spots, the Cy5 intensity was normalized with the Cy3 intensity in the same gel using the Progenesis SameSpots software version 3 (Nonlinear Dynamics, Newcastle, UK), so that gel-to-gel variations were eliminated (Fig. 1B). All samples were examined in triplicate gels and the mean normalized intensity value was used for the analysis.

2.6. *Data analysis*

The spot intensity data were subjected to scatter plot, hierarchical clustering analysis and Wilcoxson test using the Expressionist software (GeneData, Basel, Switzerland).

2.7. *Mass spectrometric protein identification*

The proteins corresponding to the spots of interest were identified by mass spectrometry [20]. In brief, 100 μg of protein samples were labeled with Cy5, and separated by 2D-PAGE. Then proteins were recovered and digested with modified trypsin (Promega, Madison, WI). The trypsin digests were subjected to liquid chromatography coupled with tandem mass spectrometry equipped with a nanoelectrospray ion source (Finnigan LTQ linear ion trap mass spectrometer, Thermo Electron Co., San Jose, CA). The Mascot software (version 2.2; Matrix Science, London, UK) was used to search for the mass of the peptide ion peaks against the SWISS-PROT database (Homo sapiens, 471472 sequences in Sprot_57.5.fasta file). Proteins with a Mascot score of 34 or more were considered as positively identified. When multiple proteins were identified in a single spot, the proteins with the highest number of peptides were considered as those corresponding to the spot (Supplementary Table 3).

2.8. *Western blotting*

Protein samples were separated by sodium dodecyl sulfate (SDS)-PAGE and subsequently blotted on a nitrocellulose membrane. Western blotting was performed using antibodies to CapG (1:500; Proteintech Group, Inc, Chicago, IL) and actin (1:250; Sigma, Saint Louis, MO), and horseradish peroxidase-conjugated secondary antibodies (1:1,000; GE Healthcare Biosciences). The immunocomplex was detected by the enhanced chemiluminescence system (ECL Plus; GE Healthcare Biosciences) and LAS-3000 (Fuji Photo Film , Tokyo, Japan).

2.9. *Immunohistochemistry*

Immunohistochemical staining for CapG was performed on formalin-fixed and paraffin-embedded tissue sections using a polymer-based method (Envision Dual Ling System-HRP, Dako, DK-2600 , Glostrup, Denmark). The sections were deparaffinized, dehydrated and treated with 10 ml/l H_2O_2 in methanol for 30 min to remove endogenous peroxidase activity. For antigen retrieval, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 121 °C for 10 min. The antibody to CapG (Proteintech Group, Chicago, IL) was used at a dilution of 1:500. The sections were visualized using 3,30-diaminobenzidine tetrahydrochloride as a chromogen. Finally, the sections were couterstained with hematoxylin. Staining was assessed by two independent observers in a blinded fashion for clinical data (H.O. and N.M.). Possible discrepancies between the observers were solved by discussion and re-examination.

2.10. *Statistical analysis*

Associations between the results of immunohistochemistry and clinicopathological factors were assessed by the $\chi 2$ test. Cumulative survival rates and survival curves were calculated by the Kaplan–Meier method [21]. The log-rank test was performed for the comparison of survival curves. Cox's proportional hazard model was performed to estimate the hazard ratio (HR) and 95% confidence interval (CI) of outcome. Multivariate analyses were performed using risk factors for outcome. Risk factors with p value less than 0.05 were considered to be significant. Statistical analyses were performed using the SPSS 11.0 statistical package (SPSS, Chicago, IL).

3. Results

The generated protein expression profiles of the cholangiocarcinoma cell lines and tumors examined consisted of 3571 protein spots (Supplementary Table 2). The spot numbers in Supplementary Table 2 were defined using Progenesis SameSpot, and commonly used for all gels. We monitored the system reproducibility by running Sample 3 (Table 1) twice. The scatter plot showed that the intensity value of 91.2% of protein spots was scattered within a 2-fold value difference, and that the correlation coefficient of the paired intensity for all protein spots was 0.8167, demonstrating the high reproducibility of our profiling method using 2D-DIGE (Fig. 1C). We compared the profiles of the groups with different response to GEM treatment. The result of comparative studies is summarized as a heat-map in Fig. 2. Among the 3571 protein spots detected in total, we identified 17 spots whose intensity was different (more than two fold difference, Wilcoxson $p<0.05$) between the sensitive and resistant cell line group, and 38 spots whose intensity was different between the sensitive and resistant xenograft group. We also found 117 protein spots whose intensity was different between the effective and non-effective group of cases. Total number of protein spots identified was 172 as certain identical protein spots repeatedly showed the intensity differences. We identified 156 distinct gene products corresponding to all these 172 protein spots by mass spectrometry (Supplementary Table 3). The results of the expression study and protein identification are demonstrated as a heatmap in Fig. 2, and the localization of the identified protein spots and the results of protein identification are exhibited in our database (Genome Medicine Database of Japan Proteomics, GeMDBJ Proteomics, https://gemdbj.nibio.go.jp/dgdb/DigeTop.do) and Supplementary Table 3. Hierarchical clustering showed that protein samples were grouped according to the response to GEM treatment on the basis of the intensity of 172 protein spots. Three proteins, cytokeratin

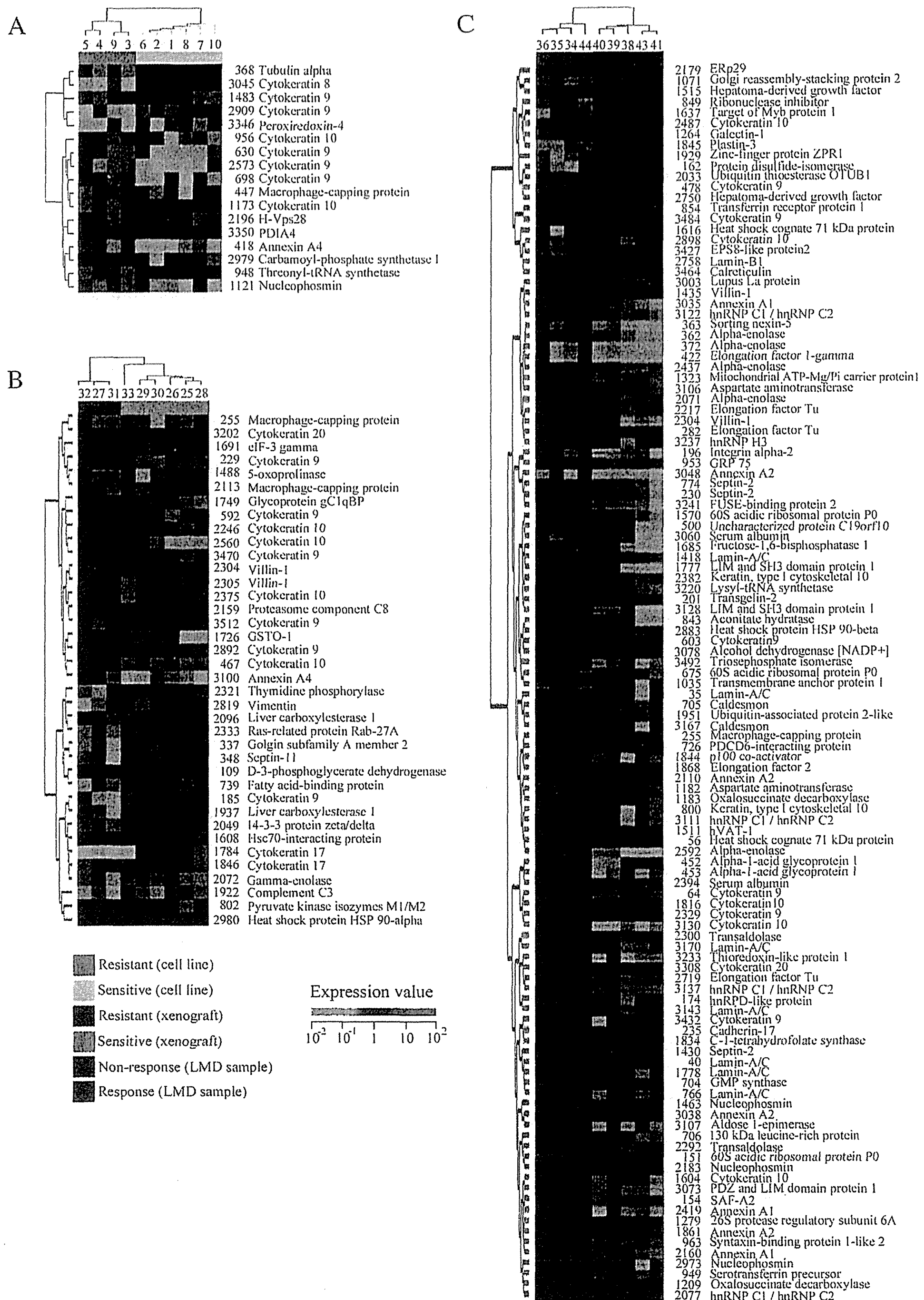


Fig. 2 – Results of protein expression study by 2D-DIGE and protein identification by mass spectrometry. (A) cell lines, (B) xenografts, and (C) primary tumors. The protein samples were grouped according to the response to GEM treatment based on the intensity of 17, 38, and 117 protein spots in (A), (B) and (C), respectively. The numbers of the sample and protein spots correspond to those in Table 1 and Supplementary Table 3.

9 (KRT9), cytokeratin 10 (KRT10) and macrophage capping protein (CapG), were commonly detected as proteins with different expression between the groups compared using cell lines, xenografts, and surgically resected tumors (Fig. 2). Although macrophage-capping protein could be detected as the difference proteins at three positions, namely spots 255, 447 and 2113 (Fig. 3A), the expression of these three species was different among samples. Specifically, cultured cancer cells expressed only spot 447 species, while the xenografts of these cells showed expression of spot 255 and 2113 isoforms. In contrast, the cancer tissues in surgical specimens showed expression of spot 255 only. These observations were summarized in Fig. 3B.

We further validated the relationship of the expression of these three proteins with response to GEM treatment by western blotting. As laser microdissection did not yield adequate amounts of protein from the primary tumors, we only examined cell lines and xenografts. Western blotting showed that the expression of CapG was inversely correlated with response to GEM treatment. This finding was correlated significantly with the corresponding 2D-DIGE results (Fig. 4A) in both the cell line (correlation coefficient: 0.509 in spot No. 447) and the xenograft (correlation coefficient: 0.503 in spot No.255, 0.652 in spot No. 2213) sample subset. We found that the Western blotting data for KRT9 and KRT10 did not correlate with 2D-DIGE data in either the cell line or the xenograft samples (data not shown).

We performed immunohistochemistry to evaluate CapG expression in cell lines, xenografts and surgically resected primary tumors. The intensity of CapG staining was defined as follows: 2+, strong cytoplasmic and/or nuclear staining in ≥30% cancer cells; 1+, faint or moderate cytoplasmic staining and no nuclear staining in ≥50% cancer cells, if 2+ cancer cells <30%; 0, faint or moderate cytoplasmic staining and no nuclear staining in <50% cancer cells, if 2+ cancer

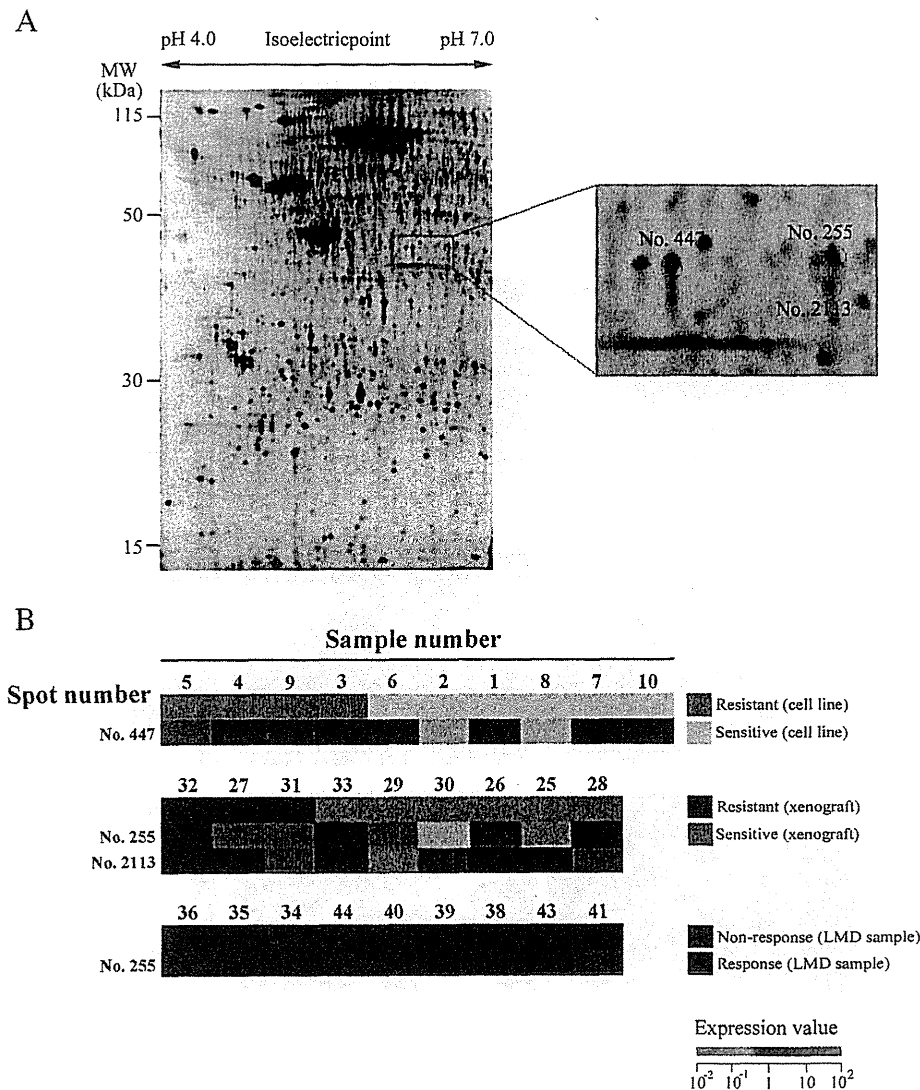
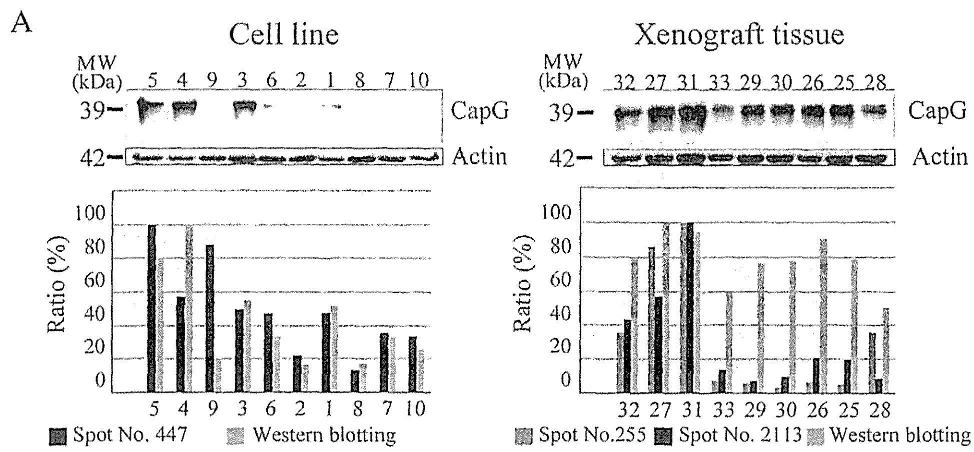


Fig. 3 – Intensity and localization of protein spots for CapG. A. The localization of CapG isoforms on the 2D image. The number of spots corresponds to those in Fig. 2. The 2D image was created by the internal control sample. B. Intensity of protein spots for CapG was exhibited by heat-maps. The protein spots with statistically (Wilcoxson p value less than 0.05) and significantly (more than 2 fold) different intensity between the groups are demonstrated with the spot number. Note that the protein spots for CapG with different intensity were unique to the sample sets.



B

CapG stained tumor cells

Nuclear staining
and/or
Strong cytoplasmic staining: more than 30%

Nuclear staining
and/or
Strong cytoplasmic staining: less than 30%

Weak cytoplasmic staining in
≥ 50% cells

Weak cytoplasmic staining in
< 50% cells

IHC Cell line

Sample 4

Sample 9

Sample 10

—100 μm

IHC Xenograft tissue

Sample 31

Sample 32

Sample 30

—200 μm

IHC Primary tumor

Sample 36

Sample 44

Sample 40

—200 μm

2+ 1+ 0

CapG positive

CapG negative

Table 2 – Immunohistochemistry results based on the criteria shown in Fig. 4.

Sample number [a]	Cell line [b,e]	GEM	CapG [d]			CapG IHC [d]
		Sensitive or resistant [c,f]	No staining (%)	Weak staining (%)	Strong staining (%)	
Cell lines						
5	NCC-CC1	Resistant	0	10	90	2+ (positive)
4	OZ	Resistant	70	0	30	2+ (positive)
9	NCC-BD2	Resistant	50	50	0	1+ (positive)
3	TKKK	Resistant	30	30	40	2+ (positive)
6	NCC-BD1	Sensitive	80	0	20	0 (negative)
2	Hucct1	Sensitive	80	15	5	0 (negative)
1	TGBC24TKB	Sensitive	20	30	50	2+ (positive)
8	NCC-CC4-1	Sensitive	100	0	0	0 (negative)
7	NCC-CC3-2	Sensitive	60	40	0	0 (negative)
10	NCC-CC3-1	Sensitive	80	20	0	0 (negative)
Xenografts						
32	NCC-CC1	Resistant	0	70	30	2+ (positive)
27	OZ	Resistant	0	50	50	2+ (positive)
31	TKKK	Resistant	0	10	90	2+ (positive)
33	NCC-CC4-1	Sensitive	80	5	15	0 (negative)
29	NCC-CC3-1	Sensitive	100	0	0	0 (negative)
30	NCC-CC3-2	Sensitive	100	0	0	0 (negative)
26	TGBC24TKB	Sensitive	70	20	10	0 (negative)
25	Hucct1	Sensitive	60	40	0	0 (negative)
28	NCC-BD1	Sensitive	90	10	0	0 (negative)

Primary tumor tissues with recurrence undergoing gemcitabine therapy after surgery

Sample number [a]	Duration of SD [g]	CapG [d]			CapG IHC [d]
		No staining (%)	Weak staining (%)	Strong staining (%)	
34	LT6	0	60	40	2+ (positive)
35	LT6	0	20	80	2+ (positive)
36	LT6	0	30	70	2+ (positive)
44	LT6	0	90	10	1+ (positive)
38	MT6	20	60	20	1+ (positive)
43	MT6	100	0	0	0 (negative)
41	MT6	100	0	0	0 (negative)
39	MT6	70	30	0	0 (negative)
40	MT6	100	0	0	0 (negative)

[a] Sample numbers correspond to those in GeMDBJ Proteomics (https://gemdbj.nibio.go.jp/dgdb/DigeTop.do).
[b] The characters of cell lines were described in our previous paper (16).
[c] *In vitro* assay for determining the sensitivity for the treatment with gemcitabine was detailed in our previous paper (16).
[d] CapG, macrophage-capping protein. IHC, immunohistochemistry. The diagram of CapG classification is described in Fig. 4.
[e] These cell lines provided the material used for transplantation.
[f] Determining the sensitivity was based on the drug sensitivity of the original cell lines.
[g] The duration of the period in which the disease was stable (SD) after treatment with gemcitabine; LT6, less than 6 months; MT6, more than 6 months.

cells <30%. Samples with an immunohistochemical score of 1+ or 2+ were defined as positive and the others as negative (Fig. 4B). Under these criteria, we divided the cases into the CapG positive and negative groups. As shown in Table 2, all four "resistant" cell lines (100%), and one of six "sensitive" cell lines (17%) were CapG positive. All three resistant xenografts (100%) were CapG positive, and all six sensitive xenografts (100%) were CapG negative. All four samples from the non-effective group (100%) and one of five samples from the effective group (20%) were CapG positive. These immunohistochemistry

Fig. 4 – (A) Western blotting. The sample numbers correspond to those in Table 1. Upper panel; Western blotting image. Lower panel; the corresponding quantified data of 2D-DIGE and Western blotting. The ratio of standardized intensity of protein spots for CapG in 2D-DIGE and the intensity of protein bands for CapG in Western blotting are demonstrated. (B) The diagram demonstrates the scoring system used for CapG staining. CapG staining was scored as 0, 1+, or 2+. A score of 1+ or 2+ was defined as positive, and the score of 0 was defined as negative. Typical images for CapG positive and negative staining are shown.