The combination of GC has also been studied by many researchers for the treatment of BTC (Park *et al*, 2006; Eckel and Schmid, 2007; Pasetto *et al*, 2007; Lee *et al*, 2008). So far, the largest randomised Phase III study has been the recent UK ABC-02 study, in which the efficacy and safety of gemcitabine 1000 mg m^{-2} alone *vs* the combination of gemcitabine 1000 mg m^{-2} plus cisplatin 25 mg m^{-2} was evaluated by British research groups (Cancer Research UK and University College London). That study was initiated as a randomised phase II study with gemcitabine alone *vs* GC (UK ABC-01 study) and then was expanded to a phase III study (ABC-02 study) (Valle *et al*, 2009a, b).

Our study was planned to follow-up on an earlier study of gemcitabine monotherapy conducted in Japanese BTC patients (Okusaka *et al*, 2006). Given the encouraging results from the UK ABC-01 study, we conducted this study to (1) evaluate both gemcitabine monotherapy and the GC combination in Japanese BTC patients, and (2) determine whether benefits similar to those observed in the UK study could be obtained for the combination regimen.

The primary objective of the study was to compare the 1-year survival rate in patients with BTC who received one of these two therapies. The secondary objectives included response rate, progression-free survival (PFS) and assessment of safety.

MATERIALS AND METHODS

Study design

This was a multicentre, randomised phase II study to evaluate the efficacy and safety of GC combination compared with single-agent gemcitabine in chemotherapy-naive patients with locally advanced or metastatic BTC. Patients were randomised to either single-agent gemcitabine 1000 mg m^{-2} on days 1, 8 and 15 of a 28-day cycle (G-arm) or cisplatin 25 mg m^{-2} followed by gemcitabine 1000 mg m^{-2} on days 1, 8 of a 21-day cycle (GC-arm). Randomisation was stratified by primary site (gallbladder cancer or other BTC) and the presence or absence of primary tumour.

Eligibility criteria

Eligible patients met the following criteria: histologically confirmed unresectable locally advanced or metastatic cancer of the biliary tract; no history of earlier chemotherapy; performance status of 0 or 1; a life expectancy of at least 3 months; at least 20 years of age at the time of study entry; adequate function of major organs (haemoglobin $\geqslant$10 g per 100 ml, white blood cells $\geqslant$3000/mm^3, neutrophils $\geqslant$1500/mm^3, platelets $\geqslant$100 000/mm3, AST/ALT/ALP $\leqslant$3 times upper limit of normal (ULN), total bilirubin $\leqslant$2 times ULN, $\leqslant$3 times ULN for patients with obstructive jaundice or metastases to the liver, serum creatinine $\leqslant$1.5 times ULN, creatinine clearance or 24-h creatinine clearance $\geqslant$45 ml min^{-1}).

This study followed the ethical principles that have their origins in the Declaration of Helsinki, and was conducted in accordance with the protocol, the 'ordinance on Good Clinical Practice' and related regulations. Written informed consent was obtained from all patients who were considered eligible for participation in this study before enrolment. The Efficacy and Safety Evaluation Committee, an independent review board, was consulted if any efficacy and safety issues arose in the study.

Study treatment

The assigned treatment was given for a minimum of 12 weeks (at least four cycles in the GC-arm and three cycles in the G-arm) and continued to a maximum of 48 weeks (up to 16 cycles in the GC-arm and up to 12 cycles in the G-arm), unless disease progression (PD) was evident, an intolerable adverse event occurred or the patient was required to withdraw from the study.

Efficacy and safety assessment

All patients who received at least 1 dose of the study drug were included in the efficacy and safety assessment. Response rate was evaluated according to the Response Evaluation Criteria in Solid Tumors. Evaluation of tumours after patient randomisation was performed every 6 weeks until PD. Adverse events were graded according to the Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE v3.0).

Statistical design and analysis

The sample size was calculated by the selection method of Simon (Simon *et al*, 1985), which is based on the proposition that GC combination therapy is selected if the 1-year survival rate for the GC-arm is higher than that for the gemcitabine arm. We assumed a 1-year survival rate of 25% for the G-arm and 35% for GC-arm (Okusaka *et al*, 2006; Park *et al*, 2006). With these assumptions, 30 patients per arm were needed to appropriately select the combination therapy with a probability of $\geqslant$80%. To optimise safety and efficacy information, the sample size was set to 42 patients per arm.

The Kaplan–Meier method was used to estimate 1-year survival (primary outcome), PFS and 6-month PFS rates (secondary outcomes) for each treatment arm; 95% confidence intervals (CIs) were calculated. A Cox proportional hazards model was used to calculate the hazard ratio, 95% CI and its two-tailed *P*-value. Fisher's exact test was used to compare the patient characteristics, response and disease control rates, and toxicities between the two treatment arms. The exact CIs were calculated based on binomial distributions.

RESULTS

Patients

This study was carried out from September 2006 to October 2008 at nine study centres in Japan. Eighty-four patients were randomised to either gemcitabine monotherapy (G-arm) or GC combination (GC-arm). One patient assigned to the GC-arm was not treated because the general condition of the patient deteriorated before study treatment. All of the remaining 83 patients, 41 in the GC-arm and 42 in the G-arm, received at least 1 dose of study treatment. Efficacy and safety were evaluated for each of these 83 patients (Figure 1). Demographic variables (Table 1) were well balanced between the two treatment arms, except for patients with ampullary carcinoma (4 in GC-arm, 0 in G-arm).

Drug exposure and duration of the treatments

A total of 247 (median 6.0) and 203 (median 4.0) cycles were administered in the GC-arm and G-arm, respectively. Relative dose intensities were 78.9% for gemcitabine and 79.0% for cisplatin in the GC-arm, and 87.4% for gemcitabine in the G-arm. Three patients in the GC-arm and two patients in the G-arm completed 48 weeks treatment.

Efficacy

A total of 83 patients were evaluable for tumour response according to the protocol, 41 in the GC-arm and 42 in the G-arm. No complete tumour responses were observed. In total, eight patients in the GC-arm had a partial response (PR) compared with five patients in the G-arm (PR 19.5 *vs* 11.9%). In addition,

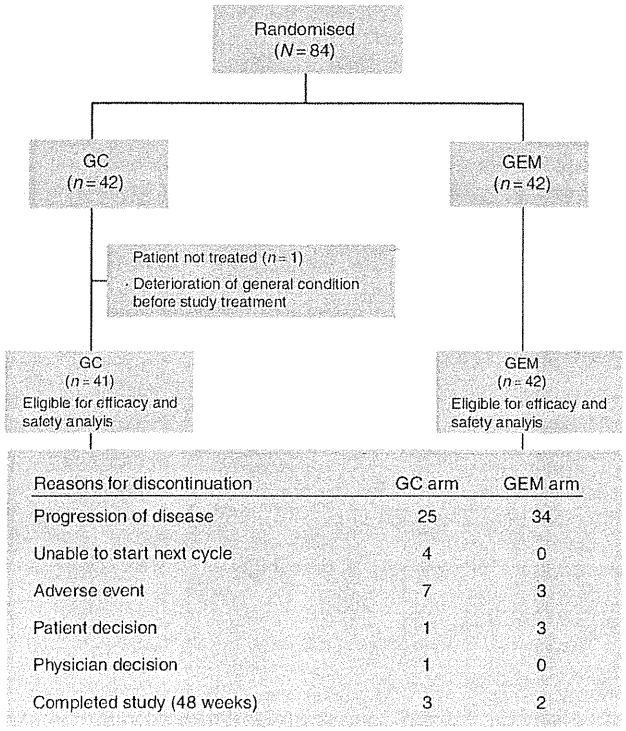

Figure 1 CONSORT diagram. Disposition of patients. GC = gemcitabine–cisplatin combination; GEM = gemcitabine alone.

20 patients had stable disease in the GC-arm *vs* 16 patients in the G-arm (SD 48.8 *vs* 38.1%). The disease control rate (CR + PR + SD) was 68.3% (95% CI: 51.9, 81.9) *vs* 50.0% (95% CI: 34.2, 65.8) in favour of the combination therapy. The 1-year survival rate (39.0 *vs* 31.0%), median survival time (11.2 months *vs* 7.7 months) and median PFS (5.8 months *vs* 3.7 months) were better for the GC-arm *vs* G-arm (Figure 2). The hazard ratio between the GC and G-arms was 0.69 (95% CI: 0.42, 1.13) for overall survival (OS) and 0.66 (95% CI: 0.41, 1.05) for PFS (Table 2).

As shown in Table 3, the prognosis for patients with gallbladder cancer was worse than that for patients with non-gallbladder cancer; however, the median survival times were longer with the GC combination in gallbladder cancer patients (9.1 months *vs* 6.7 months), as well as in patients with non-gallbladder cancer (13.0 months *vs* 8.0 months). The prognosis for patients with primary tumours was worse than that for patients without primary tumours; however, the GC therapy showed longer median survival time in both patient subgroups (9.4 months *vs* 7.4 months in the patients with primary tumours, 16.1 months *vs* 12.7 months in the patients without primary tumours).

Safety

All adverse events observed in this study were predictable and manageable based on the safety profile of GC. As shown in Table 4, the most common grade 3 or higher adverse events (≥25%) were neutropenia (56.1%), thrombocytopenia (39.0%), haemoglobin decrease (36.6%), RBC decrease (34.1%), leukopenia (29.3%) and γ-GTP increase (29.3%) in the GC-arm, and neutropenia (38.1%) and γ-GTP increase (35.7%) in the G-arm. The incidence of haematotoxicity was higher in the GC-arm; grade 3 or more serious C-reactive protein increase was detected only in the monotherapy arm.

Table 1 Patient characteristics

Characteristic	GC (*N* = 41) *n* (%)	GEM (*N* = 42) *n* (%)	*P*-value
Gender			
Male	18 (43.9)	21 (50.0)	0.662
Female	23 (56.1)	21 (50.0)	
Age (year)			
Median	65.0	66.5	0.0812[a]
Range	43–80	49–78	
PS			
0	34 (82.9)	28 (66.7)	0.129
1	7 (17.1)	14 (33.3)	
Primary tumour sites			
Extraheptic bile duct	8 (19.5)	11 (26.2)	0.239
Intraheptic bile duct	14 (34.1)	14 (33.3)	
Gallbladder	15 (36.6)	17 (40.5)	
Ampulla	4 (9.8)	0 (0.0)	
Metastatic sites			
Liver	22 (53.7)	20 (47.6)	0.663
Regional lymph nodes	23 (56.1)	28 (66.7)	0.372
Distant lymph nodes	19 (46.3)	18 (42.9)	0.827
Lung	8 (19.5)	7 (16.7)	0.782
Peritoneum	7 (17.1)	7 (16.7)	1.000
Bone	0 (0.0)	1 (2.4)	1.000
Others	3 (7.3)	3 (7.1)	1.000
Initial onset or recurrence			
Initial onset	30 (73.2)	32 (76.2)	0.804
Recurrence after surgery	11 (26.8)	10 (23.8)	
Histological type			
Adenocarcinoma	39 (95.1)	41 (97.6)	0.616
Adenosquamous cancer	2 (4.9)	1 (2.4)	
Disease stage (gallbladder cancer, extrahepatic bile duct cancer, ampulla cancer)			
IIA	0 (0.0)	0 (0.0)	1.000
IIB	3 (7.3)[b]	2 (4.8)[b]	
III	2 (4.9)	2 (4.8)	
IV	16 (39.0)	17 (40.5)	
Recurrence after surgery	6 (14.6)	7 (16.7)	
Disease stage (intrahepatic bile duct cancer)			
II	0 (0.0)	1 (2.4)[b]	0.389
IIIA	0 (0.0)	1 (2.4)	
IIIB	0 (0.0)	0 (0.0)	
IIIC	0 (0.0)	2 (4.8)	
IV	9 (22.0)	7 (16.7)	
Recurrence after surgery	5 (12.2)	3 (7.1)	
Biliary drainage			
No	25 (61.0)	24 (57.1)	0.824
Yes	16 (39.0)	18 (42.9)	
Previous therapy			
No	30 (73.2)	28 (66.7)	0.855
Surgery	11 (26.8)	12 (28.6)	
Radiotherapy	0 (0.0)	1 (2.4)	
Surgery and radiotherapy	0 (0.0)	1 (2.4)	

Abbreviations: GC = gemcitabine and cisplatin; GEM = gemcitabine; PS = performance status. [a]*t*-test. [b]Patients were diagnosed as having unresectable disease with marked regional node metastases involving the proper hepatic artery and/or main portal vein.

There were no treatment related deaths. Most of the patients recovered from the above adverse events by reducing or discontinuing the study treatment.

Post-study chemotherapy

Thirty patients in the GC-arm received post-study chemotherapy including S-1, tegafur/gimeracil/oteracil potassium (19 patients), gemcitabine (10 patients) and tegafur/uracil (1 patient). In the G-arm, 33 patients received post-study chemotherapy including S-1 (20 patients), gemcitabine (11 patients), cisplatin/fluorouracil (1 patient) and doxorubicin/tegafur/uracil (1 patient).

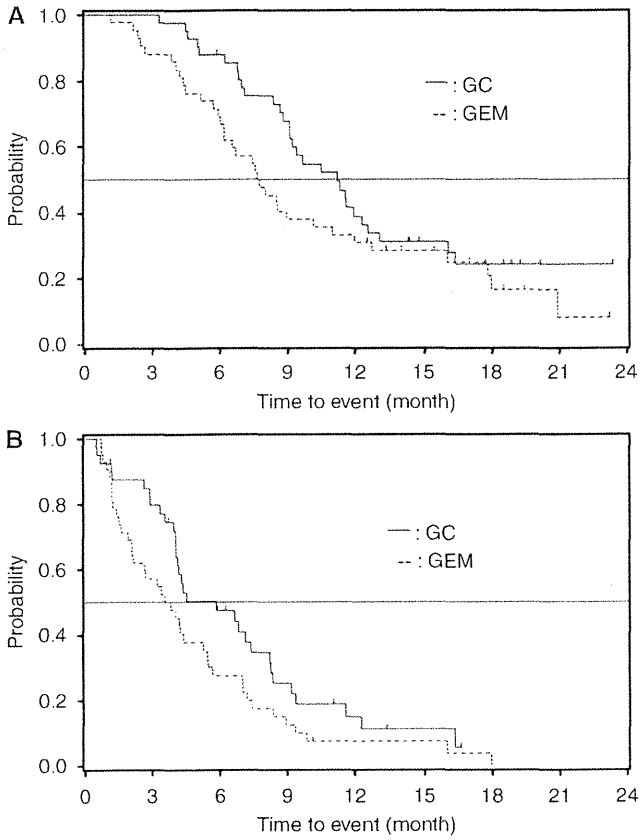

Figure 2 Kaplan–Meier curve of overall survival and progression-free survival. (**A**) Overall survival. (**B**) Progression-free survival. GC = gemcitabine–cisplatin combination; GEM = gemcitabine alone; CI = confidence interval.

DISCUSSION

Although this study (BT22 study) showed that gemcitabine monotherapy and the GC combination were both active in Japanese patients with advanced BTC, a superior benefit was obtained with the combination treatment. In the GC/G-arms, the 1-year survival rate was 39.0%/31.0%, median survival time was 11.2/7.7 months and median PFS time was 5.8/3.7 months (Table 2).

The UK ABC-02 study, which was conducted with the same dose and regimen as this study (Valle *et al*, 2009b), showed a similar benefit for the GC combination. The respective median survival/PFS times in that study were 11.7/8.5 months in their GC-arm, and 8.2/6.5 months in their G-arm.

The hazard ratios reported in the ABC-02 study for OS (0.68, 95% CI: 0.53, 0.86) and PFS (0.70, 95% CI: 0.56, 0.88) compared well with the respective values from our study: 0.69 (95% CI: 0.42, 1.13) and 0.66 (95% CI: 0.41, 1.05). As the number of patients was based on Simon's selection method (Simon *et al*, 1985), this study was not designed to compare and identify statistical significant differences between the two treatment arms. These hazard ratios

Table 3 Overall survival time by stratification factor

Median survival time (months) (95% CI)	GC (*N* = 41)	GEM (*N* = 42)	*P*-value
Tumour site			
Gallbladder	9.1 (6.9, 11.6)	6.7 (4.2, 11.0)	0.675
Non-gallbladder	13.0 (9.2, ***)	8.0 (6.1, 16.0)	0.110
Primary tumour			
Presence of primary tumour	9.4 (8.7, 11.6)	7.4 (5.9, 8.5)	0.253
Absence of primary tumour	16.1 (12.3, ***)	12.7 (6.5, ***)	0.389

Abbreviations: GC = gemcitabine and cisplatin; GEM = gemcitabine; CI = confidence interval. ***denotes upper limits are not available.

Table 2 Summary of time-to-event end points: overall response and survival

	GC (*N* = 41) *n* (%)	GEM (*N* = 42) *n* (%)	*P*-value
Overall response rate			
Complete response (CR)	0 (0.0)	0 (0.0)	
Partial response (PR)	8 (19.5)	5 (11.9)	
Stable disease (SD)	20 (48.8)	16 (38.1)	
Progressive disease (PD)	9 (22.0)	17 (40.5)	
Not evaluable (NE)	4 (9.8)	4 (9.5)	
Response rate (95% CI)	19.5% (8.8, 34.9)	11.9% (4.0, 25.6)	0.380
Disease control rate (CR+PR+SD) (95% CI)	68.3% (51.9, 81.9)	50.0% (34.2, 65.8)	0.119
Overall survival			
1-year survival rate (95% CI)	39.0% (23.7, 54.4)	31.0% (17.0, 44.9)	
Median survival time (95% CI)	11.2 months (9.1, 12.5)	7.7 months (6.1, 11.0)	
Hazard ratio (95% CI)	0.69 (95% CI: 0.42, 1.13)		0.139
Progression-free survival (PFS)			
Median PFS (95% CI)	5.8 months (4.1, 8.2)	3.7 months (2.1, 5.3)	
Hazard ratio (95% CI)	0.66 (95%CI: 0.41, 1.05)		0.077
6-Months PFS rate (95% CI)	47.4% (31.4, 63.4)	27.7% (14.0, 41.5)	

Abbreviations: GC = gemcitabine and cisplatin; GEM = gemcitabine; CI = confidence interval.

Table 4 Summary of maximum toxicity grades[a] (incidence ⩾30%)

	GC ($N=41$)			GEM ($N=42$)			
	Maximum toxicity grade			Maximum toxicity grade			
Events	**Grade 3 (%)**	**Grade 4 (%)**	**All grades (%)**	**Grade 3 (%)**	**Grade 4 (%)**	**All grades (%)**	***P*-value**
Haematological							
WBC count decreased	29.3	0	87.8	19.0	0	69.0	0.061
Haemoglobin decreased	26.8	9.8	85.4	9.5	7.1	85.7	1.000
Neutrophil count decreased	39.0	17.1	82.9	28.6	9.5	69.0	0.200
Platelet count decreased	26.8	12.2	80.5	4.8	2.4	76.2	0.791
RBC decreased	34.1	0	75.6	14.3	0	78.6	0.798
Haematocrit decreased	4.9	0	58.5	0	0	54.8	0.826
Non-haematological							
Anorexia	0	0	80.5	4.8	0	61.9	0.090
Nausea	0	0	68.3	0	0	42.9	0.027
Fatigue	0	0	58.5	2.4	0	50.0	0.511
AST increased	17.1	0	53.7	14.3	2.4	52.4	1.000
ALT increased	24.4	0	51.2	16.7	0	52.4	1.000
Vomiting	0	0	48.8	0	0	23.8	0.023
GGT increased	29.3	0	46.3	31.0	4.8	50.0	0.827
Pyrexia	0	0	43.9	4.8	0	57.1	0.190
LDH increased	0	0	36.6	0	0	35.7	1.000
Constipation	0	0	36.6	0	0	33.3	0.820
ALP increased	7.3	0	31.7	16.7	0	40.5	0.495
Weight decreased	0	0	31.7	0	0	31.0	1.000
Diarrhoea	2.4	0	31.7	0	0	26.2	0.634
Blood sodium decreased	17.1	0	31.7	9.5	0	19.0	0.214
C-reactive protein increased	0	0	26.8	7.1	0	52.4	0.025

Abbreviations: ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; GC = gemcitabine and cisplatin; GEM = gemcitabine; GGT = γ-glutamyltransferase; LDH = lactate dehydrogenase; RBC = red blood cell; WBC = white blood cell. [a]Events were graded according to CTCAE v3.0.

strongly suggest that the GC combination has superior benefit compared with single-agent gemcitabine, even though there were no statistical significant differences in survival and PFS between the two arms in our study.

Although there have been many single-arm Phase II studies of the GC combination for BTC (Thongprasert *et al*, 2005; Kim *et al*, 2006; Charoentum *et al*, 2007; Meyerhardt *et al*, 2008; Valle *et al*, 2009a), these results have never been distilled to one fixed dose and regimen of GC. Many previous studies of GC combination reported relatively higher response rates, but with more serious treatment-related adverse events (Thongprasert *et al*, 2005; Kim *et al*, 2006; Charoentum *et al*, 2007; Meyerhardt *et al*, 2008). In the phase II study conducted by Thongprasert *et al* (2005), 17.85% of the patients who were treated with the GC combination required dose reduction, and in another Phase II study recently conducted by Meyerhardt *et al* (2008), dose reductions and study withdrawals were required for 50% of the patients who received the combination therapy. In our study, we also observed more frequent adverse events with the doublet (Table 4). However, as shown in Figure 1, only seven patients (17%) discontinued from the study because of adverse events and four patients (9.7%) required dose adjustments in the GC-arm.

Overall, the toxicity observed in this study was manageable. Although interstitial pneumonia was detected in one patient from each of the arms, both patients recovered with appropriate treatment. One grade 3 renal failure and one grade 2 peripheral neuropathy were observed in GC-arm, in line with similar events seen in previous studies of the GC combination (Thongprasert *et al*, 2005; Kim *et al*, 2006; Charoentum *et al*, 2007; Meyerhardt *et al*, 2008; Valle *et al*, 2009a). It is to be noted that despite the higher incidence of haematotoxicity in patients receiving the combination therapy, drug-caused myelosuppression did not result in febrile neutropenia or bleeding. Grade 3 or greater increases in C-reactive protein were observed only in the gemcitabine monotherapy-arm, also suggesting that the combination therapy did not increase neutropenic infections.

In this study, we stratified patients into those with gallbladder cancer and those with other BTCs. Gallbladder cancer has been reported to have a different biological behaviour (Kim *et al*, 2006; Doval *et al*, 2004; Jarnagin *et al*, 2006); furthermore, a pooled analysis by Eckel and Schmid (2007) revealed a higher response rate to chemotherapy and shorter OS for gallbladder cancer compared with other BTCs. As shown in Table 3, patients with gallbladder cancer showed worse survival than patients with other BTCs, this being consistent with previous reports (Eckel and Schmid, 2007; Wagner *et al*, 2009). It is important to note that median survival times were longer with the GC combination in patients with gallbladder cancer (9.1 months *vs* 6.7 months), as well as in patients with non-gallbladder cancer (13.0 months *vs* 8.0 months), suggesting that the combination therapy has greater benefit than monotherapy in gallbladder cancer and other BTC patients.

Another stratification factor used for this study was the presence or absence of a primary tumour, not a commonly used stratification factor in clinical trials for advanced BTC. Locally advanced or metastatic cancer, the stratification factor used in the UK ABC-01 and UK ABC-02 studies, is more commonly used, as both of these have been shown to affect OS in advanced BTC (Park *et al*, 2009). However, considering the importance of surgical resection of the primary tumour, we decided to use this as a stratification factor for patients in this study. As shown in Table 3, patients with primary tumours showed remarkably worse survival than patients without primary tumours. However, because of the limited number of patients in our subanalyses, the results should be viewed with caution, and the usefulness of this prognostic factor should be evaluated in future studies. We will continue our efforts

in collaboration with the UK ABC-02 study group to identify prognostic factors in a larger population, which may significantly affect clinical studies in BTC.

Despite the heterogeneous nature of BTC and the ethnic differences reported for this tumour type (Goodman and Yamamoto, 2007; Aljiffry *et al*, 2009), the outcomes from this study showed striking similarity with the large-scale phase III study (UK ABC-02) results. This suggests that cisplatin 25 mg m^{-2} plus gemcitabine 1000 mg m^{-2} on days 1 and 8 of a 21-day cycle would be beneficial in the treatment of advanced BTC.

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Conflict of interest

TO, KN, NM, SO, SK and JF have received honoraria, and YN, MK, JF and SN are employed by Eli Lilly Japan.

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Cisplatin and Etoposide as First-line Chemotherapy for Poorly Differentiated Neuroendocrine Carcinoma of the Hepatobiliary Tract and Pancreas

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Objective: The combination chemotherapy consisting of cisplatin and etoposide, one of the standard regimens for small cell lung cancer, has been widely used to treat extrapulmonary poorly differentiated neuroendocrine carcinomas. However, there were no prior reports limited to the hepatobiliary tract and pancreas as the primary sites.

Methods: We reviewed the cases in our database from October 1995 to January 2009 and retrospectively examined the clinical data of patients, with unresectable or recurrent poorly differentiated neuroendocrine carcinoma arising from the hepatobiliary tract and pancreas, who received combination chemotherapy with cisplatin and etoposide as the first-line treatment. The chemotherapy regimen consisted of cisplatin 80 mg/m^2 given intravenously on day 1 and etoposide 100 mg/m^2 intravenously on days 1–3, repeated every 3–4 weeks.

Results: Twenty-one patients were treated with the above regimen of cisplatin and etoposide combination chemotherapy. The primary tumor site was the liver in 2 patients, gallbladder in 8 patients, pancreas in 10 patients and ampulla of Vater in 1 patient. Although no complete responses were obtained, three patients had partial responses, resulting in an overall response rate of 14%. Median progression-free survival was 1.8 months, and median overall survival was 5.8 months. The major adverse events were myelosuppression and gastrointestinal toxicities, with Grade 3 or 4 neutropenia (90%), nausea (33%) and anorexia (24%).

Conclusions: Cisplatin and etoposide combination as the first-line chemotherapy for hepatobiliary or pancreatic poorly differentiated neuroendocrine carcinoma had only marginal antitumor activity and relatively severe toxicity compared with previous studies on extrapulmonary poorly differentiated neuroendocrine carcinoma treated with the same regimen.

Key words: cisplatin – etoposide – neuroendocrine carcinoma – chemotherapy

INTRODUCTION

Neuroendocrine tumors are rare tumors that exhibit a variety of morphologic, functional and behavioral characteristics (1). The aggressiveness of these tumors varies greatly depending on the histological degree of differentiation, from well-differentiated neuroendocrine tumors to poorly differentiated neuroendocrine carcinomas (PD-NECs).

No standard treatment for unresectable extrapulmonary PD-NECs has been established yet. However, combined chemotherapy with cisplatin and etoposide, one of the standard regimens employed for the treatment of small cell lung cancer (SCLC), has been used widely for the treatment of extrapulmonary PD-NECs, because the genetic, pathological and clinical features of PD-NECs overlap with those of SCLC (2–6). The previous reports, in general, refer to a

wide variety of extrapulmonary sites of origin of the primary tumors, partly because the rarity of the disease precludes clinical studies devoted to each individual primary origin of the tumors. Thus, there have been no prior reports of treatment limited to neuroendocrine tumors arising from the hepatobiliary and pancreatic region as primary sites.

It is well established that adenocarcinomas arising from the hepatobiliary tract or pancreas have a worse prognosis when compared with that of gastric or colorectal adenocarcinomas, despite the histologies being similar. It remains to be determined whether these tumors of different primary origins can be included within the same group for treatment.

Therefore, it has not yet been clarified whether combined chemotherapy with cisplatin and etoposide might be as effective against hepatobiliary and pancreatic PD-NECs as it is for miscellaneous extrapulmonary PD-NECs. We report our experience of combined chemotherapy with cisplatin and etoposide as the first-line chemotherapy for patients with unresectable or recurrent PD-NECs, focusing on the tumors arising from the hepatobiliary tract and pancreas.

PATIENTS AND METHODS

Patients

Between October 1995 and January 2009, in total, 25 patients with PD-NEC arising from the hepatobiliary tract and pancreas were treated at the National Cancer Center Hospital, Tokyo, Japan. Of these 25 patients, 21 received the combination of cisplatin and etoposide as the first-line chemotherapy. Before the chemotherapy, tumor specimen obtained by a fine-needle biopsy or a surgical resection was pathologically diagnosed as PD-NECs according to the WHO classification (7,8). Typically, tumor tissue showed a dense proliferation of round or polygonal tumor cells with hyperchromatic nuclei and pale to eosinophilic granular cytoplasm, arranged in sheets, nests and cords. Extensive necrosis and mitotic figures were frequently observed. Immunohistochemically, the tumor cells expressed endocrine markers, such as chromogranin A, synaptophysin, neuron-specific enolase (NSE) and/or CD56. A Ki-67 proliferation index >15% was documented in the 21 patients receiving the cisplatin plus etoposide combination chemotherapy.

Treatment Schedule

Cisplatin, 80 mg/m^2, was administered intravenously (IV) over 2 h on the first day with adequate hydration. Etoposide, 100 mg/m^2/day, was administered IV over 2 h on days 1–3. This treatment was repeated every 3–4 weeks for a maximum of six cycles unless disease progression or unacceptable toxicity occurred. In two patients, a modified schedule with split-dose administration of cisplatin at a dose of 25 mg/m^2/day IV on days 1–3 and a reduced dose of etoposide 80 mg/m^2/day IV on days 1–3 was selected from the first cycle because of advanced age and poor performance status (9).

Antiemetic prophylaxis with 5-HT3 antagonists plus dexamethasone was used at the physician's discretion. Recombinant human granulocyte colony-stimulating factor was administered if patients developed febrile neutropenia.

Response and Toxicity Evaluations

Tumor assessments by computed tomographic (CT) scan of the abdomen were carried out at baseline and every cycle according to the Response Evaluation Criteria in Solid Tumors (RECIST). CT scan of the chest was carried out at the baseline and every cycle if a chest X-ray as a screening test detected lung metastases. Responses were to be confirmed by repeated assessments carried out no less than 4 weeks apart. In addition, tumor markers of carcinoembryonic antigen (CEA), cancer antigen (CA)19-9, NSE and progastrin-releasing peptide (ProGRP) were measured every cycle. All adverse events were reviewed based on medical records and evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0.

Statistical Analysis

Overall survival was measured from the date of initial treatment to the date of death or the date of the last follow-up. Death from any cause was considered an event. Survival curves were constructed using the Kaplan–Meier method. Statistical analyses were performed using Dr. SPSS II (SPSS Japan Inc., Tokyo, Japan).

RESULTS

Patient Characteristics

The characteristics of the 21 treated patients are listed in Table 1. The median age of the patients was 57 years, with an almost equal gender distribution. One patient (5%) had metastatic recurrent disease after surgery with curative intent, and 20 (95%) had unresectable metastatic disease at the initial diagnosis. Of the 21 patients, 20 (95%) had elevated serum NSE level and 4 (19%) had elevated serum ProGRP level. The primary tumor sites included the pancreas in 10 patients (48%), gallbladder in 8 (38%), liver in 2 (10%) and ampulla of Vater in 1 (5%). Two patients with multiple liver tumors without a definite primary site were classified as having a liver origin. The most common metastatic site was the liver. Other common sites were lymph nodes and the peritoneum.

Treatment

In total, 57 cycles were administered to the 21 patients with a median of 2 cycles per patient (range, 1–6 cycles). Eight

Table 1. Patient characteristics ($n = 21$)

Characteristics	n (%)
Age (years)	
Median	57
Range	30–70
Sex	
Male	11 (52)
Female	10 (48)
ECOG performance status	
0	9 (43)
1	10 (48)
2	2 (10)
Primary tumor site	
Liver	2 (10)
Gallbladder	8 (38)
Pancreas	10 (48)
Ampulla of Vater	1 (5)
Metastatic site	
Liver	17 (81)
Lung	2 (10)
Spleen	1 (5)
Bone	1 (5)
Adrenal gland	1 (5)
Pleural	1 (5)
Lymph node	11 (52)
Peritoneum/ascites	11 (52)
CEA	
Abnormal	13 (62)
Normal	8 (38)
CA19-9	
Normal	13 (62)
Abnormal	8 (38)
NSE (ng/ml)	
Median	143.1
Range	6–1930
ProGRP[a] (U/ml)	
Median	25.5
Range	11.9–63 090

Abnormal carcinoembryonic antigen (CEA) and CA19-9 represented ≥5 ng/ml and ≥37 U/ml, respectively. ECOG, Eastern Cooperative Oncology Group; NSE, neuron-specific enolase; ProGRP, pro-gastrin-releasing peptide. [a]One patient did not have pre-treatment data examination.

patients (38%) required dose reductions during therapy. Of these patients, three required 20–25% dose reductions for both cisplatin and etoposide due to febrile neutropenia and renal dysfunction, three required a 20% dose reduction of etoposide alone due to febrile neutropenia and the remaining two required a 20% dose reduction of cisplatin alone due to serum creatinine level elevation. The median relative intensities of the doses of cisplatin and etoposide (calculated as the actual dose delivered divided by the intended dose of 3-week interval regimen) were 79% and 73%, respectively. The reasons for treatment discontinuation were radiological progressive disease in 15 patients, clinical progressive disease in 1 patient, unacceptable toxicities in 2 (gastrointestinal toxicity of prolonged Grade 2 nausea and anorexia in one, and renal toxicity as indicated by decreased creatinine clearance to <35 ml/min in the other), cytoreductive surgery in 1 and refusal of treatment by 1 (mental suffering). As for the patient who underwent cytoreductive surgery, she could not maintain response duration until the next course. In addition, she had multiple liver metastases with the maximum size of >13 cm produced abdominal discomfort.

After treatment discontinuation, eight patients received second-line chemotherapy: gemcitabine monotherapy was administered to four patients, irinotecan monotherapy to three, and combination chemotherapy with cisplatin, vincristine, doxorubicin and etoposide (CODE therapy) to one. Among them, one patient, who developed disease progression after one cycle of cisplatin and etoposide, achieved a partial response after two cycles of second-line chemotherapy with gemcitabine. Three patients were treated employing other therapeutic modalities, i.e. cytoreduction surgery, allogeneic peripheral blood stem cell transplantation and chemoembolization for liver metastases. The remaining nine patients received only supportive care.

Efficacy

At the time of analysis, 2 patients were alive with disease and 19 had died of their disease. All patients were assessable for tumor response. Although no patient achieved a complete response, two with gallbladder and one with pancreatic PD-NECs achieved a partial response, giving an overall response rate of 14% (95% confidence interval, 3–36%). Ten patients (48%) had shown stable disease and the remaining eight (38%) had progressive disease. The duration of the three objective responses were 2.4, 3.1 and 3.5 months. During treatment, the serum NSE level was reduced by >50% in 15 (75%) of 20 patients who had shown a pre-treatment level of ≥15 ng/ml. All patients were included in the survival assessment. Median progression-free survival, median overall survival and the 1-year survival rate were 1.8, 5.8 months and 5%, respectively (Fig. 1). Median progression-free survival and overall survival in the pancreas group ($n = 10$) were 1.5 and 6.2 months, whereas those in the hepatobiliary tract group ($n = 11$) were 3.0 and 5.8 months, although the differences between both groups did not appear to be statistically significant.

Adverse Events

All 21 patients were assessed for toxicities, as listed in Table 2. The most common toxicities were leukopenia and

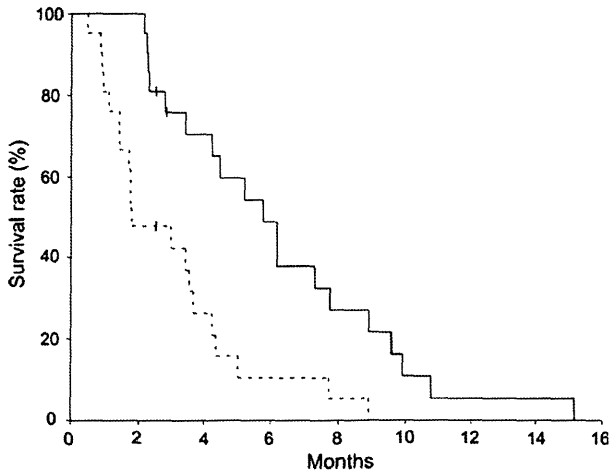

Figure 1. Overall survival (continuous line) and progression-free survival (dotted line) in the 21 patients.

neutropenia. Grade 3 or 4 leukopenia and neutropenia occurred in 15 (71%) and 19 (90%) patients, respectively, and febrile neutropenia in 8 (38%). As to non-hematological toxicities, vomiting of all grades was seen in 81% of the patients, whereas Grade 3 nausea and anorexia occurred in 33% and 24%, respectively. Although these gastrointestinal toxicities were frequently observed after cisplatin administration, most were manageable with appropriate medical treatment and only one patient needed to discontinue therapy due to gastrointestinal toxicity of prolonged Grade 2 nausea and anorexia. No other unexpected severe toxicities were observed during the treatment and there were no treatment-related deaths.

Table 2. Adverse events

	Grade				Grade 3/4, *n* (%)
	1	2	3	4	
Hematological toxicity					
Leukopenia	1	5	7	8	15 (71)
Neutropenia	1	1	2	17	19 (90)
Anemia	4	11	6	0	6 (29)
Thrombocytopenia	8	2	5	0	5 (24)
Non-hematological toxicity					
Bilirubin	3	1	3	1	4 (19)
AST	7	8	3	1	4 (19)
ALT	5	6	3	2	5 (24)
Creatinine	6	4	0	0	0
Fatigue	11	8	0	0	0
Anorexia	2	12	5	0	5 (24)
Nausea	4	9	7	0	7 (33)
Vomiting	7	10	0	0	0
Diarrhea	2	0	0	0	0
Mucositis	1	0	0	0	0
Alopecia	4	14	—	—	—
Neurological sensory	1	0	0	0	0
Febrile neutropenia	—	—	8	0	8 (38)

AST, aspartate aminotransferase; ALT, alanine aminotransferase.

DISCUSSION

In 1991, Moertel et al. (4) reported an objective response rate of 67% to combined chemotherapy with cisplatin and etoposide in 18 patients with anaplastic neuroendocrine tumors, which are analogous to the currently described extrapulmonary PD-NECs, with a median survival of 19 months. Mitry et al. (5) reported a response rate of 42% and median survival of 15 months in 41 patients with extrapulmonary PD-NECs treated with the same combination regimen. In these reports, not only tumors arising from the hepatobiliary and pancreatic regions, but also from the gastrointestinal, head and neck, and tracheal regions were included as extrapulmonary tumors. To the best of our knowledge, this is the first study of the efficacy of cisplatin plus etoposide focusing solely on tumors arising from the hepatobiliary and pancreatic regions.

In the current study, focusing on primary neuroendocrine tumors arising from the hepatobiliary and pancreatic regions, a response rate of 14% and median survival of 5.8 months were obtained in response to combined cisplatin plus etoposide therapy. Although the response rate and prognosis were extremely poor when compared with those reported by previous studies using the same combination of agents for extrapulmonary PD-NECs, when considering the finding that 75% of the patients showed a >50% decrease in the serum NSE levels, combined cisplatin plus etoposide may be considered to exert some degree of activity. However, whether this result may be comparable to that obtained with other treatment regimen for hepatobiliary and pancreatic PD-NECs is not yet clear, because few studies until date have reported on the efficacy of other regimens for this disease.

Malignant tumors arising from the hepatobiliary and pancreatic regions metastasize easily to the liver, becoming a typical cause of fatal visceral crisis; this anatomic nature may be one of the reasons for the relatively poor prognosis of these tumors. In fact, liver metastasis is a well-documented poor prognostic factor in patients with neuroendocrine tumors (10–14). The incidence of liver metastasis was 81% in the current study. Moreover, 52% had ascites as evidence of peritoneal dissemination, which is also generally recognized as a poor prognostic factor.

In the studies conducted to date, chemotherapeutic regimens for extrapulmonary PD-NECs have been patterned after those used for SCLC. However, these two entities, SCLC and extrapulmonary PD-NECs, may exhibit some differences at the molecular level. For example, Bcl-2 overexpression is observed at a high rate (75–95%) in SCLC

specimens, whereas only 33% of gastroenteropancreatic PD-NECs show this finding (15,16). Unlike SCLC, extrapulmonary PD-NECs show retention of both the short arms of chromosome 3, as revealed by restriction-fragment-length polymorphism studies and cytogenetic analyses (17). Since such cytogenetic differences between these tumors do exist, their clinical features and outcomes with the same treatment may also eventually diverge.

Neuroendocrine tumors also have other histological components in some cases (15,18–23). Such patients with PD-NECs arising from the gastric, colorectal and pancreatic regions generally have an adenocarcinoma component, whereas esophageal PD-NECs show a squamous cell carcinoma component. Thus, the nature of the non-neuroendocrine components in the PD-NECs also seems to depend on the primary site of the tumors. Two potential cells of origin of PD-NECs have been reported: pre-existing neuroectodermal cells and pluripotent epithelial stem cells, the latter appearing to be the more convincing at present (24–26). This cell of origin of the PD-NECs may explain the intermixing of adenocarcinoma or squamous cell carcinoma components in these tumors. It is well known that adenocarcinomas arising from the hepatobiliary tract and pancreas are less sensitive to chemotherapy and have a poor prognosis compared with adenocarcinomas arising from other organs. Likewise, the theory that PD-NECs arise from pluripotent epithelial stem cells may explain why hepatobiliary and pancreatic PD-NECs are less sensitive to chemotherapy and have a poor prognosis when compared with previous reports for miscellaneous extrapulmonary PD-NECs. In fact, it is interesting that elevated serum CEA and CA19-9 levels were confirmed in 38% of the patients in the current study, as both are widely used tumor markers of adenocarcinoma. In addition, one of these patients showed a partial response to gemcitabine monotherapy started after the detection of progressive disease in response to combined therapy with cisplatin and etoposide. Hence, there is a possibility that the tumor in this case showed a mixed histology consisting of neuroendocrine carcinoma and adenocarcinoma components, and that the adenocarcinoma component was refractory to the combination of cisplatin and etoposide and responsive to gemcitabine monotherapy. This may warrant the use of cytotoxic agents that are effective against both the PD-NEC component and the non-neuroendocrine carcinoma components, depending on the primary sites of the tumors.

In conclusion, the current study showed that the combination of cisplatin and etoposide exerted only marginal antitumor activity and relatively severe toxicity against PD-NECs of the hepatobiliary tract and pancreas, when compared with the treatment outcomes suggested by previous reports for extrapulmonary PD-NECs. The retrospective design of this study poses an inherent limitation. A prospective study is considered to be preferable to confirm the efficacy. Notwithstanding, because PD-NECs have an extremely poor prognosis and unsatisfactory treatment outcomes in response to combined chemotherapy with cisplatin plus etoposide, further development of novel treatment is necessary to improve the prognosis.

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Conflict of interest statement

None declared.

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Establishment of six new human biliary tract carcinoma cell lines and identification of MAGEH1 as a candidate biomarker for predicting the efficacy of gemcitabine treatment

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The aim of this study was to establish new biliary tract carcinoma (BTC) cell lines and identify predictive biomarkers for the potential effectiveness of gemcitabine therapy. Surgical specimens of BTC were transplanted directly into immunodeficient mice to establish xenografts, then subjected to *in vitro* cell culture. The gemcitabine sensitivity of each cell line was determined and compared with the genome-wide gene expression profile. A new predictive biomarker candidate was validated using an additional cohort of gemcitabine-treated BTC cases. From 55 BTC cases, we established 19 xenografts and six new cell lines. Based on their gemcitabine sensitivity, 10 BTC cell lines (including six new and four publicly available ones) were clearly categorized into two groups, and MAGEH1 mRNA expression in the tumor cells showed a significant negative correlation with their sensitivity to gemcitabine. Immunohistochemically, MAGEH1 protein was detected in three (50%) out of six sensitive cell lines, and four (100%) out of four resistant cell lines. In the validation cohort of gemcitabine-treated recurrence cases, patients were categorized into "effective" and "non-effective" groups according to the RECIST guidelines for assessment of chemotherapeutic effects. MAGEH1 protein expression was detected in two (40%) out of five "effective" cases and all four (100%) "non-effective" cases. We have established a new BTC bioresource that covers a wide range of biological features, including drug sensitivity, and is linked with clinical information. Negative expression of MAGEH1 protein serves as a potential predictive marker for the effectiveness of gemcitabine therapy in BTC. (*Cancer Sci* 2010; 101: 882–888)

Biliary tract carcinoma (BTC) has a poor prognosis, and most cases are diagnosed at advanced stages when patients present with overt symptoms. Previous studies have reported that surgical resection is the only curative treatment for BTC patients,[1–4] and no standard chemotherapy regimens have been established for inoperable cases or cases of recurrence after surgical resection.[5,6] Exceptionally, gemcitabine (2′-deoxy-2′-difluorodeoxycytidine), a deoxycytidine analog with structural and metabolic similarities to cytarabine, has been reported to be clinically effective and is considered a first-line chemotherapy for BTC, although its associated response rates (8–60%) and median overall survival (6.3–16 months) are not satisfactory.[7] It has been reported that both intrinsic and acquired resistance are important factors in the failure of gemcitabine treatment in patients with pancreatic cancer.[8] However, there have been few attempts to clarify the molecular mechanisms of gemcitabine resistance, and no data are currently available for BTC.

One factor preventing better understanding of drug resistance at the cellular and molecular levels in BTC is that only a few BTC cell lines are available for such analyses. Additionally, the construction and utility of an animal experimental model is essential for validating the *in vitro* data for these cell lines, but no such model has been established. Therefore, there is an urgent need to establish BTC cell lines from a wide range of clinical cases and apply them for translational research aimed at connecting basic research with clinical trials. In the present study, we successfully prepared 19 xenograft models from surgically resected BTC samples, and established six new cell lines. Using these new resources, we searched for molecular biomarkers associated with gemcitabine sensitivity. We also validated the efficacy of one candidate molecule, MAGEH1, as a surrogate biomarker of gemcitabine response by immunohistochemical analysis of an additional clinical cohort of gemcitabine-treated BTC.

Materials and Methods

Establishment of xenografts and tumor cell lines. The study included 55 patients with BTC who underwent radical surgery with curative intent at the National Cancer Center Hospital (Tokyo, Japan) between 2005 and 2008. The main tumor nodule was located in the lower, middle, and upper thirds of the extrahepatic bile duct, the hilar bile duct, and intrahepatic area in 4, 11, 2, 4, and 34 patients, respectively. Tumor specimens were transported to the Surgical Pathology department immediately after surgical resection, and tissue in excess of that needed for diagnosis was used for this study. The tumor tissues were washed in physiological saline, cut into small pieces (2–4 mm^3 fragments), then implanted subcutaneously into SCID mice. Congenital athymic female C.B17/Icr-scid(scid/scid) mice (CLEA Japan, Tokyo, Japan), 5–7 weeks old, were bred and housed under specific pathogen-free conditions at the National Cancer Center Research Institute Animal Center. Tumor growth to a size of 1–2 cm after maintaining the animals for 1–2 months was regarded as engraftment, and the tumors were passaged a maximum of three to five times. Xenografts in mice were passaged similarly to the transplantation of surgical

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specimens, and the tumors were subjected to cell culture after each passage. For establishment of cell lines, the xenograft tumor tissues were washed in Isozin (Meiji, Tokyo, Japan) and physiological saline, cut into small pieces, then plated into 6 cm dishes containing RPMI medium supplemented with 10% FC), 2 mM L-glutamine, 100 mg/mL streptomycin sulfate, and 100 ID/mL penicillin G sodium. Some surgical specimens were directly subjected to cell line preparation. Contaminating fibroblasts were periodically removed by wiping under microscopic observation. The cells were incubated at 37°C in 5% CO_2 in air, and the medium was changed once or twice a week. A solution of 0.05% trypsin and 0.53 mM EDTA (1×; Gibco™/Invitrogen Corporation, Carlsbad, CA, USA) was used for passaging the cells (1:3 split). Each cell line underwent repeated passage more than 20 times. Established cell lines were implanted subcutaneously into SCID mice to make xenografts for further analyses.

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 Ethics Committee of the National Cancer Center. This study was approved by the Ethical Committee of the National Cancer Center.

Biliary tract carcinoma cell lines obtained from cell banks. Four human BTC cell lines derived from Japanese patients (TKKK, OZ, TGBC24TKB, and HuCCT1) were purchased from Riken Bioresource Center (Tsukuba, Japan) or from the Japanese Collection of Research Bioresources (Osaka, Japan). The TKKK cell line was derived from intrahepatic cholangiocarcinoma, and the OZ, TGBC24TKB, and HuCCT1 cell lines from extrahepatic bile duct carcinoma.

Chemicals. Gemcitabine was obtained from Eli Lilly Pharmaceuticals (Indianapolis, IN, USA). All other chemicals were of analytical grade and commercially available.

Cytotoxicity assays for gemcitabine. The cytotoxicity of gemcitabine for each cell line was assessed by a modified 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay with CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI, USA). Tumor cells (2000 cells/well) in the exponential growth phase were grown in 96-well plates. Twenty-four hours after plating, the cells were incubated in the presence of each concentration (0 (control)–100 μM) of gemcitabine for another 72 h at 37°C in a humidified atmosphere of 5% CO_2 in air. After treatment, 20 μL CellTiter 96 AQueous One Solution Reagent was dropped into each well in the plates and the absorbance at 490 nm was recorded. Absorbance values were expressed as a percentage of untreated controls, and IC_{50} was calculated.

Gene expression analysis. Total RNA was extracted from 10 BTC cell lines using an RNeasy Micro Kit (Qiagen, Velencia, CA, USA) in accordance with the manufacturer's instructions. The total RNA yields and purity were determined spectrophotometrically by measuring the absorbance of aliquots at 260 and 280 nm. cDNA and Cy3-labeled cRNA were synthesized using a Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). The labeled cRNA probe was hybridized to an oligonucleotide microarray (Whole Human Genome 44K Array; Agilent Technologies) covering more than 41 000 human transcripts. Array hybridization and washing were carried out according to the recommended protocols, and microarrays were scanned using a DNA Microarray Scanner (Agilent Technologies) and analyzed using Gene Spring software (Agilent Technologies).

Quantitative RT-PCR. One microgram of total RNA was converted to cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) in accordance with the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was carried out using LightCycler 480 (Roche) in accordance with the manufacturer's instructions. For standardization of the amount of RNA, expression of GAPDH in each sample was quantified. (Primers are shown in Table S1.)

Mutation analysis of *p53* and *KRAS* genes. Each exon of the *p53* and *KRAS* genes (exons 5–8 of *p53* and exons 1–2 of *KRAS*) was amplified from genomic DNA of each cell line and gel-purified. Direct sequencing was carried out using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). (Primers are shown in Table S1.)

Assessment of response to gemcitabine in cases of recurrent BTC. Among the 100 patients who underwent surgery for BTC between September 26, 2003, and October 2, 2007, 34 developed recurrent tumors and received chemotherapy, and were followed for 6 months or longer. Among these patients, 24 who were treated with gemcitabine alone were selected for this study. The mean duration of postoperative follow-up in these 24 patients was 627 days. We further excluded 15 patients from the analysis because: (i) the drug administration period was less than 1 month in three patients; (ii) the diagnosis of tumor recurrence was not consistent between the oncologist and the radiologist in three patients; (iii) we were unable to obtain an accurate judgement of the efficacy of gemcitabine treatment in five patients; (iv) the histological diagnosis was an uncommon type of adenocarcinoma (bile duct cystadenocarcinoma, solid adenocarcinoma, and combined carcinoma) in three patients; and (v) preoperative therapy (radiation therapy) had been carried out in one patient. The effect of chemotherapy was assessed by an oncologist and a radiologist (T.O. and H.O., respectively) in accordance with the RECIST guidelines for assessment of chemotherapeutic effects.[9] None of the patients was judged as showing a complete response or a partial response. The effect of chemotherapy was categorized as "effective" or "non-effective". The "effective" group included patients whose efficacy state was stable disease for 6 months or more during chemotherapy. The "non-effective" group included patients whose efficacy state was stable disease for 5 months or less, or progressive disease during chemotherapy.

Immunohistochemical reactivity of MAGEH1 in human tumor xenografts and surgically resected specimens. Immunohistochemical analysis of MAGEH1 expression on formalin-fixed, paraffin-embedded sections of tumor xenograft tissues and surgical specimens was done using the polymer-based method (Envision+Dual Link System-HRP; Dako, Glostrup, Denmark) in accordance with the manufacturer's instructions. For antigen retrieval, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 121°C for 10 min. We used a rabbit anti-MAGEH1 polyclonal antibody (ab64784; Abcom, Cambridge, Massachusetts, USA) at a dilution of 1:500. Staining intensity was independently evaluated by two pathologists (H.O. and T.S.) without knowledge of the clinical data. Using the expression in normal hepatocytes or pancreatic duct epithelial cells as a positive control, we classified cases as MAGEH1-positive when more than 50% of tumor cells were positively stained. If the tumor showed varying degrees of differentiation, staining intensity was evaluated in the area with the most dominant type of differentiation.

Statistical analysis. The unpaired *t*-test was used for assessment of the microarray data. Microarray and qRT-PCR data were analyzed by Pearson's correlation test.

Results

Establishment and characterization of BTC xenografts and cell lines. To establish useful BTC resources, we subcutaneously transplanted 55 BTC samples (4, 11, 2, 4, and 34 cases of lower, middle, and upper thirds of the extrahepatic bile duct carcinoma, hilar bile duct carcinoma, and intrahepatic cholangiocarcinoma, respectively) into 435 immunocompromised (SCID) mice.

Table 1. Clinicopathological features of original biliary tract tumors

Xenograft	Pathological diagnosis of original tumor	Age (years)/Sex	Histologic type	Prognosis (Survival [days])	Cell line
1	CCC	70/F	Adeno, mod	Death (402)	NCC-CC1
2	CCC	71/F	Adeno, mod	Death (175)	NCC-CC3-1/-2
3	CCC	59/M	Adeno, mod	Alive (219)	NCC-CC4-1
4	Middle BDCa	58/F	Adeno, mod	Death (299)	NCC-BD1
5	Lower BDCa	77/F	Adeno, mod	Alive (316)	NCC-BD2
6	Hilar BDCa	48/M	Adeno, well	Death (500)	NA
7	CCC	54/F	Adeno, mod	Death (181)	NA
8	CCC	56/M	Adeno, mod	Death (319)	NA
9	CCC	73/M	Adeno, mod	Death (53)	NA
10	CCC	54/M	Adeno, mod	Alive (655)	NA
11	CCC	45/F	Adeno, mod	Alive (623)	NA
12	CCC	72/M	Muc	Alive (647)	NA
13	Middle BDCa	54/M	Adeno, mod	Alive (535)	NA
14	CCC	69/M	Adeno, mod	Death (174)	NA
15	Hilar BDCa	70/M	Adeno, mod	Alive (355)	NA
16	Middle BDCa	67/M	Adeno, mod	Alive (450)	NA
17	CCC	78/M	Adeno, mod	Alive (299)	NA
18	Middle BDCa	66/F	Adeno, mod	Alive (198)	NA
19	CCC	66/M	Adeno, mod	Death (168)	NA

Adeno, adenocarcinoma; CCC, cholangiocellular carcinoma; F, female; hilar BDCa, hilar bile duct carcinoma; lower BDCa, lower third of extrahepatic bile duct carcinoma; M, male; middle BDCa, middle third of extrahepatic bile duct carcinoma; mod, moderately differentiated; muc, mucinous adenocarcinoma; well, well differentiated; NA, not applicable.

Table 2. Mutation status of *p53* and *KRAS* genes of established novel biliary tract carcinoma cell lines

Cell line	*KRAS* (exons 1–2)		*p53* (exons 5–8)	
	Nucleotide change	Amino acid change	Nucleotide change	Amino acid change
NCC-BD1	G37C	G13C	C457T, A463C,G467C	P153S, T155P, R156P
NCC-BD2	WT	WT	Homozygous deletion	No product
NCC-CC1	G35T	G12V	G524A	R175H
NCC-CC3-1	G35A	G12D	WT	WT
NCC-CC3-2	G35A	G12D	WT	WT
NCC-CC4-1	WT	WT	WT	WT

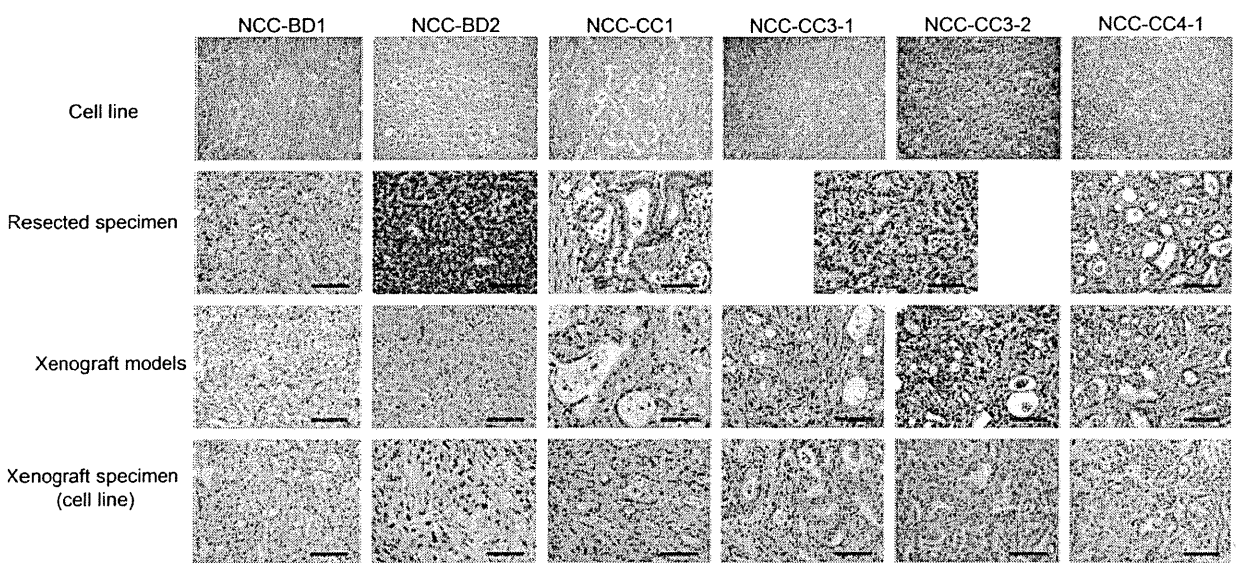

Fig. 1. Cell morphology and tumor histology of primary specimen/xenograft of established new biliary tract carcinoma cell lines. *In vitro* cell morphology and tumor histology (H&E staining) of resected primary specimens, xenografts of primary tumor samples and xenografts of cell lines are shown. Scale line = 200 μm.

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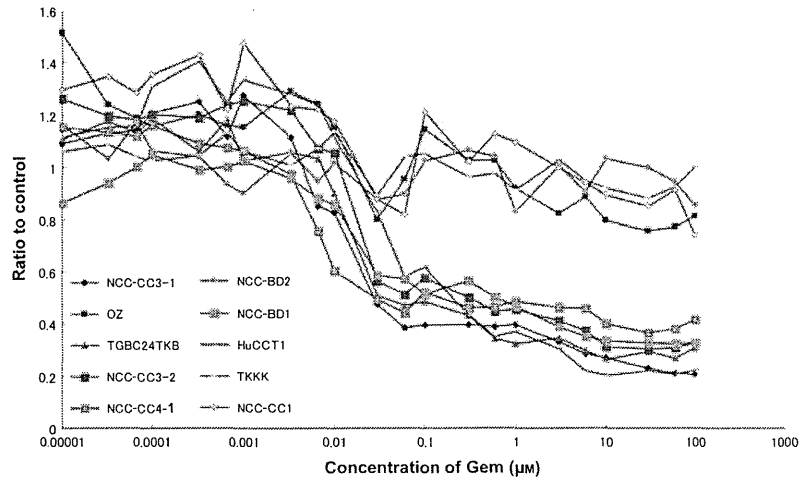

Fig. 2. Sensitivity to gemcitabine (Gem) in 10 biliary tract carcinoma cell lines. Ratio of cell proliferation compared to the control (treated with DMSO) at each concentration (μM) of Gem was plotted. Note that 10 cell lines are clearly segregated into two groups (Gem-sensitive and Gem-resistant) with distinct Gem sensitivity.

Nineteen xenograft models (1, 4, 0, 2, and 12 cases of lower, middle, and upper thirds of the extrahepatic bile duct carcinoma, hilar bile duct carcinoma, and intrahepatic cholangiocarcinoma, respectively) were obtained, and six cell lines including two subclones were established through xenograft models (five cell lines) or directly from a surgical specimen (one cell line). The cell lines were designated as NCC-BD1, NCC-BD2, NCC-CC1, NCC-CC3-1, NCC-CC3-2, and NCC-CC4-1, respectively. Four cell lines were derived from intrahepatic BTC and two from extrahepatic BTC (Table 1). Other clinicopathological features of the patients from whom the cell lines were obtained are summarized in Table 1.

Mutation analysis of the *KRAS* and *p53* genes revealed frequent (3/5, 60%) alterations in them. It also confirmed that these new cell lines were of human origin and that two subclones, NCC-CC3-1 and NCC-CC3-2, shared the same *KRAS* mutation (Table 2). The morphology and histology of the established cell lines and primary tumors, and xenografts of primary tumor and cell lines, are shown in Figure 1. As NCC-BD2 cells were unable to form tumors in mice, we used a cell block of this cell line. Comparing the morphological features between primary tumors and cell lines, we observed considerable conservation of tumor histology (Fig. 1), suggesting that the established cell lines could be considered representative of each original primary.

Classification of 10 BTC cell lines by gemcitabine sensitivity. We then attempted to evaluate whether these new cell lines could be used for revealing novel biomarkers for drug sensitivity. For this purpose, we first determined the gemcitabine sensitivity of 10 BTC cell lines including four commercially available BTC cell lines. The relative survival ratios of the 10 BTC cell lines in response to various doses of gemcitabine are shown in Figure 2. The IC_{50} value for each cell line was calculated, and the results are summarized in Table 3. Interestingly, as can be seen in Figure 2, on the basis of drug sensitivity, we were able to classify these cell lines into two groups: a gemcitabine-sensitive group that included NCC-BD1, NCC-CC3-1, NCC-CC3-2, NCC-CC4-1, HuCCT1, and TGBC24TKB cells (the IC_{50} values being 0.6, 0.03, 0.06, 0.03, 0.2, and 0.03 μM respectively) and a gemcitabine-resistant group that included NCC-BD2, NCC-CC1, TKKK, and OZ cells, whose IC_{50} values were beyond the range of our measurement (>100 μM). As all of the newly established cell lines were from chemotherapy-naïve tumors, this result suggests that BTC cells possess intrinsic molecular mechanism associated with gemcitabine sensitivity.

Significant differences in mRNA expression between groups sensitive and resistant to gemcitabine. To further elucidate the molecular differences between the groups sensitive and resistant to gemcitabine, we investigated the genome-wide mRNA expression in all the cell lines. By comparing the sensitive group with the resistant group, we isolated genes that showed significant differences in expression between the two (Table 4). These included genes associated with cell signaling (*SEC23A*, *RRAS2*, and *BMP8B*) or telomere maintenance (*TERF1*), or genes whose functions were unknown (*NOL10*, *CCDC117*, and *ZSWIM6*). All were candidate biomarkers associated with gemcitabine sensitivity, and among them we focused on MAGEH1 (melanoma antigen family H 1) because: (i) mRNA expression of MAGEH1 in the resistant group was more than five times higher than in the sensitive group; (ii) MAGEH1 is a transmembrane protein that is easily accessible to antibody; and (iii) there was a significant difference in its expression between the two groups ($P = 0.000093$). We then validated the differential expression of MAGEH1 between the two groups by qRT-PCR. As shown in Figure 3, the data for MAGEH1 expression obtained by qRT-PCR, which was normalized with GAPDH expression, was highly correlated with DNA microarray data (coefficient of correlation, 0.847) and also differed significantly ($P = 0.009$) between the sensitive and resistant groups.

MAGEH1 expression in gemcitabine-treated BTC cases. Finally, we tested whether MAGEH1 expression is correlated with clinical response to gemcitabine treatment by immunohistochemical analysis of clinical cases. Before analyzing the clinical samples, we tested the anti-MAGEH1 antibody in xenograft tumor samples. Three cell lines (50%) out of the six sensitive cell lines and

Table 3. Gemcitabine IC_{50} values and assessment of reactive cytotoxicity of biliary tract carcinoma cell lines

Cell line	IC_{50} (μM)	Drug sensitivity
NCC-BD1	0.60	S
NCC-BD2	>100	R
NCC-CC1	>100	R
NCC-CC3-1	0.03	S
NCC-CC3-2	0.06	S
NCC-CC4-1	0.03	S
TKKK	>100	R
OZ	>100	R
Hucct1	0.20	S
TGBC24TKB	0.03	S

R, resistant; S, sensitive.

Table 4. List of genes differentially expressed between gemcitabine sensitive and resistant groups of biliary tract carcinoma cell lines

Gene symbol	Average expression (R)	Average expression (S)	Ratio (R/S)	*P*-value†	Chromosome locus
TIMELESS	1.866235575	0.858141402	2.174741332	1.45E-05	12q12-q13
SEC23A	1.601411675	0.796303448	2.011057064	2.34E-05	14q21.1
MAGEH1	2.100036325	0.397001692	5.289741503	9.28E-05	Xp11.21
NOL10	1.482213925	0.854618707	1.734356987	0.000201766	2p25.1
RRAS2	0.221456871	1.54467481	0.143367956	0.000429397	11p15.2
BMP8B	1.7544659	0.572194878	3.066203432	0.000440394	1p35-p32
TERF1	1.422439425	0.778783987	1.826487767	0.000451224	8q13
SEC23A	1.5599122	0.633786226	2.461259234	0.0004951	14q21.1
CCDC117	1.71272665	0.699035142	2.45012954	0.000557389	22q12.1
C14orf107	0.490823853	1.299093433	0.377820286	0.000632072	14q22.3
ZSWIM6	0.508965063	1.33793895	0.380409781	0.000753833	5q12.1
RPL34	0.52856332	1.102003908	0.479638335	0.000934328	4q25

†Obtained using the unpaired *t*-test. R, resistant group; S, sensitive group.

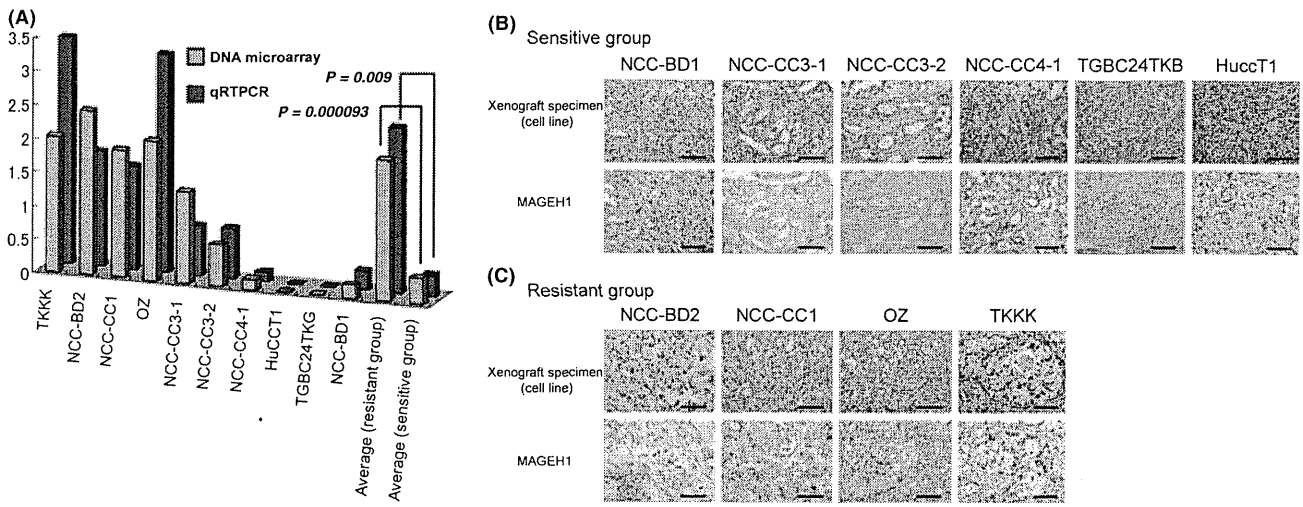

Fig. 3. (A) MAGEH1 mRNA expression in 10 biliary tract carcinoma cell lines. Relative expression of MAGEH1 mRNA compared to GAPDH expression in each cell line was quantified by microarray (blue columns) and quantitative RT-PCR (red columns). MAGEH1 expression was significantly different between gemcitabine (Gem)-sensitive and Gem-resistant groups. (B,C) Immunohistochemical analysis of MAGEH1 protein in xenograft specimens of 10 biliary tract carcinoma cell lines. Tumor histology (H&E staining) of xenograft specimens of cell lines, split into Gem-sensitive (B) and Gem-resistant (C) groups, and MAGEH1 protein expression detected by anti-MAGEH1 antibody in the same area are shown. All three cell lines that lacked MAGEH1 expression belong to the Gem-sensitive group. Scale line = 200 μm.

all four cell lines (100%) in the resistant group were positive for MAGEH1 expression (Fig. 3).

We selected nine recurrent BTC cases treated with gemcitabine alone, which were fully evaluated for drug effects by imaging diagnosis, as described in the "Materials and Methods" section, and whose tumor samples had been sufficiently examined and pathologically diagnosed. After clinical evaluation, we identified five "effective" cases and four "non-effective" cases (Table S2). We examined MAGEH1 protein expression in surgical specimens of the primary tumor in these nine cases. As shown in Figure 4, two (40%) of five "effective" cases were positive, and all four "non-effective" cases (100%) were positive.

Discussion

Elucidation of the molecular mechanisms determining the biological characteristics of cancer cells is one strategy for improving the clinical outcome of BTC patients, but only a few BTC cell lines serving as potent biological tools and animal models with properties resembling those of human cancer have been established. In this study, we succeeded in establishing six novel BTC cell lines including various subtypes and 19 BTC xenograft models after trying 55 cases. Despite carrying out multiple transplantations, we did not observe any marked discrepancy in cell morphology between the original tumors and the cell lines/xenografts, suggesting that this model could be stable and useful for biological studies. Moreover, we were able to fully combine the corresponding clinical information for patients and pathological archive specimens of primary tumors and xenografts for both primary tumors and cell lines with biological data on the cell lines for both basic and preclinical research. To add more clinically relevant functional data, we examined the gemcitabine sensitivities of these cell lines.

Previously, several predictive markers for the effects of gemcitabine chemotherapy have been reported in various types of tumor, including equilibrative nucleoside transporter-1 (hENT1),[10] ribonucleotide reductase subunit M2 (RRM2),[11] and heat shock protein 27 (HSP27)[12] for pancreatic carcinoma, ribonucleotide reductase subunit M1 (RRM1)[13] for non-small-cell lung cancer (NSCLC), hENT1 for ampulla of Vater carcinoma,[14] carcinoembryonic antigen-related cell adhesion

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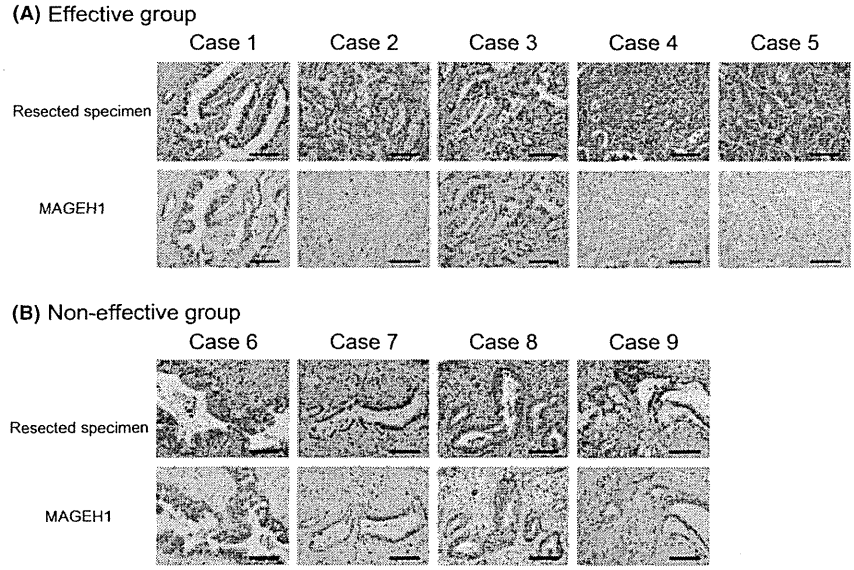

Fig. 4. Immunohistochemical analysis of MAGEH1 protein in primary tumor specimens of gemcitabine (Gem)-effective and non-effective groups. Tumor histology (H&E staining) of primary tumor specimens, split into Gem-effective (A) and Gem-non-effective (B) groups and MAGEH1 protein expression detected by anti-MAGEH1 antibody in the same area are shown. All three cases that lacked MAGEH1 expression belong to the Gem-effective group. Scale line = 200 μm.

molecule 6 (CEACAM6) for intrahepatic cholangiocarcinoma,[5] and RRM1 for biliary tract carcinoma.[15] Among these previously reported biomarkers, our microarray analysis validated that RRM2 expression was significantly ($P = 0.03$) increased (three-fold on average) in the resistant group compared to the sensitive one (data not shown). However, most of these studies analyzed a small number of cell lines (maximum two), for example, comparing a gemcitabine-sensitive cancer cell line with its subclone that had acquired gemcitabine resistance, and focused on molecules that are already known to be associated with gemcitabine transport and metabolism. No study has yet tested its efficacy in clinical samples. The present study examined the largest number of BTC cell lines to be detailed in published reports to date, including six novel ones, in relation to clinicopathological information. To discover potential biomarkers in an unbiased way, we examined genome-wide expression profiles using a microarray, identified several biomarker candidates including MAGEH1, and validated its significance in another cohort of clinical BTC cases.

MAGEH1 is a member of the melanoma antigen family (MAGE)[16]. The human MAGE family was originally identified as a tumor-specific antigen,[17] and is now classified into two subtypes (type I and type II).[18] Type I MAGE is completely silenced in normal tissues except male germ cells and placenta, whereas type II MAGE is expressed in both tumors and a fraction of normal tissues. MAGEH1 belongs to the type II MAGE family and is also expressed in normal human tissues.[16] MAGEH1 is expressed in 69% of NSCLC[19] and in 100% of renal cell carcinomas,[20] but no data for BTC have been reported. MAGEH1 associates with the intracellular domain of the p75/NGF receptor[21] and regulates the cell cycle,[19] but its precise role in cancer is largely unknown. In the Gene Expression Omnibus (GEO) database at National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/geo/), there is one set of public microarray data showing the association between MAGEH1 expression and gemcitabine resistance in NSCLC cells. Comparison of the gene expression profile of parental Calu3 cell with those of gemcitabine-resistant subclones (Calu3-GemR) revealed that the mean expression of MAGEH1 mRNA in Calu3-GemR clones was more than twice as high as that in the parental cells.[22] However, there was no significant difference between the two, probably because of the small sample size analyzed ($P = 0.2481$; Fig. S1).

We further investigated whether MAGEH1 protein expression can be used for predicting clinical response to gemcitabine treatment, as protein expression is more stable and easier to test in clinical samples than RNA expression. Consistent with the mRNA expression data, we found that MAGEH1 protein was expressed in all resistant and non-effective cases. However, MAGEH1-positive cases also included a portion of sensitive or effective cases, possibly because of post-translational regulation of MAGEH1 protein expression. Significantly, however, MAGEH1-negative cell lines and primary cases were all gemcitabine-sensitive or effective cases, suggesting that MAGEH1 expression could be used as a negative predictor of gemcitabine response. That is, if immunohistochemical staining for MAGEH1 is negative, it is highly likely that a particular case would respond to gemcitabine therapy. Based on its previously reported functions, it remains unclear why MAGEH1 expression would be inversely correlated with gemcitabine response. It could function as a regulator of gemcitabine metabolism or might simply be a surrogate marker of distinct BTC subtypes. Because we analyzed only cases for which the result of gemcitabine treatment had been assessed objectively, it was difficult to collect a large number of retrospective cases. Moreover, we were unable to examine the expression of MAGEH1 RNA in the clinical specimens by RT-PCR because only small amounts of the frozen samples were available. Therefore, further prospective analysis of a larger cohort will be necessary to determine the clinical efficacy of MAGEH1 expression as a predictive biomarker of gemcitabine response.

Recently, a report has indicated that both the amount of stroma and vascularity in the tumor are associated with gemcitabine sensitivity in pancreatic cancer.[23] It was proposed that the hypovascularity and poor vascular architecture of pancreatic ductal carcinomas might impose an additional limitation to therapeutic delivery. Therefore, it was hypothesized that disrupting the stroma of pancreatic tumors might alter the vascular network

and thereby facilitate the delivery of chemotherapeutic agents. Accordingly, we recognized that the tumors in the non-effective group showed a tendency to have more of the stromal component than the tumors in the effective group (Fig. 4). Thus the stromal component would also play an important role in drug resistance of BTC.

In spite of the limited number of cases we examined, our result is consistent with the idea that more complex mechanisms regulate the gemcitabine sensitivity of BTC. In this sense, combination of other biomarker candidates obtained from the present screening or ones discovered through different approaches such as proteomic analysis with MAGEH1 should predict the drug response more accurately. In any event, the present study has shown that our new resource with clinical annotation would be valuable for discovering new biomarkers, and future studies for identifying new therapeutic/diagnostic targets are warranted.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Mircoarray data of association between MAGEH1 and gemcitabine in non-small lung cancer from NCBI GEO database.

Table S1. Primers for mutation analysis of *p53* and *KRAS* genes.

Table S2. Clinicopathological feature of 9 patients.

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ORIGINAL RESEARCH ARTICLE

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Population Pharmacokinetics of Gemcitabine and Its Metabolite in Japanese Cancer Patients

Impact of Genetic Polymorphisms

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Abstract

Background and Objective: Gemcitabine (2′,2′-difluorodeoxycytidine) is an anticancer drug, which is effective against solid tumours, including non-small-cell lung cancer and pancreatic cancer. After gemcitabine is transported into cells by equilibrative and concentrative nucleoside transporters, it is phosphorylated by deoxycytidine kinase (DCK) and further phosphorylated to its active diphosphorylated and triphosphorylated forms. Gemcitabine is rapidly metabolized by cytidine deaminase (CDA) to an inactive metabolite, 2′,2′-difluorodeoxyuridine (dFdU), which is excreted into the urine. Toxicities of gemcitabine are generally mild, but unpredictable severe toxicities such as myelosuppression and interstitial pneumonia are occasionally encountered. The aim of this study was to determine the factors, including genetic polymorphisms of *CDA*, *DCK* and solute carrier family 29A1 (*SLC29A1* [*hENT1*]), that alter the pharmacokinetics of gemcitabine in Japanese cancer patients.

Patients and Methods: 250 Japanese cancer patients who received 30-minute intravenous infusions of gemcitabine at 800 or 1000 mg/m^2 in the period between September 2002 and July 2004 were recruited for this study. However, four patients were excluded from the final model built in this study because they showed bimodal concentration-time curves. Two patients who experienced gemcitabine-derived life-threatening toxicities in October 2006 and January 2008 were added to this analysis. One of these patients received 30-minute intravenous infusions of gemcitabine at 454 mg/m^2 instead of the usual dose (1000 mg/m^2).

Plasma concentrations of gemcitabine and dFdU were measured by high-performance liquid chromatography-photodiode array/mass spectrometry. In total, 1973 and 1975 plasma concentrations of gemcitabine and dFdU, respectively, were used to build population pharmacokinetic models using nonlinear mixed-effects modelling software (NONMEM® version V level 1.1).

Results and Discussion: Two-compartment models fitted well to plasma concentration-time curves for both gemcitabine and dFdU. Major contributing factors for gemcitabine clearance were genetic polymorphisms of *CDA*, including homozygous *CDA**3 [208G>A (Ala70Thr)] (64% decrease), heterozygous *3 (17% decrease) and *CDA* −31delC (an approximate 7% increase per deletion), which has a strong association with *CDA**2 [79A>C (Lys27Gln)], and coadministered S-1, an oral, multicomponent anti-cancer drug mixture consisting of tegafur, gimeracil and oteracil (an approximate 19% increase). The estimated contribution of homozygous *CDA**3 to gemcitabine clearance provides an explanation for the life-threatening severe adverse reactions, including grade 4 neutropenia observed in three Japanese patients with homozygous

*CDA**3. Genetic polymorphisms of *DCK* and *SLC29A1* (*hENT1*) had no significant correlation with gemcitabine pharmacokinetic parameters. Aging and increased serum creatinine levels correlated with decreased dFdU clearance.

Conclusion: A population pharmacokinetic model that included *CDA* genotypes as a covariate for gemcitabine and dFdU in Japanese cancer patients was successfully constructed. The model confirms the clinical importance of the *CDA**3 genotype.

Background

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is an anticancer drug, which is effective against solid tumours, including non-small-cell lung cancer and pancreatic cancer.[1] After gemcitabine is transported into cells by equilibrative and concentrative nucleoside transporters (ENTs and CNTs),[2,3] it is initially phosphorylated by deoxycytidine kinase (DCK) to 2′,2′-difluorodeoxycytidine monophosphate and then is further phosphorylated to its active diphosphorylated and triphosphorylated forms, dFdCDP and dFdCTP.[4,5] Gemcitabine is rapidly metabolized by cytidine deaminase (CDA) to an inactive metabolite, 2′,2′-difluorodeoxyuridine (dFdU), which is excreted into the urine.[6]

Toxicities of gemcitabine are generally mild,[7,8] but unpredictable severe toxicities such as myelosuppression and interstitial pneumonia are occasionally encountered.[9-11] In a previous paper, we reported that a single nucleotide polymorphism (SNP) of the *CDA* gene, *CDA* 208G>A (Ala70Thr), designated as *CDA**3, caused reduced CDA activity and led to increased incidences of severe neutropenia in patients receiving gemcitabine-based combination chemotherapy.[12] Moreover, all three patients who were homozygous for *CDA**3 encountered life-threatening gemcitabine-mediated toxicities, including grade 4 neutropenia.[13,14] Pharmacokinetic data available from two of these patients revealed a gemcitabine clearance of about 20% of the median clearance rate.[12,14]

In this study, we performed a population pharmacokinetic analysis of gemcitabine in Japanese cancer patients to determine which factors, including genetic factors, affect the pharmacokinetics of gemcitabine and to facilitate individualized gemcitabine-based chemotherapies.

Methods

Patients

The ethics committees of the National Cancer Center (Tokyo, Japan) and the National Institute of Health Sciences (Tokyo, Japan) approved this study. Written informed consent was obtained from all participants. 250 patients, who received 30-minute intravenous infusions of gemcitabine 800 or 1000 mg/m^2 from September 2002 to July 2004, participated in this study. Two patients[14] who experienced gemcitabine-mediated life-threatening toxicities in October 2006 and January 2008 were added to the study. One of these patients, who carried a homozygous *CDA**3 gene, received 30-minute intravenous infusions of gemcitabine 454 mg/m^2 instead of the usual dose (1000 mg/m^2). All patients in this study have been previously reported,[12,14] and their characteristics are summarized in table I.

Table I. Demographic and clinical profiles of the gemcitabine (dFdC)-treated population

Variable	Value
Sex (n; male/female)	162/86
Dose (n; mg/m^2)	
1000	243
800	4
454	1
Pancreatic cancer (n)	207
Lung cancer (n)	35
Methothelium cancer (n)	6
Monotherapy (n)	182
Combination therapy (n)	66
cisplatin	26
carboplatin	16
fluorouracil	4
S-1	10
vinorelbine	10
Age (y)[a]	62.67±9.04 [35–80]
BSA (m^2)[a]	1.56±0.17 [1.14–1.97]
Bodyweight (kg)[a]	54.56±9.76 [30–80.3]
Serum creatinine (mg/dL)[a]	0.70±0.19 [0.4–1.5]

a The values are expressed as mean±SD [range].

BSA = body surface area; **S-1** = an oral product of tegafur with gimeracil and oteracil.