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

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.

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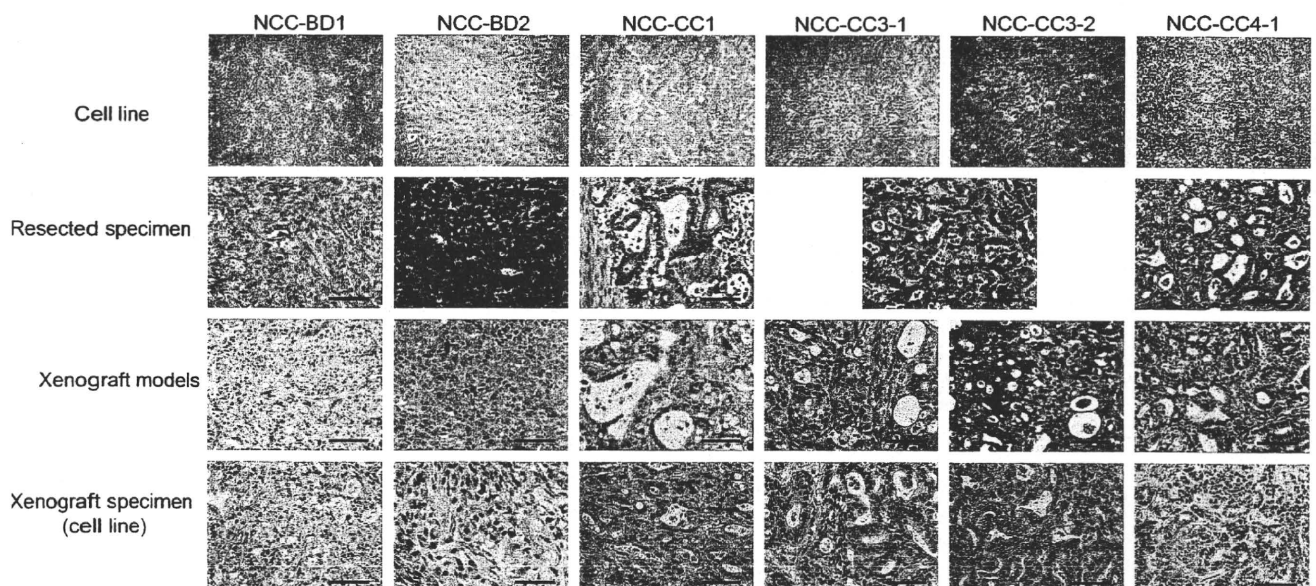
Fig. 1. Cell morphology and tumor histology of primary specimen/xenograft of established new biliary tract carcinoma cell lines. *In vitro* cell morphology and tumor histology (H&E staining) of resected primary specimens, xenografts of primary tumor samples and xenografts of cell lines are shown. Scale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

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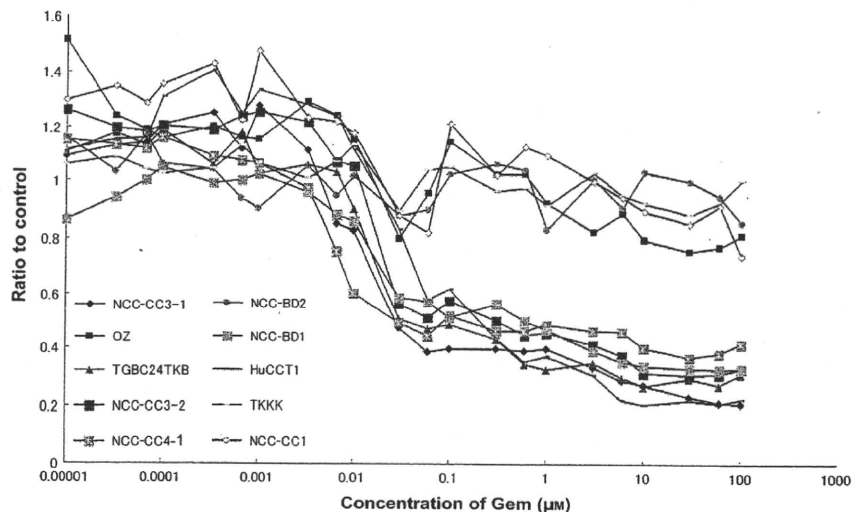
Fig. 2. Sensitivity to gemcitabine (Gem) in 10 biliary tract carcinoma cell lines. Ratio of cell proliferation compared to the control (treated with DMSO) at each concentration (μM) of Gem was plotted. Note that 10 cell lines are clearly segregated into two groups (Gem-sensitive and Gem-resistant) with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

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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.
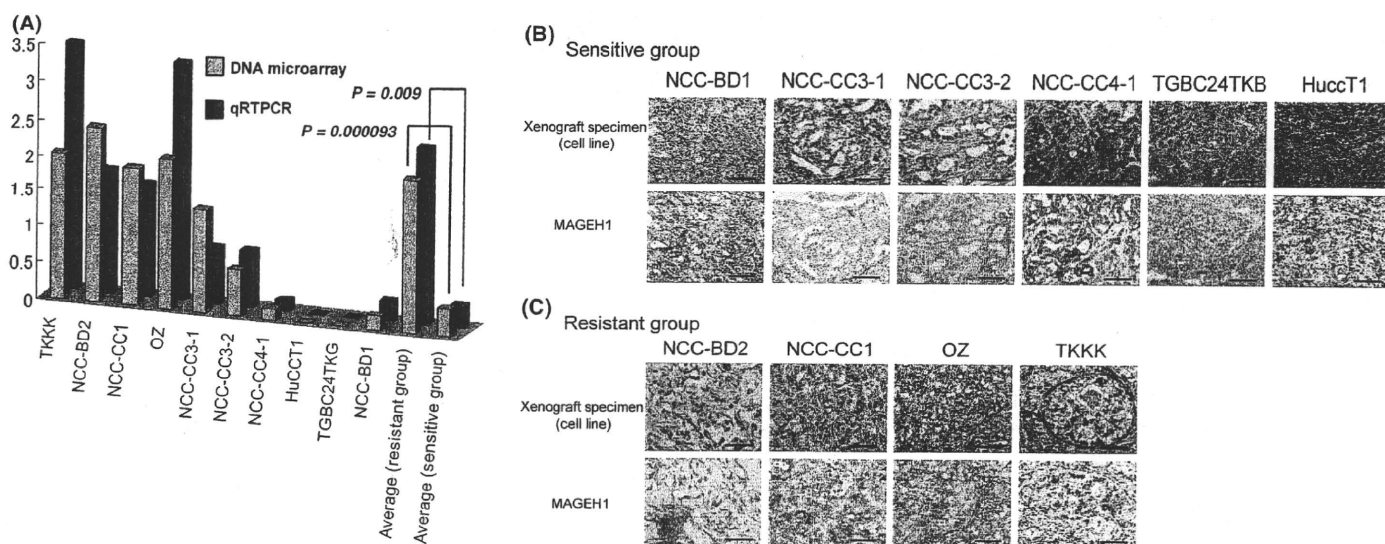
Fig. 3. (A) MAGEH1 mRNA expression in 10 biliary tract carcinoma cell lines. Relative expression of MAGEH1 mRNA compared to GAPDH expression in each cell line was quantified by microarray (blue columns) and quantitative RT-PCR (red columns). MAGEH1 expression was significantly different between gemcitabine (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).
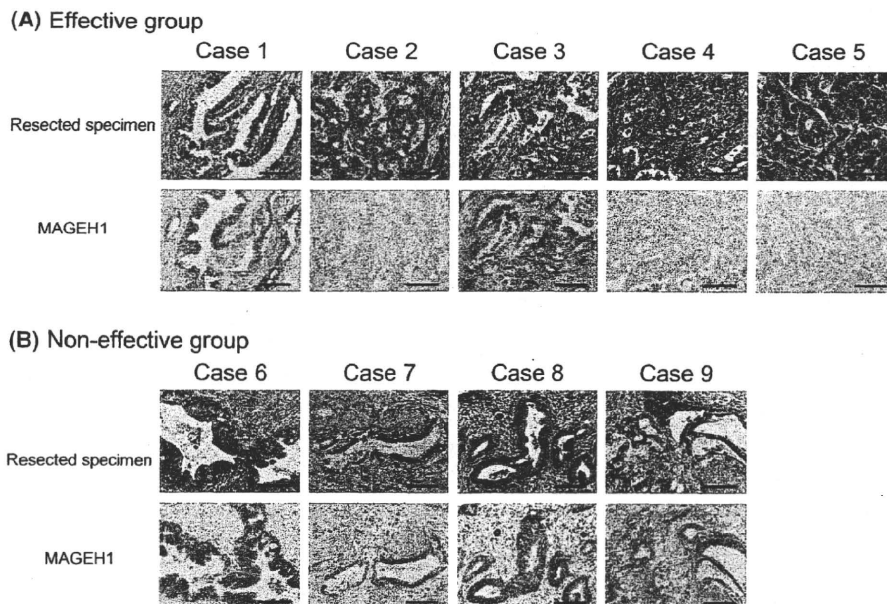
We selected nine recurrent BTC cases treated with gemcitabine alone, which were fully evaluated for drug effects by imaging diagnosis, as described in the "Materials and Methods" section, and whose tumor samples had been sufficiently examined and pathologically diagnosed. After clinical evaluation, we 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

(A) Effective group

Case 1　Case 2　Case 3　Case 4　Case 5

Resected specimen

MAGEH1

(B) Non-effective group

Case 6　Case 7　Case 8　Case 9

Resected specimen

MAGEH1

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.

Acknowledgments

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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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Clinical Trial Note

Randomized Phase II Study of Gemcitabine plus S-1 Combination Therapy vs. S-1 in Advanced Biliary Tract Cancer: Japan Clinical Oncology Group Study (JCOG0805)

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A randomized Phase II selection design trial comparing gemcitabine plus S-1 combination therapy with S-1 monotherapy for chemo-naïve unresectable or recurrent biliary tract cancer patients was started in Japan. The aim of this trial is to evaluate the efficacy and safety of the two regimens and to determine which is more promising as a test arm regimen to be compared with the current standard regimen, gemcitabine plus cisplatin, in a subsequent Phase III trial. Patients with unresectable or recurrent biliary tract cancer are randomized to either gemcitabine plus S-1 combination therapy arm or S-1 monotherapy arm. A total of 100 patients will be accrued for this study from 18 institutions over 1 year. The primary endpoint is the proportion of 1-year overall survival, and the secondary endpoints are progression-free survival, response rate and adverse events.

Key words: iliary tract cancer – gemcitabine – S-1 – randomized Phase II selection design trial

INTRODUCTION

Biliary tract cancer consists of intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma, gallbladder cancer and ampulla of Vater cancer. In Japan, it is estimated that ~18 000 patients with biliary tract cancer die annually and it is the sixth leading cause of cancer death (about 5.6% of all deaths due to cancer) (1,2).

In biliary tract cancer, curative surgical resection offers the only chance for a cure; however, most patients are initially diagnosed with unresectable disease. Moreover, many patients who undergo curative surgery develop recurrence (3).

For unresectable or recurrent biliary tract cancer, systemic chemotherapy is recognized as a standard treatment and, globally, gemcitabine, platinum analogue and fluoropyrimidine are considered as the key drugs (3,4). Although gemcitabine alone was regarded as the standard regimen for the advanced biliary cancer until recently, gemcitabine plus cisplatin (GC) has become the new standard regimen on the basis of the results of the ABC-02 trial (5), in which the superiority of GC over gemcitabine alone was shown.

Gemcitabine plus S-1 combination therapy (GS) or S-1 monotherapy is another promising regimen for unresectable or recurrent biliary tract cancer. In a Phase II trial for biliary tract cancer, S-1 monotherapy showed a better response rate (35%) (6) than gemcitabine alone (17.5%) (7) with milder toxicity. GS also showed a better response (34%) than gemcitabine alone in a Phase II trial for biliary tract cancer (8), even though the former showed much more toxicity than the latter. Therefore, we regard both regimens as promising and

planned this randomized Phase II trial to determine which regimen is more promising as the test arm regimen in a subsequent Phase III trial, in which the test arm will be compared with the current standard regimen, gemcitabine plus cisplatin.

The Protocol Review Committee of the Japan Clinical Oncology Group (JCOG) approved this protocol in December 2008 and the study was initiated in February 2009. This trial was registered at the UMIN Clinical Trials Registry as UMIN 000001685 (http://www.umin.ac.jp/ctr/index.htm).

PROTOCOL DIGESTS OF THE JCOG0805

OBJECTIVES

The aim of this study is to evaluate the safety and efficacy of the two regimens and to determine which regimen is more promising as the test arm regimen in a subsequent Phase III trial.

STUDY SETTING

The study was a multi-institutional open-label randomized Phase II selection design trial.

RESOURCES

This study is supported by Grants-in-Aid for Cancer Research (20S-3, 20S-6) Health and Labour Sciences Research Grant for Clinical Cancer Research (19–22), from the Ministry of Health, Labour and Welfare of Japan.

ENDPOINTS

The primary endpoint is the proportion of 1-year overall survival in all eligible patients. Overall survival is defined as days from randomization to death from any cause, and it is censored at the last follow-up day when the patient is alive. The secondary endpoints are progression-free survival, response rate and adverse events.

Progression-free survival is defined as days from randomization to disease progression or death from any cause, and it is censored at the latest day when the patient is alive without any evidence of progression.

ELIGIBILITY CRITERIA

INCLUSION CRITERIA

For inclusion in the study, patients are required to fulfill all of the following criteria.

(i) Clinically diagnosed with biliary tract cancer, which includes intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma, gallbladder cancer and ampulla of Vater cancer.

(ii) Recurrent or unresectable biliary tract cancer.
(iii) Histologically proven papillary adenocarcinoma, tubular adenocarcinoma, or adenosquamous carcinoma for extrahepatic cholangiocarcinoma, gallbladder cancer and ampulla of Vater cancer patients. Histologically proven adenocarcinoma for intrahepatic cholangiocarcinoma patients.
(iv) Without central nervous system metastasis.
(v) Without moderate or more severe ascites and pleural effusion.
(vi) No previous therapy against biliary tract cancer.
(vii) No previous chemotherapy or radiotherapy against any other malignancies.
(viii) ECOG performance status of 0 or 1.
(ix) Sufficient oral intake.
(x) Aged 20–79 years old.
(xi) Adequate organ functions.
(xii) Written informed consent.

EXCLUSION CRITERIA

Patients are excluded if they meet any of the following criteria.

(i) Simultaneous or metachronous (within 5 years) double cancers, with the exception of intramucosal tumor curable with local therapy.
(ii) Pregnant or lactating women or women of childbearing potential and men who want to get their partner pregnant.
(iii) Psychosis.
(iv) Requiring systemic steroid medication.
(v) Interstitial pneumonia or lung fibrosis.
(vi) Watery diarrhea.
(vii) Active bacterial or fungous infection.
(viii) Severe complication: heart failure, renal dysfunction, liver dysfunction, hemorrhagic peptic ulcer, paresis of intestine, ileus, uncontrollable diabetes mellitus etc.
(ix) Requiring the administration of flucytosine, phenytoin or warfarin potassium.
(x) Drug allergy for iodic drugs or gadolinium.

RANDOMIZATION

After confirmation of fulfillment of the eligibility criteria, registration is made by telephone or fax to the JCOG Data Center. Patients are randomized in the JCOG Data Center by a minimization method balancing the arms with institution, primary tumor (gallbladder cancer/intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma or ampulla of Vater cancer) and clinical stage (II, III/IV or recurrent).

TREATMENT METHODS

For the GS arm, 1000 mg/m^2 gemcitabine is infused on days 1 and 8, and 30 mg/m^2 S-1 is orally administered twice per day from days 1 to 14, repeated every 3 weeks.

For the S-1 monotherapy arm, 40 mg/m^2 S-1 is orally administered twice per day for 4 weeks, followed by a 2-week rest, repeated every 6 weeks.

Protocol treatments in both arms are continued until progression, unacceptable toxicity or patient refusal.

FOLLOW-UP

Enhanced abdominal computed tomography (CT)/magnetic resonance imaging, chest CT/X-rays and tumor markers (CEA and CA19-9) are evaluated at least every 6 weeks during the protocol treatment. Adverse events are evaluated at least every 2 weeks during the protocol treatment using CTCAE ver. 3.0.

STUDY DESIGN AND STATISTICAL ANALYSIS

This study is a randomized Phase II selection design trial (9) to evaluate which regimen, GS or S-1, is more promising for the test arm regimen for a subsequent Phase III trial. The regimen that shows the higher point estimate in terms of the proportion of 1-year survival will be considered to be more promising.

The frequency of toxicity is expected to be higher in GS than in S-1 monotherapy, but we expect that the frequency of severe toxicity will be almost equivalent. Therefore, we will select the more promising regimen on the basis of efficacy, namely, 1-year overall survival, as long as the levels of severe toxicity do not differ markedly between the two arms.

Sample size was determined as follows by Simon's selection design. We assumed that 1-year survival of one regimen is 30% and that of the other regimen is more than 40%. In this situation, the sample size ensuring at least 85% probability of correct selection of the more effective regimen is 98 patients, with 49 patients per arm. Considering the likelihood of some ineligible patients being enrolled, the total number of patients was set at 100.

INTERIM ANALYSIS AND MONITORING

We do not plan the interim analysis in this study. In-house monitoring will be performed every 6 months by the JCOG Data Center to evaluate the study progress and to improve the study quality.

Participating Institutions

The participating institutions (from north to south) are as follows: Sapporo-Kosei General Hospital, Tochigi Cancer Center, Jichi Medical University, Saitama Cancer Center, National Cancer Center Hospital East, Chiba Cancer Center Hospital, National Cancer Center Hospital, Kyorin University School of Medicine, Cancer Institute Hospital, Kanagawa Cancer Center, Yokohama City University Medical Center, Shizuoka Cancer Center, Aichi Cancer Center Hospital, Osaka Prefectural Hospital Organization Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka National Hospital, National Hospital Organization Shikoku Cancer Center, National Kyushu Cancer Center and Kyushu University Hospital.

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Conflict of interest statement

None declared.

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Treatment Efficacy/Safety and Prognostic Factors in Patients with Advanced Biliary Tract Cancer Receiving Gemcitabine Monotherapy: An Analysis of 100 Cases

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Abstract

Aim: The purpose of this study was to elucidate the treatment efficacy and safety of gemcitabine monotherapy, and to identify prognostic factors in patients with advanced biliary tract cancer receiving this therapy. *Method:* The data of 100 patients with advanced biliary tract cancer who were treated with gemcitabine as first-line chemotherapy were reviewed retrospectively. *Results:* One patient showed complete response (1.0%) and 6 patients showed partial response (6.0%), yielding an overall response rate of 7.0%. The main grade 3/4 toxicities were neutropenia and leukopenia. The median survival, 1-year survival rate and progression-free survival were 7.3 months, 21.6% and 3.1 months, respectively. Multivariate analysis identified a performance status of 0–1, serum C-reactive protein level of <3.0 mg/dl, serum carcinoembryonic antigen level of <10 ng/ml and serum albumin level of ≥3.5 g/dl as factors independently associated with a favorable prognosis. *Conclusions:* Gemcitabine monotherapy showed modest efficacy with manageable toxicity in patients with biliary tract cancer. These results could be useful as reference data for optimizing treatment strategies and planning future clinical trials in patients with advanced biliary tract cancer.   Copyright © 2010 S. Karger AG, Basel

Introduction

Biliary tract cancer (BTC) is uncommon in western countries, but it is a common cancer-related death in Japan, with an estimated 16,000 deaths occurring annually [1]. Surgery currently remains the only potentially curative treatment, but the majority of patients are diagnosed at an advanced stage of the disease because of the lack of early symptoms. Moreover, even in patients treated with surgical resection, the risk of recurrence is extremely high [2]. Although systemic chemotherapy is indicated

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for patients with unresectable disease, standard chemotherapeutic regimens have not been established in the last decades [2]. To improve survival, various agents were evaluated in clinical trials. Among these agents, gemcitabine was found to yield relatively favorable results [3, 4], and it has been administered worldwide either as a single agent or in combination with other agents for the treatment of BTC [2–5]. More recently, in a randomized phase III study of combined chemotherapy with gemcitabine and cisplatin versus gemcitabine monotherapy (UK ABC-02 study), median survival times were 11.7 and 8.3 months, respectively (p = 0.002) [6]. Therefore, the gemcitabine-cisplatin combination will become standard chemotherapeutic treatment for advanced BTC.

Numerous clinical trials investigating gemcitabine-based regimens have been conducted to date, but the numbers of patients have been small and only selected patients have been treated with gemcitabine. In addition, prognostic factors in BTC patients treated with gemcitabine have not yet been fully clarified. The objectives of this current study were to retrospectively review the treatment efficacy and safety of gemcitabine monotherapy, as well as to identify prognostic factors in patients with advanced BTC receiving this therapy.

Patients and Methods

Patients

One hundred sixteen patients with advanced or recurrent BTC received gemcitabine monotherapy from December 2001 to August 2007 at the National Cancer Center Hospital and National Cancer Center Hospital East. The diagnosis of BTC was confirmed histologically and/or cytologically as adenocarcinoma. Among these patients, the data of 16 patients were excluded from this analysis (a history of prior treatment in 10 patients; voluntary move to another hospital before the first tumor assessment in 3 patients and no evaluable tumor in 3 patients). A total of 100 patients had measureable lesion(s) and data were thus analyzed to elucidate the treatment efficacy and safety of gemcitabine monotherapy. The following criteria had to be met to be eligible for systemic chemotherapy, including gemcitabine monotherapy, at our institutions: Eastern Cooperative Oncology Group performance status (PS) of 0–2, adequate bone marrow function (white blood cell (WBC) count $\geq 3,000/mm^2$, absolute neutrophil count $\geq 1,000/mm^3$ and platelet count $\geq 70,000/mm^3$) and availability of written informed consent from each patient. Patients were excluded if they had severe complications. Gemcitabine was administered at a dose of 1,000 mg/m^2 by intravenous injection for 30 min on days 1, 8 and 15 of each 28-day cycle until disease progression, appearance of unacceptable toxicity or patient's refusal for treatment continuation. All patients underwent physical examination and assessment of toxicity at least once every 1 or 2 weeks until the completion of gemcitabine treatment. All patients with obstructive jaundice underwent percutaneous transhepatic or endoscopic retrograde biliary drainage before treatment. These patients were required to have serum bilirubin levels of <3.0 mg/dl and serum AST and ALT levels <5 times the upper limit of normal.

Response and Toxicity Evaluation

The antitumor effect of gemcitabine was evaluated by CT/MRI conducted every 4–8 weeks after the start of treatment. Tumor response was determined according to the Response Evaluation Criteria in Solid Tumors [7]. The size of measurable lesions was determined using enhanced CT or MRI. For this analysis, tumor response was reviewed, and the best overall response was recorded for each patient. Toxicities were graded according to the Common Terminology Criteria for Adverse Events, version 3.0.

Analysis of Prognostic Factors

Eighteen variables were selected in this study based on previous investigations [8–11] and our own clinical experience. All data were obtained just before the start of the systemic chemotherapy. The variables, which were divided into two clinically meaningful subgroups, were as follows: age (<65/$\geq$65 years), sex (male/female), PS (0–1 or 2), WBC count (<8,500/$\geq$8,500/μl), hemoglobin level (<12.0/$\geq$12.0 g/dl), platelet count (<220,000/$\geq$220,000/μl), serum albumin level (<3.5/$\geq$3.5 g/dl), serum total bilirubin level (<2.0/$\geq$2.0 mg/dl), serum lactate dehydrogenase (LDH) level (<230/$\geq$230 IU/l), serum C-reactive protein (CRP) level (<3.0/$\geq$3.0 mg/dl), biliary drainage (presence/absence) and prior surgical resection (presence/absence) as the host-related variables, primary tumor location (intrahepatic, extrahepatic, bile duct and ampulla of Vater/gallbladder), extent of disease (localized/metastatic), peritoneal dissemination (presence/absence), liver metastasis (presence/absence), serum carcinoembryonic antigen (CEA) level (<10/$\geq$10 ng/ml) and serum carbohydrate antigen 19-9 (CA 19-9) level (<1,000/$\geq$1,000 U/ml) as the tumor-related variables. Peritoneal dissemination was defined as recognition of peritoneal nodules on CT/MRI or positive cytology of group V ascites.

Statistical Analysis

Progression-free survival was calculated as the time interval from the 1st day of treatment to the date of detection of disease progression, last day of follow-up, or the date of death. Overall survival was calculated as the time interval from the 1st day of treatment to the date of death or the last day of follow-up. In univariate analysis, cumulative survival proportions were calculated by the Kaplan-Meier method and differences were evaluated by the log-rank test. Only variables that were identified as showing statistical significance in univariate analysis were included into Cox's proportional hazard regression model for multivariate analysis. p < 0.05 was considered to be statistically significant and all the tests were two-sided. All statistical analyses were performed using the SPSS statistical software package (SPSS version 11.0 for Windows).

Results

Patient Characteristics

The characteristics of the patients are shown in table 1. PS was 0 in 66 patients (66.0%), 1 in 27 patients (27.0%)
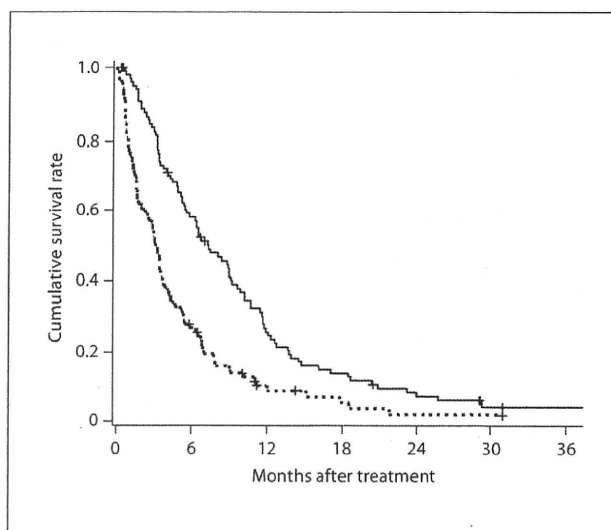
Table 1. Patient characteristics

Characteristics	Patients
Age, years, median [range]	67.5 [44–82]
Sex, n (%)	
Male	60 (60.0)
Female	40 (40.0)
PS, n (%)	
0	66 (66.0)
1	27 (27.0)
2	7 (7.0)
WBC, n/μl, median [range]	6,400 [3,200–17,200]
Hemoglobin, g/dl, median [range]	12.2 [6.2–15.3]
Platelets, n × 10^4/μl, median [range]	23.2 [7.9–56.8]
Albumin, g/dl, median [range]	3.6 [1.9–4.6]
Total bilirubin, mg/dl, median [range]	0.8 [0.2–4.1]
Lactic dehydrogenase, IU/l	
median [range]	203.0 [70.0–733.0]
CRP, mg/dl, median [range]	0.9 [0.0–26.3]
Primary tumor site, n (%)	
Intrahepatic bile duct	23 (23.0)
Extrahepatic bile duct	25 (25.0)
Gallbladder	45 (45.0)
Ampulla of Vater	7 (7.0)
Extent of disease, n (%)	
Locally advanced	20 (20.0)
Metastatic	80 (80.0)
Metastatic site, n (%)	
Liver	36 (36.0)
Lymph node	28 (28.0)
Peritoneal dissemination	25 (25.0)
Lung	16 (16.0)
Biliary drainage (+)	30 (30.0)
Prior surgical resection (+)	28 (28.0)
CEA, ng/ml, median [range]	6.5 [0.5–3,110.0]
CA 19-9, U/ml, median [range]	258.1 [0.0–827,000]

Fig. 1. Overall survival (solid line) and progression-free survival (broken line) in patients with BTC treated with gemcitabine monotherapy.

and 2 in 7 patients (7.0%). Twenty-three (23.0%) patients had intrahepatic bile duct cancer, 25 (25.0%) had extrahepatic bile duct cancer, 45 (45.0%) had gallbladder cancer, and 7 (7.0%) had cancer in the ampulla of Vater. The median number of cycles of gemcitabine monotherapy administered was 2.9 (range: 1–34). Eighteen patients (18.0%) received second-line treatment as follows: S-1 monotherapy, 7 patients; uracil/tegafur, 3 patients; uracil/tegafur + doxorubicin, 2 patients; immunotherapy, 3 patients, and other treatments, 3 patients.

Tumor Response

All the 100 patients had measureable primary or metastatic lesion(s). Of the 100 patients, complete response (CR) was achieved in 1 patient, partial response (PR) in 6 patients, stable disease (SD) was noted in 56 patients, and

progressive disease (PD) in 35 patients. The remaining 2 patients could not be assessed radiologically, but both were judged as showing clinical evidence of tumor progression. The overall response rate (RR) was 7.0% [95% confidence interval (CI), 2.9–13.9]. The data were also analyzed according to the tumor type. The overall RR in patients with cancer of the intrahepatic bile duct, extrahepatic biliary duct, gallbladder and ampulla of Vater were 4.2% (1/23), 8.0% (2/25), 8.8% (4/45) and 0.0% (0/7), respectively. The overall disease control rates (CR + PR + SD) in patients with cancer of the intrahepatic bile duct, extrahepatic bile duct, gallbladder and ampulla of Vater were 69.5% (16/23), 60.0% (15/25), 57.8% (26/45) and 85.7% (6/7), respectively.

Survival

By the time of the analysis, 91 of the 100 patients had died as a result of PD. The median follow-up of censored 9 patients was 7.0 months (range, 0.4–30.9).

The overall and progression-free survival curves are shown in figure 1. The median survival, 1-year survival rate and median progression-free survival were 7.3 months (95% CI, 5.4–9.2 months), 21.6% and 3.1 months (95% CI, 2.6–3.6 months), respectively. The median progression-free survival times in PR, SD and PD patients was 12.0 (95% CI 9.5–14.5), 4.3 (95% CI 2.6–6.0) and 0.8 (95% CI 0.6–1.0) months, respectively, and the overall

Table 2. Treatment-related adverse events (worst grade reported during the treatment period)

Adverse events	Toxicity grade				
	1	2	3	4	3/4 (%)
Hematological toxicity					
Leukopenia	24	18	9	0	9 (9.0)
Neutropenia	6	9	11	5	16 (16.0)
Thrombocytopenia	9	9	2	0	2 (2.0)
Anemia	18	20	3	0	3 (3.0)
Non-hematological toxicity					
Nausea/vomiting	13	0	1	0	1 (1.0)
Anorexia	22	2	2	0	2 (2.0)
Fatigue	25	2	0	0	0
Diarrhea	3	0	0	0	0
Rash	8	1	0	0	0
Decreased serum					
albumin level	13	6	2	0	2 (2.0)
Elevated serum AST	16	7	2	0	2 (2.0)
Elevated serum ALT	10	5	2	0	2 (2.0)
Elevated serum ALP	3	1	5	0	5 (5.0)
Hyponatremia	9	0	0	0	0
Cognitive disturbance	0	0	1	0	1 (1.0)
Biliary tract infection	1	0	2	0	2 (2.0)

AST = Aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase.

survival times were 17.1 (95% CI 14.6–19.7), 9.7 (95% CI 8.4–10.9) and 3.2 (95% CI 2.6–3.9) months, respectively.

Toxicity

The most severe hematological and non-hematological toxicities during the entire treatment period are summarized in table 2. With regard to grade 3/4 hematological toxicities, neutropenia was observed in 16 patients (16.0%), leukopenia in 9 patients (9.0%), anemia in 3 patients (3.0%) and thrombocytopenia in 2 patients (2.0%). In regard to the main grade 3/4 non-hematological toxicities, an elevated alkaline phosphatase level was observed in 5 patients (5.0%), and other adverse events occurred in <3%. Cognitive disturbance was observed in 1 patient (1.0%); however, recovery occurred in the absence of any treatment. There were no other life-threatening toxicities and no treatment-related deaths.

Univariate Analysis

Of the 18 pretreatment variables, 12 variables (female, PS 0–1, WBC count <8,500/μl, hemoglobin >12.0 g/dl, serum albumin ≥3.5 g/dl, serum total bilirubin <2.0 mg/dl, serum LDH <230 IU/l and serum CRP <3.0 mg/dl, intrahepatic, extrahepatic, bile duct and ampulla of Vater cancer, absence of peritoneal dissemination, absence of liver metastasis and serum CEA <10 ng/ml) were identified as being significantly associated with a longer survival time (table 3).

Multivariable Analysis

The 12 variables identified by univariate analysis as being of prognostic significance were subsequently incorporated in Cox's proportional hazard model for multivariate analysis, and a PS of 0–1, serum CEA <10 ng/ml, serum albumin ≥3.5 g/dl, and serum CRP <3.0 mg/dl were identified as being independently associated with a favorable prognosis (table 4).

Discussion

Gemcitabine has been used as a key drug for advanced BTC, and at present gemcitabine-based regimens are widely used as first-line treatment for advanced BTC. However, to date, reliable data of gemcitabine monotherapy based on large-scale studies are still lacking. This study shows not only efficacy and safety but also prognostic factors in a large study cohort.

Studies on gemcitabine monotherapy at doses of 800–2,200 mg/m^2 as first-line therapy for advanced BTC have reported response rates of 0–36.0%, and median survival times from 4.6 to 14.0 months [12–20]. Our overall response rate of 7.0% in BTC patients administered gemcitabine monotherapy as first-line therapy in our study was comparable to those reported from previous trials of gemcitabine monotherapy. The median survival of 7.3 months and the incidence of adverse events were also in accord with previous reports [12–20]. These findings clearly demonstrate that gemcitabine monotherapy is well tolerated in patients with advanced BTC in the clinical setting.

The study was also designed to determine prognostic factors in patients with advanced BTC administered gemcitabine monotherapy. The identification of prognostic factors can help to predict life expectancy and to select the appropriate treatment. In the current study, among the variables investigated, PS, serum CRP, serum albumin and serum CEA were found to be independently associated with patient prognosis.

PS was the strongest prognostic factor, although most of our patients (93%) had a good PS (0–1) and only 7 patients were PS 2. PS is a simple, but widely used index re-

Table 3. Univariate analysis to identify prognostic factors associated with survival in BTC patients

Variable	Patients	Median survival months	Hazard ratio (95% CI)	p value
Age				
≥65 years	59	8.4	1	0.25
<65 years	41	6.4	1.28 (0.84–1.96)	
Sex				
Female	60	9.0	1	0.02
Male	40	5.5	1.68 (1.08–2.54)	
PS				
0–1	93	8.1	1	<0.01
2	7	1.3	11.15 (4.38–28.38)	
WBC count				
≥8,500/μl	81	8.8	1	0.02
<8,500/μl	19	3.3	1.90 (1.10–3.29)	
Hemoglobin				
<12 g/dl	51	10.2	1	<0.01
≥12 g/dl	49	5.5	1.85 (1.22–2.86)	
Platelets				
≥220,000/μl	39	9.1	1	0.18
<220,000/μl	61	6.5	1.34 (0.87–2.01)	
Albumin				
≥3.5 g/dl	59	9.2	1	<0.01
<3.5 g/dl	41	5.1	2.61 (1.67–4.01)	
Total bilirubin				
<2 mg/dl	83	8.9	1	0.01
≥2 mg/dl	17	5.5	2.28 (1.19–4.37)	
LDH				
<230 IU/l	61	9.7	1	<0.01
≥230 IU/l	39	3.5	2.55 (1.64–3.94)	
CRP				
<3 mg/dl	75	9.2	1	<0.01
≥3 mg/dl	25	3.3	4.01 (2.41–6.67)	
Biliary drainage				
Absent	70	8.4	1	0.91
Present	30	6.5	1.03 (0.66–1.60)	
Prior surgical resection				
Yes	29	10.2	1	0.10
No	71	6.4	1.45 (0.93–2.27)	
Primary tumor site				
Bile duct	55	9.1	1	0.04
Gallbladder	45	6.4	1.55 (1.02–2.35)	
Extent of disease				
Localized	16	11.7	1	0.14
Metastatic	84	6.5	1.44 (0.89–2.33)	
Peritoneal dissemination				
Absent	75	8.1	1	0.04
Present	25	4.9	1.64 (1.02–2.65)	
Liver metastasis				
Absent	64	9.0	1	<0.01
Present	36	5.8	1.81 (1.17–2.79)	
CEA				
<10 ng/ml	57	9.7	1	0.01
≥10 ng/ml	43	5.8	1.73 (1.12–2.66)	
CA 19-9				
<1,000 U/ml	66	8.4	1	0.12
≥1,000 U/ml	34	5.2	1.41 (0.91–2.17)	

Bile duct = Intra- and extrahepatic bile duct/ampulla of Vater.

Table 4. Significant prognostic factors in BTC patients treated with gemcitabine determined by multivariate analysis using Cox's proportional hazard model

Variable	Hazard ratio	95% CI	p value
PS			
0–1	1		<0.01
2	5.417	2.05–14.28	
CRP			
<3 mg/dl	1		<0.01
≥3 mg/dl	2.791	1.53–5.09	
CEA			
<10 U/ml	1		<0.01
≥10 U/ml	2.138	1.36–3.36	
Albumin			
≥3.5 g/dl	1		<0.01
<3.5 g/dl	2.005	1.218–3.30	

flecting the physical condition of the patient, which has been recognized as an important prognostic factor in patients with a variety of malignancies, including BTC [10, 11]. In this study, the median survival of PS 2 patients was only 1.6 months. Thus, patients of PS 2 should not receive gemcitabine monotherapy. Serum albumin and CRP were also found to be significant prognostic factors in this study. They are interrelated, because albumin is related to systemic inflammation, which is measured by serum CRP and other cytokines [21–24]. CRP is produced by the liver and its production is induced by pro-inflammatory cytokines, such as interleukin-6 and tumor necrosis factor-α, which are involved in the pathogenesis of cachexia [25, 26]. These cytokines are associated with hypermetabolism, weight loss and anorexia and, as a result, may reflect shortened survival. Serum CEA is currently the most widely used tumor marker for other malignancies [27–30]. High serum CEA has also been shown to be a poor prognostic factor in patients with other malignancies, and in agreement with these data, our results also suggest that high serum CEA levels may reflect a high tumor burden and be related to survival in BTC patients. Contrary to our expectation, the primary site of cancer was not identified as an independent prognostic factor in our study. Several other studies have reported that involvement of the gallbladder is predictive of poor overall survival [3, 31–33]. In the current study, while involvement of the gallbladder was identified as a poor prognostic factor on univariate analysis, it was not extracted as an independent prognostic factor by multivariate analysis (p = 0.10). The reason for this discrepancy is unclear,

but we investigated as many as 18 variables that may potentially affect the prognosis and identified 4 as independent prognostic factors. Recently, several phase III trials have been conducted, besides the ABC-02 study. More effective chemotherapeutic regimens based on gemcitabine are expected to be developed in the near future. The results of this study may help to optimize the design of future clinical trials using gemcitabine.

In conclusion, gemcitabine monotherapy for advanced BTC exhibited modest efficacy with manageable toxicity. PS and serum levels of CRP, CEA and albumin were identified as independent prognostic factors. These results could be useful in predicting life expectancy, selecting the appropriate treatment strategy and designing future clinical trials for patients with advanced BTC.

Acknowledgments

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Phase I/II Study of Hepatic Arterial Infusion Chemotherapy With Gemcitabine in Patients With Unresectable Intrahepatic Cholangiocarcinoma (JIVROSG-0301)

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Objectives: No established therapy exists for unresectable intrahepatic cholangiocarcinoma (ICC). We conducted a phase I/II study to ascertain the recommended dose (RD) of hepatic arterial infusion using gemcitabine (GEM) for ICC and to assess the efficacy and safety.

Methods: For patients with unresectable ICC, GEM was administered through the hepatic artery via the port system as a 30-minute infusion on days 1, 8, and 15 every 4 weeks for 5 cycles. In phase I, dosage for levels 1, 2, and 3 was set at 600, 800, and 1000 mg/m², respectively, and was increased in 3 to 6 patients at a time. Maximum tolerated dose was defined as a dosage resulting in dose-limiting toxicity in 2 of 3 patients or 3 of 6 patients, and RD was estimated during the first cycle. In the phase II, more RD patients were added to assess tumor response and toxicity.

Results: During the phase I, 16 patients were enrolled. Maximum tolerated dose was not reached. Assuming RD at 1000 mg/m², the phase II enrolled a total of 13 patients. The following Grade 3 toxicities were observed: neutropenia 20%, increased gamma-glutamyl transpeptidase 8%, increased aspartate aminotransferase 4%, increased alanine aminotransferase 4%, increased bilirubin 4%, nausea 4%, and fatigue 4%. The tumor response rate was 7.7% (complete response 0, partial response 1, stable disease 8, and progressive disease 4).

Conclusion: Whereas the toxicity of hepatic arterial infusion with 1000 mg/m² GEM for ICC was tolerable, expected efficacy could not be obtained, thus suggesting only minimal activity.

Key Words: intrahepatic cholangiocarcinoma, hepatic arterial infusion, gemcitabine, phase I/II study, clinical trial

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Intrahepatic cholangiocarcinoma (ICC) constitutes 5% to 15% of cases of the primary hepatic cancer in Japan. It is a cancer with a relatively low incidence, but is characterized by spread from the biliary epithelium to Glisson capsule. ICC has a high incidence of lymph node metastasis and vascular invasion and also tends to invade adjacent organs, so that in a fair number of cases it is already advanced and unresectable at the time of detection.[1–3] Chemother-apy is the treatment option for unresectable ICC, but no standard therapy has been established.[4,5] Typically, drug regimens centered on 5-fluorouracil (5-FU) have been used, but recently, gemcitabine hydrochloride (GEM) has appeared promising.[6]

Hepatic arterial infusion (HAI) chemotherapy is one local therapy for unresectable malignant hepatic tumors and its anticancer effect is obtained by raising the local concentration of the anticancer agent. Local therapy also reduces systemic adverse response and can increase the effect on the hepatic lesions by infusing the active medicinal agent into a hepatic artery.[7] In Japan, HAI with percutaneous placement of a catheter-port system is highly feasible,[8–10] and HAI of GEM can be continued systematically. If a local effect for ICC supplying from the hepatic artery can be obtained with HAI of GEM, this treatment may contribute to prolonging patient survival.

With this as background, we designed a phase I and II clinical trial to evaluate HAI chemotherapy with GEM for unresectable ICC, and a multicenter study was carried out by the Japan Interventional Radiology in Oncology Study Group.

MATERIALS AND METHODS

Study Design and Patient Eligibility

A phase I and II clinical trial at multiple institutions was designed to determine the dose-limiting toxicity (DLT) and recommended dose (RD) for HAI chemotherapy with GEM to treat unresectable ICC, as well as to evaluate its safety and tumor response effect. Dose-limiting toxicity and recommended dose of hepatic arterial infusion of GEM were determined as the primary end point, and the frequency and severity of adverse events, tumor response effect in the liver only, and tumor response effect in the whole body were the secondary end points. In phase I portion, DLT was assessed and RD was estimated, and in phase II portion, cases were added at the estimated RD, and the tumor response effect was evaluated. Toxicity assessment was conducted in all patients with HAI chemotherapy.

The inclusion criteria were the following conditions for cases of unresectable ICC:

1. Cases of histologically confirmed ICC (initial tumor or recurrence after resection), which was determined to be unresectable by a hepatic surgeon at each institution, or it was judged to be the prognosis-determining factor, even when metastasis was found as extrahepatic lesions.
2. Cases that were previously untreated with GEM or that were previously treated with agents other than GEM in the past, but had received no chemotherapy for at least 4 weeks from the last session, and were not responded by the chemotherapy.
3. Cases in which measurable lesions that corresponded to the target lesions on response evaluation criteria in solid tumors were located in the liver and had maximum tumor diameters of 20 mm or more

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on computed tomography (CT) images with 10-mm slices or 10 mm or more on CT images with slices of 5 mm or less.

4. Cases in which a port-catheter system for HAI was placed percutaneously, and arterially infused contrast medium was distributed through the entire liver or at least the entire hepatic lesions and in whom it was confirmed that there was no distribution of the arterially infused contrast medium in the surrounding extrahepatic organs based on CT angiography or MR angiography from the implanted port.

5. Cases aged 20 years or more with an Eastern Cooperative Oncology Group performance status classification of 2 or less.

6. Cases in which major organ function was maintained (white blood cell count $\geq 3000/mm^3$ and $\leq 12,000/mm^3$, platelets $\geq 100,000/mm^3$, transaminase ≤ 5 times the institution's upper limit of normal, serum total bilirubin ≤ 3.0 mg/dL, serum creatinine ≤ 1.5 mg/dL, electrocardiogram not indicating the need for treatment) and in whom hepatic function was Grade 2 or less on National Cancer Institute-Common Toxicity Criteria (NCI-CTC) (version 2.0) with consideration of the influence of the hepatic lesion.

7. Cases of life expectancy of more than 8 weeks.

8. Cases in which written informed consent was obtained.

Patients excluded from the trial were the patients who scheduled for radiation therapy for the hepatic portal region because of hepatic portal region invasion or lymph node metastasis, or who had previously undergone radiation therapy; patients with concurrent infection excluding viral hepatitis, fever of 38°C or above, or who required antibiotics; patients with serious complications (intestinal paralysis, intestinal obstruction, interstitial pneumonia, pulmonary fibrosis, intractable diabetes mellitus, cardiac failure, renal failure, hepatic failure, etc); patients with other concurrent cancer; patients who could not undergo angiography because of allergy to iodinated contrast material; patients with serious mental disabilities; patients who were pregnant or may have been pregnant, and nursing mothers; and patients whose catheters for HAI chemotherapy were placed via laparotomy.

This study protocol was approved by the ethics committee of the Japanese Society of Interventional Radiology and the institutional review boards of the participating hospitals.

Treatment Protocol and Evaluation Methods

Using a percutaneously placed HAI catheter-port system, 1 course was defined as HAI of GEM on days 1, 8, and 15; a course was performed every 4 weeks for a total of 5 courses.

In phase I portion, the GEM dosage was set at Level -1, 400 mg/m^2; Level 1, 600 mg/m^2; Level 2, 800 mg/m^2; and Level 3, 1000 mg/m^2. Because the approval dosage of GEM is 1000 mg/m^2 in Japan, we defined it as the upper limit in this study. The design called for increase at each level in 3 to 6 patients from Level 1. Three patients were enrolled at each level. The study on the next dose level was not conducted until all 3 patients had completed the first cycle without any problems regarding safety and tolerance. If a DLT of any type was detected in 1 of 3 patients during the first cycle, an additional 3 patients were enrolled. If DLT was detected in more than 2 patients, the dose was defined as the maximum tolerated dose (MTD). RD was estimated to be one level below that judged to be MTD. DLT was defined as follows and judged during the first course: Grade 4 leukopenia or neutropenia; Grade 4 thrombocytopenia; nonhematologic toxicities of Grade 3 or more (excluding that from PD, nausea/vomiting, and alopecia; for patients whose pre-enrollment level of transaminase or serum total bilirubin was Grade 2, DLT was taken to be more than twice the pre-enrollment level); not meeting the criteria to start administration (same as the enrollment criteria) for the next course on day 29 because of toxicity.

In phase II portion, up to 13 patients were added at the dose found to be RD in phase I portion and the tumor response effect was judged using response evaluation criteria in solid tumors. Because HAI was being used, the target lesion was limited to hepatic lesions. Tumor size was measured on intravenous contrast-enhanced CT within 2 weeks before enrollment, and the tumor response effect was judged after the completion of courses 1, 3, and 5, and as needed.

Toxicity assessment was done in all cases using NCI-CTC (version 2.0) and the frequency of the worst grade was obtained during all courses. Physical examination and blood tests were done immediately before the start of each treatment and recorded.

Statistical Analysis

In phase I portion, the number of enrolled patients per level from Level -1 to Level 1 was minimum 6. The maximum number of patients up to Level 3, in case that MTD was reached, was 18 patients in the dose finding stage. In phase II portion, when the threshold tumor response rate was taken to be 20% and the expected efficacy rate was set at 50%, 13 patients would be needed to judge the tumor response effect under conditions of $\alpha = 0.1$ and $\beta = 0.2$, and 7 to 10 cases would need to be added at the estimated RD. For the entire study, a maximum of 25 patients was needed.

RESULTS

Patient Backgrounds

A total of 16 patients were enrolled in the phase I portion (May 2004–November 2005), and 9 patients were added for the phase II portion (February 2006–November 2006). All patients met the eligibility requirements. A summary of all 25 patients is shown in Table 1.

Phase I Portion

In phase I portion, 6 patients were registered at Level 1, 6 at Level 2, and 4 at Level 3. DLT appeared in 2 of the 6 patients at Level 1, and 2 of the 6 patients at Level 2, but DLT did not appear at Level 3. The third and fourth patients at Level 3 were registered at almost the same time. Four patients did not meet the criteria to start administration for the second course on day 29. In these 4 patients, the administration of drugs had been delayed because of Grade 1 and 2 leukopenia (n = 3) or thrombocytopenia (n = 4) in the first course. No Grade 4 hematologic toxicity or nonhematologic toxicity of Grade 3 or more was seen in the first course (Tables 2, 3). MTD was not reached up to Level 3. Accordingly, the RD was assumed to be the Level 3 dose of 1000 mg/m^2.

Phase II Portion

Nine patients were added at GEM 1000 mg/m^2. In these patients, together with the patients at Level 3 in phase I portion (total: 13 patients), the tumor response effect was complete response 0/partial response 1/stable disease 8/progressive disease 3/not evaluated 0 in the liver only, and complete response 0/partial response 1/stable disease 8/progressive disease 4/not evaluated 0 in the whole body. The response rate was 7.7% (95% confidence interval [CI], 0.2%–36.0%). Although disease control was not one of the assessment items, the disease control rate with SD added was 69% (95% CI, 38.6%–90.9%). The tumor response effect and survival in all 25 treated patients are shown in Table 4 and Figure 1.

Toxicity

The incidence of adverse events (NCI-CTC version 2.0) of Grade 3 or more in all treated cases was 20% neutropenia, 8% elevated gamma-glutamyl transpeptidase (GGT), 4% elevated aspartate aminotransferase (AST), 4% elevated alanine aminotransferase (ALT), 4% elevated bilirubin, 4% nausea, and 4% fatigue. The only

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TABLE 1. Patients' Characteristics

Phase	Phase I			Phase II	All Patients
Level of GEM Dose	Level 1	Level 2	Level 3	Estimated RD	
GEM dose	600 mg/m^2	800 mg/m^2	1000 mg/m^2	1000 mg/m^2	600, 800, 1000 mg/m^2
No. patients	6	6	4	9	25
Age (yr)					
Median (range)		64 (34–76)		56 (46–74)	58 (34–76)
Gender					
Male	3	5	3	7	18
Female	3	1	1	2	7
ECOG PS					
0	4	5	3	7	19
1	1	1	1	2	5
2	1	0	0	0	1
Previous therapy					
None	4	2	3	4	13
Resection	1	3	1	5	10
Chemotherapy	1	0	1	2	4
Embolization or ablation	0	2	0	1	3
Extrahepatic lesions					
None	3	3	2	8	16
Lymph node	3	3	2	0	8
Peritoneum	1	0	0	0	1
Lung	0	1	2	1	4
Median no. courses administered	5	4.5	4		5
Median no. administrations	15	14	12		15
Relative dose intensity	81.9%	87.3%	84.8%		84.7%

ECOG indicates Eastern Cooperative Oncology Group performance status.

TABLE 2. No. Patients With Hematologic Toxicities (Cycle 1, Phase I Portion, n = 16)

Level Dose n Grade	Level 1 600 mg/m^2 6				Levele 2 800 mg/m^2 6				Level 3 1000 mg/m^2 4			
	1	2	3	4	1	2	3	4	1	2	3	4
Leucocytes	1	2	0	0	1	3	0	0	2	1	0	0
Neutrophils	0	2	1	0	1	1	2	0	1	1	0	0
Hemoglobin	0	1	0	0	0	0	0	0	0	0	0	0
Platelets	2	2	0	0	2	1	0	0	1	1	0	0

Grade 4 event was elevated bilirubin in 1 patient in the second course, but this was accompanied by portal vein tumor thrombosis (Tables 5, 6).

Events related to the HAI procedure included difficulties with the placed catheter-port system in 5 patients (catheter obstruction in 3 patients, port damage in 2 patients), and hepatic artery occlusion in 1 patient. In 2 of the patients with catheter obstruction and the 2 patients with port damage the catheter or port was exchanged and the treatment continued. The remaining patient with catheter obstruction showed an antitumor effect of PD, so the catheter was not replaced and the treatment was stopped. In the patient with hepatic artery occlusion, a left hepatic artery occlusion occurred in the second course, which meant that the drug was not reaching the left lobe of the liver, and the treatment was discontinued.

TABLE 3. No. Patients With Adverse Events (Cycle 1, Phase I Portion, n = 16)

Level Dose n Grade	Level 1 600 mg/m^2 6				Level 2 800 mg/m^2 6				Level 3 1000 mg/m^2 6			
	1	2	3	4	1	2	3	4	1	2	3	4
Nausea	0	2	0	0	2	0	0	0	3	0	0	0
Vomiting	0	1	0	0	0	0	0	0	2	0	0	0
Fatigue	1	1	0	0	3	0	0	0	0	0	0	0
Stomatitis	0	0	0	0	1	0	0	0	0	0	0	0
Headache	0	0	0	0	1	0	0	0	0	0	0	0
Diarrhea	0	0	0	0	0	0	0	0	0	0	0	0
Fever without neutropenia	0	0	0	0	0	0	0	0	1	0	0	0
Anorexia	0	0	0	0	0	0	0	0	0	0	0	0
Alopecia	0	0	0	0	1	0	0	0	0	0	0	0
Alkaline phosphatase	2	0	0	0	1	0	0	0	1	0	0	0
Bilirubin	1	0	0	0	0	0	0	0	0	0	0	0
GGT	1	0	0	0	0	1	0	0	0	0	0	0
Hypoalbuminemia	0	0	0	0	0	0	0	0	1	0	0	0
SGOT (AST)	1	0	0	0	0	0	0	0	1	0	0	0
SGPT (ALT)	0	0	0	0	0	1	0	0	1	0	0	0
Hyperkalemia	0	0	0	0	1	0	0	0	0	0	0	0
Hyponatremia	0	0	0	0	0	0	0	0	1	0	0	0

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