where θ_2 is gemcitabine clearance for patients without *3, *CDA**3 is 0 for non-*3/non-*3, ½ for *3/non-*3 and 1 for *3/*3; $\theta_{*3hetero}$ is a parameter related to the effect of heterozygous *3 but independent of θ_{*3homo}; and *CDA**3hetero is 1 for *3/non-*3 and 0 for *3/*3 or non-*3/non-*3. Equation 3 assumes a nonlinear gene-dose effect of *CDA**3 on CL. The OFV of equation 3 (model 3) was slightly but significantly smaller than that of equation 2, which indicates that the *CDA**3 gene-dose effect is not linear.

The effects of the body surface area (BSA), bodyweight, age and sex on the CL and V_1 of gemcitabine were investigated. As shown in table III, while consideration of an effect of size on the V_1 did not improve the OFV, examination of proportionality between the CL and BSA (model 4) considerably reduced the OFV. Age and sex did not significantly affect the CL and V_1 of gemcitabine (table III), although they were significantly correlated with these parameters in our previous univariate analyses.[12] As shown in table I, 66 patients received a gemcitabine-based combination chemotherapy with either cisplatin, carboplatin, fluorouracil, S-1 (an oral anti-cancer multicomponent drug containing tegafur, gimeracil and oteracil) or vinorelbine. Among the coadministered drugs, only S-1 significantly increased CL (model 5).

The effects of genetic polymorphisms of *CDA* other than *3 on the pharmacokinetics of gemcitabine were also examined. *CDA*–31delC (rs3215400; previously described as *CDA*–33_–31delC [precisely *CDA*–33_–31 C3>C2]), *CDA* 79A>C (Lys27Gln, *2) and *CDA* IVS1+37G>A increased gemcitabine clearance, and their effects were all statistically significant (table III). A delC factor was adopted in the final model for gemcitabine because it gave the smallest p-value and OFV (model 6 in table III).

Although we previously reported that 29 genetic variations of *DCK* were detected in our patients, they were very rare except for *DCK*–360C>G and 364C>T (Pro122Ser) [the allele frequencies were 0.131 and 0.061, respectively, as shown in table II],[15] and their functions were reported to be altered.[19,20] We analysed the effects of *DCK*–360C>G and 364C>T (Pro122Ser) on gemcitabine population pharmacokinetics, but no effects were detected. Thirty-nine genetic polymorphisms of *SLC29A1* (*hENT1*), including two nonsynonymous ones, were also previously reported.[16] Although we analysed the effects of genetic polymorphisms of *hENT1* whose allele frequencies were higher than 0.05 (table II), no effects were observed in univariate analyses (data not shown).

Development of a Combined Population Pharmacokinetic Model for Gemcitabine and dFdU

Next, we added compartments for dFdU where its central compartment was connected with the central compartment of gemcitabine with a first-order metabolic rate constant (CL/V_1) (figure 2). The f_m was assumed to be 1 because >90% of administered gemcitabine was recovered in the urine as dFdU.[6] Since an extraordinarily large V_m for dFdU was obtained if the V_1 for gemcitabine was not fixed, the V_1 was fixed to the value estimated in the previous section (12.60 L). Although the sampling duration in this study was not sufficiently long for pharmacokinetic analysis of dFdU (which has a longer half-life than that of gemcitabine, as shown in figure 1b), a two-compartment model (model 7, the combined basic model for gemcitabine and dFdU) provided a better fit for the data than a one-compartment model (the ΔOFV was –3402.44). Inclusion of covariates such as the BSA, age, serum creatinine level and sex in the model significantly reduced the OFV, as shown in table III.

All covariates selected by the inclusion steps remained after the stepwise exclusion/deletion process. The final population pharmacokinetic model (model 13) for Japanese cancer patients is shown in table IV. This model indicated that gemcitabine clearance was decreased by 64% and 17% in the *3-homozygotes and heterozygotes, respectively, compared with patients without *CDA**3. The increases in gemcitabine clearance by delC were 7.5% for heterozygotes and 15% for homozygotes. If S-1 was coadministered, gemcitabine clearance increased by 19%. CL_m was reduced by 8.6% if a patient was 10 years older than the average age (62.67 years in our patient group) and by about 7.3% if the creatinine level of a patient was 0.1 mg/dL higher than the average level (0.7 mg/dL in our patient group). The V_{m1} for dFdU was decreased by 8.1%

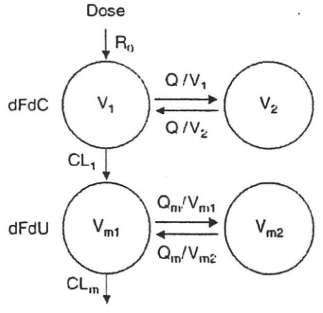

Fig. 2. Compartmental representation of gemcitabine (dFdC) and 2′,2′-difluorodeoxyuridine (dFdU) pharmacokinetics. CL_1 = clearance of gemcitabine; CL_m = clearance of the metabolite dFdU; Q = intercompartmental clearance between the central and peripheral compartments of gemcitabine; Q_m = intercompartmental clearance between the central and peripheral compartments of dFdU; R_0 = zero-order infusion rate constant; V_1 = apparent volume of distribution of the central compartment of gemcitabine; V_2 = apparent volume of distribution of the peripheral compartment of gemcitabine; V_{m1} = apparent volume of distribution of the central compartment of dFdU; V_{m2} = apparent volume of distribution of the peripheral compartment of dFdU.

Table IV. Population pharmacokinetic parameters for gemcitabine (dFdC) and 2′,2′-difluorodeoxyuridine (dFdU) in the final model

Pharmacokinetic parameter	Estimated value	CV%
Gemcitabine		
CL_1 (L/h/m^2)	$73.70 \times BSA \times (1-0.639 \times$ *3homo[a]$) \times (1-0.171 \times$ *3hetero[b]$) \times (1+0.0749 \times$ delC[c]$) \times (1+0.191 \times$ S-1[d]$)$	17.1
V_1 (L)	12.60 (Fixed)	58.9
Q (L/h)	37.50	Not estimated
V_2 (L)	9.54	25.3
dFdU		
CL_{m1} (L/h/m^2)	$11.00 \times BSA \times (1-0.00855 \times (AGE-62.67)) \times (1-0.732 \times (Cre-0.70))$	20.5
V_{m1} (L)	$15.00 \times BSA \times (1-0.00806 \times (AGE-62.67)) \times (1+0.239 \times Sex^{e})$	27.9
Q_m (L/h)	58.0	22.7
V_{m2} (L)	31.7	26.4
Residual error	SD (ε_3); 0.0844	
	CV (ε_1) and CV (ε_2); 0.200 and 0.0412, respectively	

a *3homo: 1 for homozygous *CDA**3 and 0 for others.

b *3hetero: 1 for heterozygous *CDA**3 and 0 for others.

c delC: number of *CDA*–31delC in a patient (delC = 0, 1 or 2).

d S-1: 1 for S-1 coadministered to patients and 0 for others.

e Sex: 1 for male and 0 for female.

ε = variance; **AGE** = age (years); **BSA** = body surface area (m^2); **CL_1** = clearance of gemcitabine; **CL_m** = clearance of the metabolite dFdU; **CL_{m1}** = clearance of the metabolite dFdU from central compartment; **Cre** = serum creatinine (mg/dL); **CV** = coefficient of variation (interindividual); **Q** = intercompartmental clearance between the central and peripheral compartments of gemcitabine; **Q_m** = intercompartmental clearance between the central and peripheral compartments of dFdU; **S-1** = an oral product of tegafur with gimeracil and oteracil; **V_1** = apparent volume of distribution of the central compartment of gemcitabine; **V_2** = apparent volume of distribution of the peripheral compartment of gemcitabine; **V_{m1}** = apparent volume of distribution of the central compartment of dFdU; **V_{m2}** = apparent volume of distribution of the peripheral compartment of dFdU.

if a patient was 10 years older than the average age, and was increased by 24% in males compared with females.

Evaluation of the Goodness of Fit

The observed plasma concentrations of gemcitabine and dFdU were plotted against concentrations predicted by the final model, as shown in figure 3a and b, respectively. Most gemcitabine concentrations distributed into two peaks: one peak with scattering around 25 mg/L (collected at the end of the gemcitabine infusion [30 minutes after initiation of the infusion]) and a second peak with scattering close to the point of origin. This dual peak plot was the result of very rapid gemcitabine metabolism. One point at an extremely high concentration represented the C_{max} obtained from a *3/*3 patient, who was administered 1000 mg/m^2 of gemcitabine.[12,13] For both gemcitabine and dFdU, higher plasma concentrations gave more widely scattered plots, indicating that the variation in the residual errors was proportional to the measured concentration (a constant coefficient of variation type). The slopes of the regression lines for gemcitabine and dFdU were very close to 1.0 (1.007 and 0.9908, respectively). Conditional weighted residuals (CWRES) were recently reported as a diagnostic tool for the FOCE approximation.[18] The slopes of the regression lines of CWRES for gemcitabine and dFdU against predicted plasma concentrations were very close to 0.0 (–0.00482 and –0.00926, respectively), indicating a very good fit for the constructed model. Further validation of the model by a visual predictive check or bootstrapping was not performed, because the distribution of some covariates, such as diplotypes of *CDA**3 (non-*3/non-*3 : non-*3/*3 : *3/*3 = 230 : 16 : 2), and coadministration of S-1 (in only 10 of the 248 patients) were unevenly distributed.

Discussion

Recently, Jiang et al.[21] performed population pharmacokinetic analyses on gemcitabine and dFdU, and they adopted two-compartment models for both plasma gemcitabine and dFdU pharmacokinetics. Likewise, in our study, the

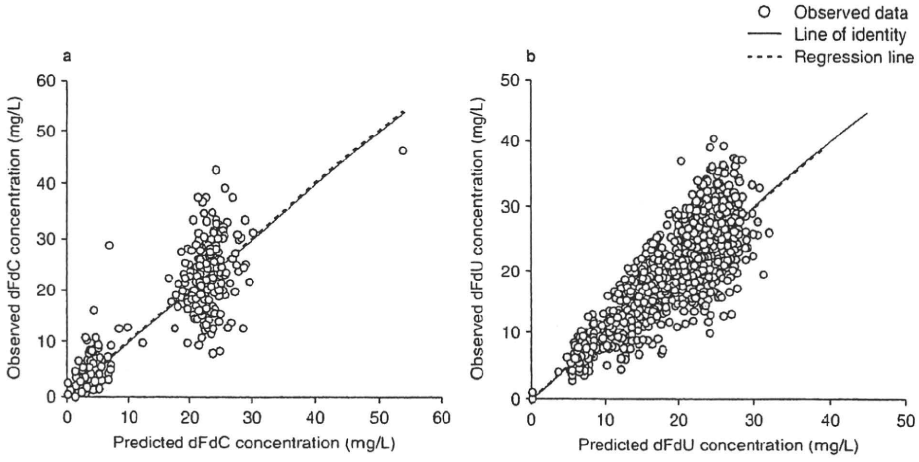

Fig. 3. Plots of observed concentrations against predicted concentrations of (a) gemcitabine (dFdC) and (b) 2′,2′-difluorodeoxyuridine (dFdU).

pharmacokinetics of gemcitabine and dFdU were effectively described by two-compartment models. The values of the estimated CL ([115.0 L/h] from a typical patient with an average BSA of 1.56 m^2), V_1 (12.60 L) and V_2 (9.54 L) were comparable to the values reported by Jiang et al.[21] (162 L/h, 15 L and 15 L, respectively). The estimated CL was slightly smaller and the V_1 was slightly larger than the values reported by Tham et al.[22] (222.8 L/h and 2.96 L, respectively). Although the reasons for these discrepancies are unknown, it should be noted that the population pharmacokinetic analyses performed by Tham et al.[22] included gemcitabine triphosphate (dFdCTP, an active form of gemcitabine) in addition to gemcitabine and dFdU, and the pharmacokinetic models applied in their study were completely different from ours.

The gemcitabine clearance in the *3/*3 patients, obtained from the model-independent analysis, was 80% less than the average clearance in patients without *3.[12,13] The effect of homozygous *3 on gemcitabine clearance, as estimated by the final population pharmacokinetic model, was a 64% decrease. This value, although slightly less than 80%, was the most significant among the covariates. Our current study also confirmed a finding from our previous report that the gene-dose effect of *CDA* was not linear. So far, we have encountered three patients with *3/*3, and all of them experienced life-threatening toxicities, including prolonged severe neutropenia.[12-14] Some of the non-*3/non-*3 and non-*3/*3 patients experienced transient grade 4 neutropenia, but only one patient required supportive treatment.[14] Thus, special attention to *3 homozygotes is advisable.

The effects of –31delC, 79A>C and IVS1+37G>A of *CDA* on gemcitabine clearance were found to be small but significant in this study (table III). All of these genotypes had slightly increased gemcitabine clearance (by <10%). The single nucleotide deletion –31delC is simultaneously present in both the haplotype *2 harbouring 79A>C and several *1 haplotypes (*1b, *1d, etc.) harbouring IVS1+37G>A in the Japanese population.[12] Thus it is reasonable that –31delC, rather than 79A>C or IVS1+37G>A, was selected as the covariate in the final model. This finding suggests that –31delC may be a functional SNP.

The haplotype analysis in our previous report[12] indicated that 208G>A, the tagging SNP of *CDA**3, is not present on a chromosome carrying –31delC, 79A>C or IVS1+37G>A. However, some patients simultaneously carried both haplotypes *2 and *3 (*2/*3). The median value of gemcitabine clearance observed in patients with *2/*3 was slightly higher than that observed in patients with *1/*3, although the difference was not statistically significant.[12]

The SNP 79A>C, a tagging SNP of the haplotype *2, results in the amino acid substitution, Lys27Gln.[12] A recent study[23] has suggested that the average enzymatic activity of CDA was significantly lower in cytoplasmic extracts of red blood cells obtained from patients with homozygous 79A (Lys27) than in those from patients with 79C (Gln27). Furthermore, it was reported that *CDA* 79A, the major allele, was a predictive marker of better response, more severe toxicity, longer time to disease progression and overall survival in Caucasian patients with advanced non-small-cell lung cancer who were treated with cisplatin and gemcitabine.[24] Haplotype *2 harbouring 79A>C also harbours –31delC, which has an incomplete association with the intron SNP IVS1+37. Our findings may explain the effects of 79A>C observed in Caucasian patients, since 79A>C is closely linked with –31delC, and the single nucleotide deletion –31delC in the 5′-untranslated region is responsible for increased clearance, a decreased AUC and less response to gemcitabine. This speculation warrants further study.

Although the effects of sex and age on model-independent pharmacokinetic parameters of gemcitabine were detected in our previous univariate analysis,[12] they were not significant in the current multivariate analysis. On the other hand, a significant effect of coadministered S-1, an oral derivative of fluorouracil, was revealed (approximately 20% higher clearance than in patients treated with gemcitabine monotherapy). In this study, nine of ten patients were coadministered S-1 in the morning a couple of hours before gemcitabine treatment. It might be noted that thymidylate synthase inhibitors such as fluorouracil can upregulate expression of hENT1, a major transporter of gemcitabine.[25] Moreover, Nakahira et al.[26] recently reported that significant increases in hENT1 expression and gemcitabine uptake were observed after S-1 treatment in mice. However, since the study duration was too short for S-1 to reveal the effects on expression of hENT1 in our study, the clinical significance of coadministration of S-1 and gemcitabine should be further investigated. In this study, four patients received fluorouracil after treatment of gemcitabine, and no effects of fluorouracil on the pharmacokinetics of gemcitabine were observed.

The metabolite dFdU is inactive and is eliminated mostly by renal excretion.[27] However, its pharmacokinetic parameters can be surrogate biomarkers of gemcitabine exposure or CDA activity because they correlate well with pharmacokinetic parameters of gemcitabine (data not shown). Serum creatinine levels and age were shown to significantly affect the clearance of dFdU. The association between dFdU clearance and renal function was also reported by Jiang et al.[21]

Conclusion

We performed population pharmacokinetic analyses of gemcitabine and dFdU in Japanese cancer patients. Clearance of gemcitabine was decreased by *CDA* 208G>A (Ala70Thr, *3) and was slightly increased by *CDA*–31delC and coadministration with S-1. Clearance of dFdU was influenced by renal function and age.

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Phase I/II study of the pharmacokinetics, safety and efficacy of S-1 in patients with advanced hepatocellular carcinoma

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S-1, an oral fluoropyrimidine derivative, has been shown to be clinically effective against various solid tumors, and preclinical studies have demonstrated activity against hepatocellular carcinoma. We conducted a phase I/II study in patients with advanced hepatocellular carcinoma to examine the pharmacokinetics, recommended dose, safety and efficacy of S-1. In phase I, the administered dose of S-1 was approximately 64 mg/m^2 per day in three patients (level 1) and approximately 80 mg/m^2 per day in six patients (level 2). There was no dose-limiting toxicity at level 1, but two patients had dose-limiting toxicity at level 2 (grade 3 anorexia and grade 2 rash requiring eight or more consecutive days of rest). The recommended dose was finally estimated to be 80 mg/m^2 per day. There were no significant differences in the pharmacokinetics of S-1 between patients with Child-Pugh A and those with B. In phase II, five of 23 patients (21.7%) had partial responses. The median progression-free survival and overall survival were 3.7 and 16.6 months, respectively. The most common toxicities of grade 3 or 4 were elevated serum aspartate aminotransferase levels, hypochromia and thrombocytopenia. In conclusion, S-1 showed an acceptable toxicity profile and promising antitumor activity for hepatocellular carcinoma, warranting further evaluation in randomized clinical trials. (*Cancer Sci* 2010; 101: 2606–2611)

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Outcomes remain poor because the disease is usually advanced and associated with hepatic impairment at diagnosis, and because of the high rate of recurrence resulting from either intrahepatic metastases from the primary tumor or multicentric lesions. As for therapy, surgical resection and percutaneous ethanol injection (PEI) or radiofrequency ablation (RFA) are considered the mainstays of treatment in patients with potentially curable disease. Transcatheter arterial chemoembolization (TACE) is the treatment of choice for noncurative HCC. Despite numerous clinical trials of a wide variety of cytotoxic agents, survival remains dismal in HCC.[1] Recently, sorafenib, an oral multi-kinase inhibitor that targets mainly Raf kinases and receptor tyrosine kinases associated with angiogenesis (vascular endothelial growth factor receptor [VEGFR]-2/-3 and platelet-derived growth factor receptor [PDGFR]-β), provided a significant survival benefit in patients with advanced HCC enrolled in placebo-controlled, randomized, phase III trials, including Asian as well as European subjects.[2,3] An initial phase I study in Japanese patients with HCC associated mainly with hepatitis C virus (HCV) infection showed promising antitumor activity and a favorable tolerability profile.[4] However, further improvement in the treatment of advanced HCC is essential.

S-1 is a novel, orally administered drug that combines tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP) and oteracil potassium (Oxo) in a molar concentration ratio of 1:0.4:1.[5] CDHP is a competitive inhibitor of dihydropyrimidine dehydrogenase (DPD), a metabolizing enzyme of 5-fluorouracil (5-FU) that is expressed in the liver. Inhibition of DPD by CDHP results in prolonged effective concentrations of 5-FU in plasma and tumor tissue.[6] Oxo, a competitive inhibitor of orotate phosphoribosyltransferase, inhibits the phosphorylation of 5-FU in the gastrointestinal tract, thereby reducing serious 5-FU-related gastrointestinal toxicity.[7] Clinically, S-1 has been shown to be effective against a variety of solid tumors, with response rates ranging 21–49% in late phase II studies conducted in Japan.[8] S-1 has yet to be evaluated in patients with HCC. However, in nude rats with human HCC xenografts, S-1 has been confirmed to have antitumor activity.[9]

Patients with HCC usually have various degrees of liver dysfunction because of associated liver disease and replacement of liver tissue by tumor, leading to pathophysiological changes that influence drug disposition. Decreased hepatic blood flow, extrahepatic and intrahepatic blood shunting and hepatocyte loss also alter drug metabolism, and decreased protein synthesis reduces drug binding to plasma proteins. In fact, the maximal tolerated dose (MTD) of 5-FU given as a 5-day continuous infusion in patients with HCC is approximately 50% of that in patients with normal organ function, and patients with cirrhosis have significantly lower clearance of 5-FU than those without cirrhosis.[10] We therefore conducted a multicenter phase I/II study to evaluate the pharmacokinetics, safety and efficacy of S-1 monotherapy in patients with advanced HCC.

Materials and Methods

Eligibility. Eligible patients had histologically or cytologically proved HCC that was not amenable to treatment by resection, liver transplantation, RFA, PEI or percutaneous microwave coagulation therapy (PMCT) and was not expected to respond to TACE. A hypervascular mass on computed tomography (CT) or magnetic resonance imaging (MRI) associated with a serum alpha-fetoprotein level or a serum protein induced by vitamin K absence or antagonist (PIVKA-II) level of more than the upper limit of normal (ULN) was considered a sufficient non-invasive diagnostic criterion for HCC. At least one measurable lesion on CT or MRI (not including necrotic lesions caused by prior treatment) was required. Other eligibility criteria included: age of at least 20 years; Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2; estimated life expectancy of at least 60 days; adequate

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Clinical trial registration: this trial was not registered in the clinical trial database because it was an early phase trial and not a controlled study.

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hematological function (white blood cells [WBC] ≥3000/mm^3, hemoglobin ≥ 9.0 g/dL, platelets ≥ 7.0 × 10^4/mm^3); adequate hepatic function (aspartate aminotransferase [AST] and alanine aminotransferase [ALT] ≤5 times the ULN, total bilirubin ≤2.0 mg/dL, serum albumin ≥2.8 g/dL, prothrombin activity ≥40%); adequate renal function (serum creatinine ≤ULN); and a Child-Pugh class of A or B. Prior treatment for HCC, such as resection, liver transplantation, RFA, PEI, PMCT and TACE was permitted if the treatment had been performed 30 or more days before registration in the study. Patients were excluded if they had: tumor involving more than 50% of the liver; brain or bone metastasis or vascular invasion of the main trunk and first-order branch(es) of the portal vein, hepatic veins, hepatic arteries or bile duct; severe complications; other malignancies; or inability to comply with the protocol requirements. Written informed consent was obtained from each patient. The study was approved by the local institutional review boards at all participating centers.

Study design. S-1 was supplied by Taiho Pharmaceutical Co., Ltd (Tokyo, Japan) in capsules containing 20 or 25 mg of FT. Individual doses were calculated according to body surface area. The calculated dose was rounded to derive the daily dose and the number of capsules to be dispensed per patient. At each dose level, S-1 was administered orally twice daily (after breakfast and dinner) for 28 consecutive days, followed by a 14-day recovery period. Each treatment cycle was 42 days. If grade 3 or higher hematological toxicity, grade 2 or higher non-hematological toxicity, grade 3 or higher elevations of AST or ALT, or grade 2 or higher increases in the serum creatinine concentration occurred, treatment with S-1 was temporarily suspended, the dose of S-1 was reduced, or both (minimum dose, 50 mg/day). Treatment continued until there was evidence of disease progression, or if the recovery period exceeded 28 days, the patient requested treatment to be discontinued or unacceptable toxicity developed and treatment was terminated at the discretion of the investigator. Drug compliance and accountability were carefully monitored; patients were requested to record their intake of S-1 and other medications in a diary.

During phase I, the starting dose of S-1 (level 1) was approximately 64 mg/m^2 per day twice daily (80% of the standard dose), level 2 was approximately 80 mg/m^2 per day and level 0 was approximately 50 mg/m^2 per day (80% of level 1). Patients were enrolled in cohorts of three for each dose level. The dose was escalated according to the cohort and was not increased in the same patient. If none of the first three patients had dose-limiting toxicity (DLT) during the first cycle, the dose was increased to level 2. If one or two of the first three patients had DLT, three additional patients were entered at the same dose level; if only one or two of the first six patients at level 1 had DLT, the dose was increased to level 2; if all of the first three patients or three or more of the first six patients had DLT, the dose was decreased to level 0; if none of the first three patients had DLT at level 0 or level 2, three additional patients were assigned to receive the same dose level. The DLT was defined as any of the following: (i) hematological toxicity ≥grade 4; (ii) non-hematological toxicity ≥grade 3; (iii) AST, ALT ≥15 times the ULN; or (iv) a rest period of 8 or more consecutive days was required. The recommended dose (RD) determined in the phase I part of this study was used in phase II.

Pharmacokinetics. Blood samples (5 mL) were obtained from each patient assigned to receive level 2 in the phase I part of the study. The samples were taken before and 1, 2, 4, 6, 8, 10 and 12 h after administration of S-1 on days 1 and 8 of the first treatment cycle. Plasma was separated from the whole-blood samples by centrifugation and stored at −20°C until analysis. Plasma FT concentrations were measured by high-performance liquid chromatography with ultraviolet detection. Plasma concentrations of 5-FU, CDHP and Oxo were measured by gas chromatography-negative ion chemical ionization mass spectrometry, as described previously.[11]

Pharmacokinetic data, including the maximum plasma concentration (C_{max}, ng/mL), time to reach C_{max} (T_{max}, h), area under the plasma-concentration–time curve for 0–12 h (AUC_{0-12}, ng h/mL) and the elimination half-life ($T_{1/2}$, h) were calculated by noncompartment model analysis using WinNonlin software, version 4.1 (Pharsight, Cary, NC, USA).

Assessment of efficacy and toxicity. All patients who received at least one dose of the study drug were included in the evaluations of response and toxicity. During each course of treatment, tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) by computed tomography (CT) or magnetic resonance imaging (MRI), with a slice thickness of no more than 5 mM.[12] The primary efficacy end-point in the phase II part of this study was the overall response rate, assessed on the basis of changes in tumor dimensions. The other end-points were overall survival (OS) and progression-free survival (PFS). The PFS was defined as the interval between the date of initiating treatment and the date on which disease progression was first confirmed or the date of death from any cause. Overall survival was defined at the interval from the date of initiating treatment to the date of death from any cause. Median OS and median PFS were

Table 1. Patient characteristics

	Level 1 (n = 3)	Level 2 (n = 23)
	n (%)	n (%)
Median age (range) (years)	67.0 (63–68)	68.0 (45–78)
Gender		
Male	2 (66.7)	21 (91.3)
Female	1 (33.3)	2 (8.7)
Virus marker		
HBs (+)	1 (33.3)	3 (13.0)
HCV (+)	1 (33.3)	14 (60.9)
HBs(−), HCV(−)	1 (33.3)	6 (26.1)
Child-Pugh classification		
A	3 (100)	16 (69.6)
B	0 (0)	7 (30.4)
Stage		
Stage II	1 (33.3)	3 (13.0)
Stage III	1 (33.3)	10 (43.5)
Stage IVB	1 (33.3)	10 (43.5)
Vascular invasion	0 (0)	2 (8.7)
ECOG PS		
0	3 (100)	21 (91.3)
1	0 (0)	2 (8.7)
Pretreatment		
TA(C)E	2 (66.7)	17 (73.9)
Surgery	1 (33.3)	8 (34.8)
RFA	0 (0)	7 (30.4)
HAI	2 (66.7)	6 (26.1)
PEI	0 (0)	4 (17.4)
Radiation	0 (0)	4 (17.4)
PMCT	0 (0)	3 (13.0)
Systemic chemotherapy	0 (0)	3 (13.0)
BCLC staging		
Early	0 (0)	1 (4.3)
Intermediate	2 (66.7)	11 (47.8)
Advanced	1 (33.3)	11 (47.8)

BCLC, Barcelona Clinic Liver Cancer Group; ECOG, Eastern Cooperative Oncology Group; HAI, hepatic arterial infusion; HBs, hepatitis B surface antigen; HCV, hepatitis C virus antibody; PEI, percutaneous ethanol injection; PMCT, percutaneous microwave coagulation therapy; PS, performance status; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization.

Table 2. Toxic effects

Toxicity	Level 1 (n = 3)		Level 2 (n = 23)		Child Pugh A (n = 16)		Child Pugh B (n = 7)	
	All grades	≥G3	All grades	≥G3	All grades	≥G3	All grades	≥G3
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
All adverse events	3 (100.0)	0 (0.0)	23 (100.0)	10 (43.5)	16 (100.0)	8 (50.0)	7 (100.0)	2 (28.6)
Hematological								
Erythropenia	1 (33.3)	0 (0.0)	21 (91.3)	1 (4.3)	14 (87.5)	1 (6.3)	7 (100.0)	0 (0.0)
Hypochromia	1 (33.3)	0 (0.0)	19 (82.6)	4 (17.4)	12 (75.0)	4 (25.0)	7 (100.0)	0 (0.0)
Leukopenia	2 (66.7)	0 (0.0)	18 (78.3)	1 (4.3)	12 (75.0)	1 (6.3)	6 (85.7)	0 (0.0)
Lymphopenia	2 (66.7)	0 (0.0)	12 (52.2)	3 (13.0)	7 (43.8)	3 (18.8)	5 (71.4)	0 (0.0)
Neutropenia	1 (33.3)	0 (0.0)	17 (73.9)	1 (4.3)	12 (75.0)	1 (6.3)	5 (71.4)	0 (0.0)
Reduced hematocrit	1 (33.3)	0 (0.0)	19 (82.6)	1 (4.3)	12 (75.0)	1 (6.3)	7 (100.0)	0 (0.0)
Reduced prothrombin content	1 (33.3)	0 (0.0)	19 (82.6)	0 (0.0)	14 (87.5)	0 (0.0)	5 (71.4)	0 (0.0)
Thrombocytopenia	1 (33.3)	0 (0.0)	18 (78.3)	4 (17.4)	12 (75.0)	4 (25.0)	6 (85.7)	0 (0.0)
Non-hematological								
Elevated alkaline phosphatase	0 (0.0)	0 (0.0)	8 (34.8)	1 (4.3)	7 (43.8)	1 (6.3)	1 (14.3)	0 (0.0)
Elevated lactate dehydrogenase	0 (0.0)	0 (0.0)	15 (65.2)	0 (0.0)	9 (56.3)	0 (0.0)	6 (85.7)	0 (0.0)
Elevated serum AST	1 (33.3)	0 (0.0)	8 (34.8)	4 (17.4)	6 (37.5)	3 (18.8)	2 (28.6)	1 (14.3)
Elevated serum bilirubin	0 (0.0)	0 (0.0)	18 (78.3)	3 (13.0)	13 (81.3)	2 (12.5)	5 (71.4)	1 (14.3)
Hyponatremic	0 (0.0)	0 (0.0)	8 (34.8)	0 (0.0)	5 (31.3)	0 (0.0)	3 (42.9)	0 (0.0)
Reduced cholinesterase	2 (66.7)	0 (0.0)	18 (78.3)	0 (0.0)	13 (81.3)	0 (0.0)	5 (71.4)	0 (0.0)
Reduced serum albumin	0 (0.0)	0 (0.0)	18 (78.3)	2 (8.7)	12 (75.0)	1 (6.3)	6 (85.7)	1 (14.3)
Reduced total protein	0 (0.0)	0 (0.0)	11 (47.8)	0 (0.0)	8 (50.0)	0 (0.0)	3 (42.9)	0 (0.0)
Anorexia	1 (33.3)	0 (0.0)	18 (78.3)	2 (8.7)	13 (81.3)	1 (6.3)	5 (71.4)	1 (14.3)
Ascites	0 (0.0)	0 (0.0)	7 (30.4)	0 (0.0)	3 (18.8)	0 (0.0)	4 (57.1)	0 (0.0)
Diarrhea	0 (0.0)	0 (0.0)	10 (43.5)	0 (0.0)	8 (50.0)	0 (0.0)	2 (28.6)	0 (0.0)
Fatigue	0 (0.0)	0 (0.0)	19 (82.6)	2 (8.7)	13 (81.3)	2 (12.5)	6 (85.7)	0 (0.0)
Pigmentation	0 (0.0)	0 (0.0)	20 (87.0)	0 (0.0)	14 (87.5)	0 (0.0)	6 (85.7)	0 (0.0)
Rash	0 (0.0)	0 (0.0)	8 (34.8)	0 (0.0)	5 (31.3)	0 (0.0)	3 (42.9)	0 (0.0)
Stomatitis	0 (0.0)	0 (0.0)	7 (30.4)	0 (0.0)	5 (31.3)	0 (0.0)	2 (28.6)	0 (0.0)

Dosage level, level 1, 2 (n = 3, 23); AST, aspartate aminotransferase.

Table 3. Efficacy in patients who received dose level 2

	Child-Pugh A (n = 16)	Child-Pugh B (n = 7)	Total (n = 23)
Partial response†	4	1	5
Stable disease‡	5	2	7
Progressive disease	7	3	10
Not evaluable	0	1	1
Response rate (90%CI)§ (%)	–	–	23.1 (9.0–40.4)
Response rate (95%CI) (%)	25.0 (7.3–52.4)	14.3 (0.4–57.9)	23.1 (7.5–43.7)
Median PFS (95% CI) (months)	3.3 (2.3–5.1)	3.7 (2.5–7.4)	3.7 (2.5–5.1)
Median OS (95% CI) (months)	17.8 (14.0–NA)	14.5 (9.6–18.7)	16.6 (14.0–24.5)
1-year survival (95% CI) (%)	–	–	69.6 (50.8–88.4)
1.5-years survival (95% CI) (%)	–	–	43.0 (22.6–63.5)
Disease control rate¶			
6W (95% CI) (%)	–	–	47.8 (26.8–69.4)
12W (95% CI) (%)	–	–	26.1 (10.2–48.4)
24W (95% CI) (%)	–	–	21.7 (7.5–43.7)

†Partial response was re-evaluated after at least 4 weeks in patients with a partial response. ‡Stable disease was reassessed after at least 6 weeks. §Response rate (90% CI) is a primary end-point. ¶Disease control rates were respectively estimated by dividing the number of patients with no disease progression by the total number of patients. Disease control was defined as a response of complete response, partial response or stable disease. CI, confidence interval; NA, not available; OS, overall survival; PFS, progression-free survival.

estimated using the Kaplan–Meier method. Physical findings and the results of serum chemical and urine analyses were assessed at 2-week intervals; vital signs were assessed as necessary. Patients were observed until death or at least 1 year after registration to determine survival status. The severity of all adverse events was evaluated according to the Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE, Ver. 3.0). The duration of all adverse events and their relation to S-1 were initially assessed by the attending physicians. Subsequently, an independent review committee reviewed data on objective response and adverse events.

Statistical considerations. With the response rate as the primary end-point, a total sample size of at least 23 patients was estimated to be required in the phase II portion to allow the

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study to have a one-sided 5% significance level of 0.05 and a power of 70%, assuming a threshold response rate of 5% and an expected response rate of 20%.

Results

Patient characteristics and treatment. Between May 2006 and April 2007, a total of 26 patients (nine in phase I and 17 in phase II) were enrolled at four centers in Japan. All patients were eligible for the evaluation of toxicity and efficacy. The first six patients who received dose level 2 (80 mg/m^2 per day) during the phase I part of this study were included in the phase II assessment, along with the 17 other patients (a total of 23 patients in the phase II assessment). The characteristics of patients are summarized in Table 1. At the study entry, 11 of 26 (42.3%) had metastatic disease. Six patients (23.1%) had single extrahepatic metastases (lung metastases, three patients; lymph node metastasis, three patients). Four patients had two sites of metastases, including the lung, lymph nodes and adrenal glands. Of the 26 patients, 23 had received some prior treatment, including three who had received systemic chemotherapy.

Dose-limiting toxicity and RD. None of the three patients who received dose level 1 (64 mg/m^2 per day) in the phase I part of the study had DLT. At dose level 2 (80 mg/m^2 per day), one patient with Child-Pugh class B had grade 3 anorexia during the first course of treatment, but the other two patients in the same cohort had no DLT. Three additional patients were enrolled to confirm safety, and one patient with Child-Pugh class B had a grade 2 rash; recovery required eight or more consecutive days of rest. Because two of the six patients who received level 2 had DLT, level 2 was defined as the RD for the phase II part of the study.

Treatment delivered. Twenty-three patients received a total of 85 cycles of treatment at dose level 2 (median, three cycles per patient; range, 1–15). The dose of S-1 was reduced in seven patients (30.4%) or a total of nine cycles (10.6%). The most common reasons for dose reductions were rash in four patients, and elevated serum bilirubin concentrations and anorexia in two patients each (some overlap among patients). Treatment was delayed because of toxicity in 12 patients (20 cycles), most often in cycles 1 or 2. The most common reasons for toxicity-related treatment delays were fatigue (five patients), rash (four patients) and elevated serum bilirubin concentrations (three patients). The reasons for terminating treatment were progressive disease in 19 patients (82.6%), adverse reactions in two patients (8.7%) and other reasons in two patients (8.7%; one required 28 or more consecutive days of rest, and one withdrew consent).

Toxicity. Drug-related adverse events occurring in all 26 patients in the phase I/II portion of the study are shown in Table 2. Treatment with S-1 was generally well tolerated throughout the study. Grade 3 or 4 toxicity occurred in 10 of the 23 patients (43.5%) who received level 2. Most toxic effects were laboratory abnormalities. There was no grade 3 or 4 toxicity at level 1. The most common grade 3 or 4 hematological toxic effects were hypochromia (17.4%), thrombocytopenia (17.4%) and lymphopenia (13.0%); the most common grade 3 or 4 nonhematological toxic effects were elevated serum AST levels (17.4%) and elevated serum bilirubin concentrations (13.0%).

Efficacy. A response could be evaluated in 26 patients in the phase I/II portion of the study. In the phase I part of the study (dose level 1), one patient had a partial response, one had progressive disease and the other was not evaluable. Of the 23 patients in the phase II part of the study, five (21.7%; 90% confidence interval [CI], 9.0–40.4%) responded to treatment Among the 23 patients in whom a response could be evaluated, five had a partial response, seven had stable disease, and 10 had progressive disease (Table 3). The remaining patient underwent imaging studies, but treatment was completed after one course, and continuation of stable disease for at least 6 weeks could not be

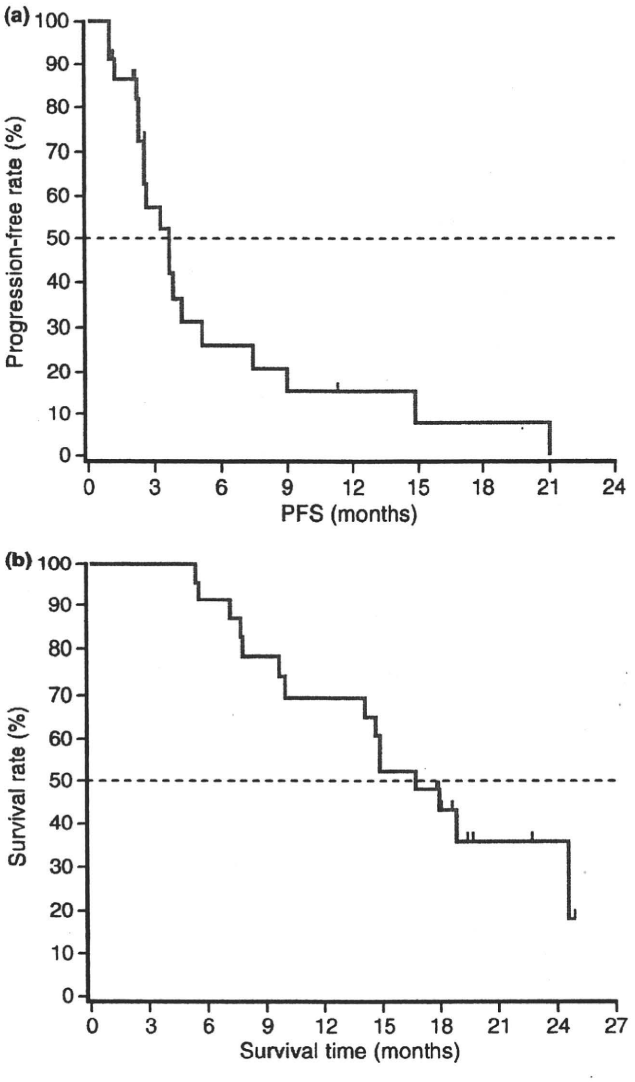

Fig. 1. Progression-free survival (PFS) (a) and overall survival (b) in patients who received dose level 2 of S-1 (n = 23). The median progression-free survival and overall survival were 3.7 and 16.6 months, respectively.

Table 4. Pharmacokinetics of FT, 5-FU, CDHP and Oxo on day 1 and day 8 in patients with HCC who received dose level 2

		C_{max} (ng/mL)	T_{max} (h)	$AUC_{0\text{-}12}$ (ng h/mL)	$T_{1/2}$ (h)
FT	Day 1	2032 ± 437	3.3 ± 1.0	17070 ± 5139	10.1 ± 2.8
	Day 8	4365 ± 1712	3.7 ± 0.8	42399 ± 18137	12.7 ± 5.0
5-FU	Day 1	114.5 ± 35.5	4.3 ± 0.8	695.3 ± 223.6	2.3 ± 1.0
	Day 8	145.8 ± 31.4	4.3 ± 0.8	936.6 ± 292.3	2.4 ± 1.0
CDHP	Day 1	267.2 ± 76.8	3.3 ± 1.0	1424.8 ± 414.2	3.3 ± 0.9
	Day 8	281.0 ± 113.8	3.3 ± 1.0	1694.4 ± 603.5	3.4 ± 0.9
Oxo	Day 1	38.5 ± 1.8	3.7 ± 0.8	231.6 ± 69.8	4.0 ± 2.1
	Day 8	33.4 ± 9.5	4.0 ± 0.0	241.5 ± 115.6	4.0 ± 2.0

Parameters are represented as mean ± SD. CDHP, 5-chloro-2,4-dihydroxypyridine; 5-FU, 5-fluorouracil; FT, tegafur; Oxo, oteracil potassium.

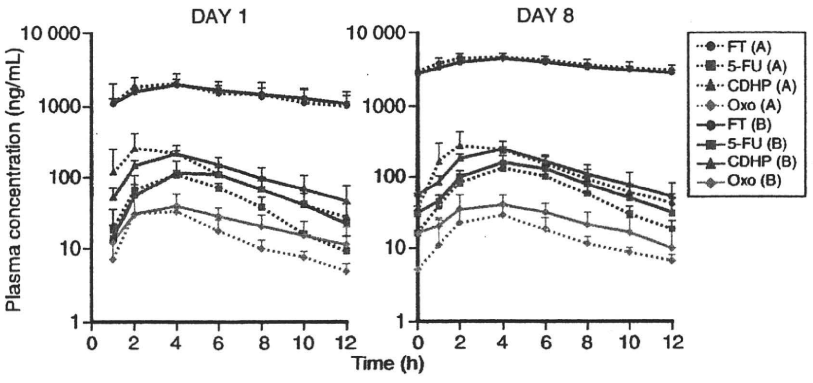

Fig. 2. Plasma-concentration–time profiles of tegafur (FT), 5-fluorouracil (5-FU), 5-chloro-2,4-dihydroxypyridine (CDHP) and oteracil potassium (Oxo) on day 1 and day 8 were similar in patients with Child-Pugh class A ($n = 3$) and those with Child-Pugh class B ($n = 3$).

confirmed. The duration of the five responses was 42, 147, 188, 238 and 371 days, respectively.

The median PFS was 3.7 months (95% CI, 2.5–5.1 months). The disease control rates at 6, 12 and 24 weeks were 47.8% (95% CI, 26.8–69.4%), 26.1% (95% CI, 10.2–48.4%) and 21.7% (95% CI, 7.5–43.7%), respectively. The PFS and OS are shown in Figure 1. The median OS was 16.6 months (95% CI, 14.0–24.5 months). Survival rates were 69.6% (95% CI, 50.8–88.4%) at 1 year and 43.0% (95% CI, 22.6–63.5%) at 1.5 years.

Pharmacokinetic analysis. Table 4 shows the pharmacokinetic data for the components of S-1 and 5-FU at level 2 on days 1 and 8. Compared with day 1, the C_{max} and $AUC_{0\text{-}12}$ of FT increased markedly on day 8; however, these increases were within the expected range given the slow elimination of FT, and repeated administration of S-1 had no effect on the T_{max} or $T_{1/2}$ of FT. There was no evidence of accumulation of 5-FU, CDHP or Oxo on day 8.

Figure 2 compares the plasma-concentration–time profiles of S-1 components and 5-FU between patients with Child-Pugh class A and those with Child-Pugh class B on days 1 and 8. The plasma-concentration–time profiles of FT, 5-FU, CDHP and Oxo were similar in patients with Child-Pugh class A and those with Child-Pugh class B on both days.

Discussion

There has been no established standard therapy for patients with advanced HCC refractory to surgery, transplantation, local ablation and TACE.[13,14] Some cytotoxic regimens have produced encouraging response rates, but survival benefits have been minimal compared with control groups, at the cost of clinically unacceptable adverse effects.[1,15]

S-1 is an anticancer drug consisting of FT, CDHP and Oxo. The conversion of FT to 5-FU is mediated mainly by hepatic cytochrome CYP2A6.[16] 5-FU is rapidly metabolized by DPD in the liver after the intravenous administration of 5-FU alone, but S-1, which includes a DPD inhibitor (i.e. CDHP), produces prolonged, effective concentrations of 5-FU in the blood. Thus, the liver plays an important role in the metabolism of FT.

The RD of S-1 in patients with HCC was estimated to be 80 mg/m^2 per day in phase I, which is similar to the dose recommended for the treatment of other solid tumors. However, in patients with HCC, Ueno *et al.*[10] reported that the DLT of 5-FU administered as a 5-day continuous infusion was stomatitis. Moreover, the MTD was equivalent to approximately 50% of that of 5-FU in patients with normal organ function,[10] suggesting that 5-FU-related gastrointestinal toxicity was reduced by Oxo in the formulation of S-1. We did not determine the MTD in this study because S-1 was approved for the treatment of other cancers. The pharmacokinetic properties of S-1 components and 5-FU in patients with HCC were similar to those in patients with pancreatic cancer or biliary tract cancer.[17,18]

Hematological toxic effects and symptomatic events such as pigmentation (87.0%), fatigue (82.6%), anorexia (78.3%) and ascites (30.4%) were more common than previously reported for S-1 in patients with other cancers. Nonetheless, severe toxic effects were comparable among patients with HCC and those with other cancers. Nonhematological toxic effects related to hepatic function were also more frequent than reported previously for S-1 in patients with other types of cancer, but such effects may have been caused by differences in underlying liver disease.

The pharmacokinetics of S-1 did not obviously differ between patients with Child-Pugh class A and those with Child-Pugh class B, suggesting that hepatic dysfunction associated with Child-Pugh class B did not affect the pharmacokinetics of S-1 components or 5-FU. The sample size of the pharmacokinetic evaluations was small because the primary end-point was to determine the RD as the evaluation of DLT in phase I. At dose level 2, DLT occurred in two patients with Child-Pugh class B (Grade 3 anorexia in one, and a Grade 2 rash requiring 8 or more consecutive days of rest in the other). There was no DLT at level 1 (given only to patients with Child-Pugh class A). However, the patient who had DLT of grade 3 anorexia had renal dysfunction at baseline, and the plasma 5-FU concentrations in this patient on day 8 were higher than those in other patients, perhaps contributing to the development of DLT (data not shown). In addition, there were no obvious differences in the incidence or grade of drug-related adverse events between patients with Child-Pugh class A and those with Child-Pugh class B, consistent with the results of pharmacokinetic analysis. These results suggested that there were no clinically meaningful differences in pharmacokinetics or safety according to Child-Pugh class or between patients with HCC and those with other cancers, and that S-1 was well tolerated in patients with HCC, similar to patients with other cancers. However, our study had several limitations: only a very small number of patients with Child-Pugh class B were included; among the patients with Child-Pugh class B, the score was heterogeneous, ranging from 7 to 9; and only patients with better scores were studied. Therefore, extra care should be taken when S-1 is given to patients with Child-Pugh class B.

As for efficacy, five of 23 patients had partial responses at dose level 2. Compared with previously reported response rates obtained with single-agent chemotherapy in patients with HCC, our results are good. In particular, the median OS appeared to be longer than that obtained with other agents in non-Japanese studies. The reason for the better OS in Japanese patients might be similar to that previously reported for sorafenib.[4] The median OS in our study was similar to that in a Japanese phase I study of sorafenib.[4] In studies of sorafenib in non-Japanese and

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Japanese patients with HCC, the median TTP and response rates were comparable, but the median OS was 15.6 months in Japanese patients compared with only 9.2 months in non-Japanese patients.[4] Differences in various treatments, including hepatic arterial infusion chemotherapy, and the palliative care of patients with progressive disease who had conditions such as hepatic decompression and variceal bleeding might be related to the longer survival time in Japanese rather than non-Japanese patients with HCC.

In conclusion, our results suggested that S-1 is effective and has an acceptable toxicity profile in patients with advanced HCC. Nonetheless, S-1 should be used with caution in the presence of liver dysfunction. Sorafenib has been established to be a standard treatment for advanced HCC. Perhaps, systemic chemotherapy with S-1 plus molecular-targeted therapies such as sorafenib will further improve survival in patients with advanced HCC or monotherapy with S-1 will be useful as a second-line regimen for chemotherapy.

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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

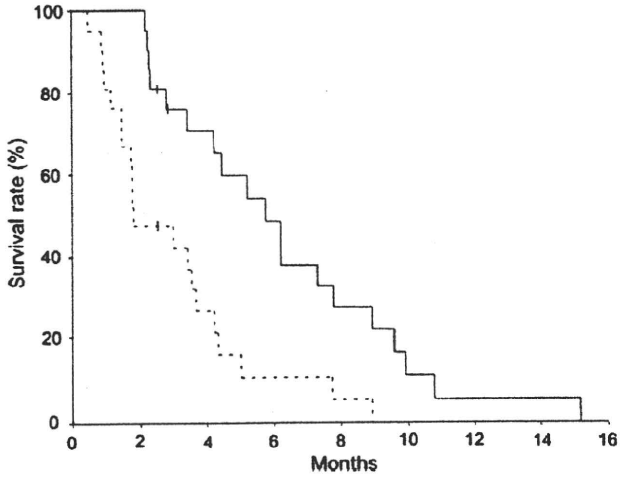

Figure 1. Overall survival (continuous line) and progression-free survival (dotted line) in the 21 patients.

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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Survival Prediction for Pancreatic Cancer Patients Receiving Gemcitabine Treatment*[S]

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Although gemcitabine monotherapy is the standard treatment for advanced pancreatic cancer, patient outcome varies significantly, and a considerable number do not benefit adequately. We therefore searched for new biomarkers predictive of overall patient survival. Using LC-MS, we compared the base-line plasma proteome between 29 representative patients with advanced pancreatic cancer who died within 100 days and 31 patients who survived for more than 400 days after receiving at least two cycles of the same gemcitabine monotherapy. Identified biomarker candidates were then challenged in a larger cohort of 304 patients treated with the same protocol using reverse-phase protein microarray. Among a total of 45,277 peptide peaks, we identified 637 peaks whose intensities differed significantly between the two groups ($p < 0.001$, Welch's t test). Two MS peaks with the highest statistical significance ($p = 2.6 \times 10^{-4}$ and $p = 5.0 \times 10^{-4}$) were revealed to be derived from α_1-antitrypsin and α_1-antichymotrypsin, respectively. The levels of α_1-antitrypsin ($p = 8.9 \times 10^{-8}$) and α_1-antichymotrypsin ($p = 0.001$) were significantly correlated with the overall survival of the 304 patients. We selected α_1-antitrypsin ($p = 0.0001$), leukocyte count ($p = 0.066$), alkaline phosphatase ($p = 8.3 \times 10^{-8}$), and performance status ($p = 0.003$) using multivariate Cox regression analysis and constructed a scoring system (nomogram) that was able to identify a group of high risk patients having a short median survival time of 150 days (95% confidence interval, 123–187 days; $p = 2.0 \times 10^{-15}$, log rank test). The accuracy of this model for prognostication was internally validated and showed good calibration and discrimination with a bootstrap-corrected concordance index of 0.672. In conclusion, an increased level of α_1-antitrypsin is a biomarker that predicts short overall survival of patients with advanced pancreatic cancer receiving gemcitabine monotherapy. Although an external validation study will be necessary, the current model may be useful for identifying patients unsuitable for the standardized therapy. ***Molecular & Cellular Proteomics 9:695–704, 2010.***

Invasive ductal adenocarcinoma of the pancreas is one of the most aggressive and lethal malignancies (1). It is the fifth leading cause of cancer-related death in Japan and the fourth in the United States, accounting for an estimated >23,000 deaths per year in Japan and >33,000 in the United States (2, 3). Because the majority of patients have distant metastases even at their first presentation (4, 5), the main therapeutic modality for pancreatic cancer is systemic chemotherapy, and gemcitabine monotherapy is the current standard (6). Gemcitabine treatment has significantly improved the median survival time of patients with advanced pancreatic cancer (7). However, the outcome of the treatment varies significantly among individuals, and a considerable portion of patients do not appear to benefit significantly from it. It therefore seems necessary to assess the efficacy and adverse effects of the drug before administration and tailor the treatment accordingly for each person.

We previously identified a predictive biomarker for hematologic toxicity, which is one of the most frequent and potentially life-threatening adverse effects associated with gemcitabine monotherapy (8). As a next step, we performed a large scale proteome analysis in this study to identify biomarkers predictive of patient survival after gemcitabine monotherapy. Several factors and their combinations have been reported to correlate significantly with outcome in patients with advanced pancreatic cancer receiving gemcitabine, such as performance status, metastases, serum albumin, alkaline phosphatase, and peripheral leukocyte count (9–11). Unfortunately, however, the accuracy of survival prediction based on these conventional prognostic factors seems unsatisfactory (9).

In recent years, there has been considerable interest in applying advanced proteomics technologies to the discovery of predictive biomarkers (12, 13). We and others have

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TABLE I

Clinical and laboratory data of patients with short term or long term survival

Survival time was calculated from the date of starting gemcitabine therapy until the date of death from cancer. Wilcoxon test was applied to assess differences in values. 5-FU, 5-fluorouracil; LAPC, locally advanced pancreatic cancer; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; C_{max}, peak concentration; AUC, area under the curve.

	Short term survivor (<100 days)	Long term survivor (>400 days)	*p*
No. of patients	29	31	
Sex (no. of patients)			0.361[a]
Male	21	19	
Female	8	12	
Age, mean (S.D.) (years)	63 (7)	67 (8)	0.123
ECOG performance status (no. of patients)			0.008[a]
0	8	20	
1	18	11	
2	3	0	
Body surface area, mean (S.D.) (m^2)	1.59 (0.17)	1.54 (0.15)	0.333
Prior therapy			0.438[a]
None	27	27	
Chemoradiotherapy using 5-FU for LAPC	2	4	
Clinical stage[b]			0.697[a]
IVa	2	3	
IVb	27	28	
Subsequent line chemotherapy after gemcitabine			0.045[a]
None	29	27	
Yes	0	4	
Leukocytes, mean (S.D.) ($\times 10^3/mm^3$)	7.6 (3.6)	5.2 (1.3)	0.002
Platelets, mean (S.D.) ($\times 10^4/mm^3$)	24.5 (7.6)	20.2 (4.6)	0.020
Hemoglobin, mean (S.D.) (g/dl)	11.7 (1.6)	11.7 (1.5)	0.491
Albumin, mean (S.D.) (g/dl)	3.4 (0.4)	3.7 (0.3)	0.014
Creatinine, mean (S.D.) (mg/dl)	0.70 (0.23)	0.68 (0.23)	0.726
AST, mean (S.D.) (IU/liter)	40 (25)	26 (15)	0.010
ALT, mean (S.D.) (IU/liter)	51 (44)	27 (19)	0.037
ALP, mean (S.D.) (units/liter)	728 (632)	337 (160)	0.026
Pharmacokinetic parameters of gemcitabine			
C_{max}, mean (S.D.) (μg/ml)	24.02 (7.52)	24.91 (6.22)	0.610
AUC, mean (S.D.) (h·μg/ml)	10.24 (2.83)	10.75 (2.32)	0.270
α_1-Antitrypsin,[c] mean (S.D.)	64.6 (66.8)	16.9 (7.9)	0.0003
α_1-Antichymotrypsin,[c] mean (S.D.)	706.4 (416.0)	389.0 (216.5)	0.0005
Tumor response[d]			<0.0001[a]
Complete response	0	0	
Partial response	0	1	
Stable disease	2	22	
Progressive disease	24	0	
Not evaluable	3	8	

[a] Calculated by χ^2 test.

[b] According to Ref. 23.

[c] Intensity of the corresponding peak measured by quantitative mass spectrometry.

[d] Evaluated after the first two cycles of gemcitabine monotherapy.

successfully applied MALDI MS-based protein profiling techniques for predicting the efficacy of chemoradiotherapy and molecular targeting therapy (14, 15). Two-dimensional image converted analysis of liquid chromatography and mass spectrometry (2DICAL)[1] is a new LC-MS-based proteomics platform that was developed in our laboratory (16). 2DICAL can quantify protein content accurately across a theoretically unlimited number of samples without isotope labeling and thus has considerable advantages over conventional LC-MS-based methods for clinical studies (17). The predictive biomarker protein for hematologic toxicity described above was identified using 2DICAL (8).

It has been generally accepted that tumor responses do not always correlate with the outcome of patients (10, 18, 19). The rates of complete and partial responses (Response Evaluation Criteria in Solid Tumors guideline) to gemcitabine mono-

[1] The abbreviations used are: 2DICAL, two-dimensional image converted analysis of liquid chromatography and mass spectrometry; AIC, Akaike's information criterion; CC, correlation coefficient; CI, confidence interval; CV, coefficient of variance; ECOG, Eastern Cooperative Oncology Group; NCC, National Cancer Center; ID, identification; FDR, false discovery rate.

therapy are limited to ~10% (20–22), and the majority of pancreatic cancers do not show significant tumor regression. Given that the ultimate goal of gemcitabine therapy for pancreatic cancer is to achieve prolonged survival, it would be desirable to stratify patients according to survival rather than tumor response (9). In the present study, using 2DICAL, we compared the base-line plasma proteome of two extreme populations of patients who had shown distinct clinical courses after identical gemcitabine treatment.

EXPERIMENTAL PROCEDURES

Patients—Samples were collected from a total of 304 patients who had all been included in our previous study (8). All patients had metastatic (stage IVb; $n = 285$) or locally advanced (stage IVa; $n = 19$) (23) histologically or cytologically proven pancreatic ductal adenocarcinoma and had received at least two cycles of gemcitabine monotherapy (1,000 mg/m^2 intravenously over 30 min on days 1, 8, and 15 of a 28-day cycle). Two hundred eighty-one patients (92%) received gemcitabine as a first line therapy (supplemental Table S1).

Two hundred sixty-two patients (86%) were treated consecutively at the National Cancer Center (NCC) Hospital (Tokyo, Japan) between September 2002 and June 2007, and 42 patients (14%) were treated consecutively at the NCC Hospital East (Kashiwa, Japan) between September 2002 and July 2004. Survival times were determined as of May 2008. During this period, 248 patients (82%) died, and 56 patients (18%) were censored. Tumor response was evaluated after the first two cycles of gemcitabine using the Response Evaluation Criteria in Solid Tumors guideline.

Sample Preparation—Blood was collected before the first administration of gemcitabine. Plasma or serum was separated by centrifugation at 1,050 × *g* for 10 min at 4 °C and frozen until analysis as reported previously (8, 24). Macroscopically hemolyzed samples were excluded from the current analysis. Two hundred fifty-two plasma samples (83%) were collected from the NCC Hospital and Hospital East, and 52 serum samples (17%) were collected from the NCC Hospital. Written informed consent was obtained from all patients before blood sampling. The protocol of this retrospective study was reviewed and approved by the institutional ethics committee boards of the NCC (Tokyo, Japan) and the National Institute of Health Sciences (Tokyo, Japan).

LC-MS—Samples were blinded, randomized, and passed through an IgY-12 High Capacity Spin Column (Beckman Coulter, Fullerton, CA) in accordance with the manufacturer's instructions. The flow-through portion was digested with sequencing grade modified trypsin (Promega, Madison, WI) and analyzed in triplicate using a nanoflow high performance LC system (NanoFrontier nLC, Hitachi High Technologies, Tokyo, Japan) connected to an electrospray ionization quadrupole time-of-flight mass spectrometer (Q-Tof Ultima, Waters, Milford, MA). LC-MS run order was also randomized to eliminate any potential bias.

MS peaks were detected, normalized, and quantified using the in-house 2DICAL software package as described previously (16). A serial identification (ID) number was applied to each of the MS peaks detected (1–45,277). The stability of LC-MS was monitored by calculating the correlation coefficient (CC) and coefficient of variance (CV) of every triplicate measurement. The mean CC and CV ± S.D. for all 45,277 peaks observed in the 60 triplicate runs were as high as 0.970 ± 0.022 and as low as 0.056 ± 0.017, respectively.

Protein Identification by MS/MS—Peak lists were generated using the Mass Navigator software package (version 1.2) (Mitsui Knowledge Industry, Tokyo, Japan) and searched against the NCBInr database (downloaded on May 20, 2008) using the Mascot software package (version 2.2.1) (Matrix Science, London, UK). The search parameters used were as follows. A database of human proteins was selected.

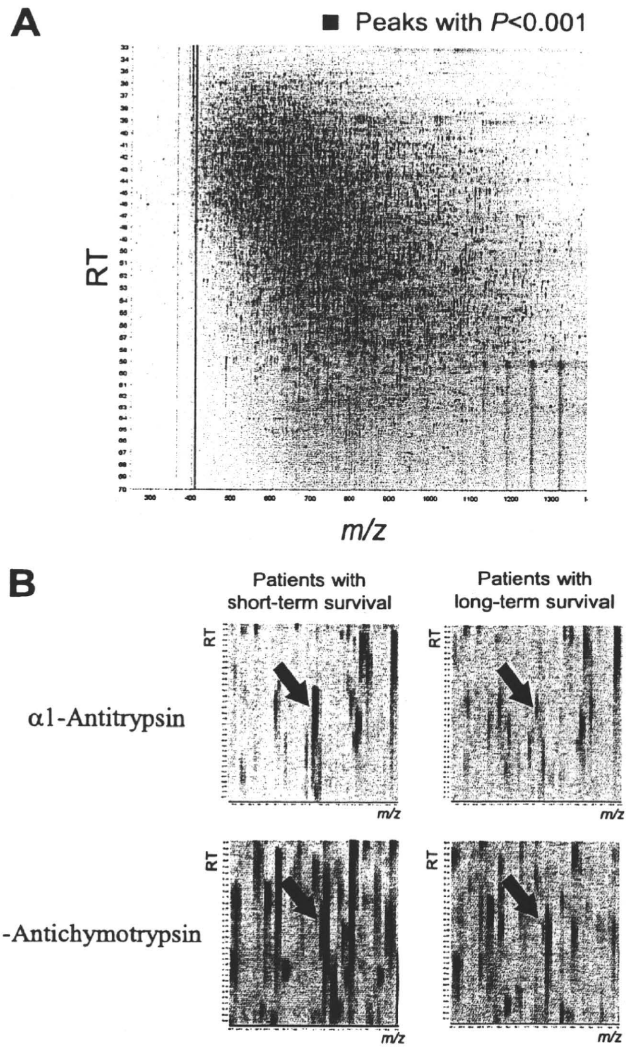

FIG. 1. *A*, two-dimensional display of all (>45,000) the MS peaks. The 637 MS peaks whose mean intensity differed significantly between patients with short term and long term survival ($p < 0.001$, Welch's *t* test) are highlighted in *red*. *B*, two MS peaks with the smallest *p* values (*upper*, $p = 2.57 \times 10^{-4}$; *bottom*, $p = 5.03 \times 10^{-4}$) in representative patients with short term (*left*) and long term (*right*) survival. *RT*, retention time.

Trypsin was designated as the enzyme, and up to one missed cleavage was allowed. Mass tolerances for precursor and fragment ions were ±2.0 and ±0.8 Da, respectively. The score threshold was set to $p < 0.05$ based on the size of the database used in the search. If a peptide matched to multiple proteins, the protein name with the highest Mascot score was selected.

Western Blot Analysis—Primary antibodies used were rabbit polyclonal antibody against human α_1-antitrypsin (Dako, Glostrup, Denmark), rabbit polyclonal antibody against human α_1-antichymotrypsin (Dako), and mouse monoclonal antibody against human complement C3b-α (Progen, Heidelberg, Germany). Ten microliters of partitioned sample was separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane. The membrane was then incubated with the primary antibody and subsequently with the relevant horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG as described previously (25, 26). Blots were developed using an ECL detection system (GE Healthcare).