A total of 35 patients documented disease progression at the time of analysis. The initial sites of disease progression are listed in Table 2. The pattern of failure was distant metastases in 33 patients (94%), local–regional recurrence in one patient (3%) and both in one patient (3%). The median progression-free interval and the median survival time were 4.4 and 9.5 months, respectively. The overall 1- and 2-year survival rates were 28 and 23%, respectively (Figure 1).

Toxicity

The acute toxicity is summarised in Table 3. The haematological toxicity was relatively brief and reversible in most patients. Grade 3–4 leucopenia and neutropenia occurred in 22 (52%) and 14 (33%) of the patients, respectively. Grade 3 thrombocytopenia occurred in one patient (2%) on the day after the chemoradiotherapy completion. The patient, who showed grade 4 anaemia, suffered catastrophic duodenal bleeding requiring embolisation under angiography. She exhibited cholangitis and sepsis subsequently and died on day 63.

The most common nonhaematological toxicity was anorexia, which was observed in 38 patients (90%). In total, 14 patients (33%) required intravenous hyperalimentation. In all, 33 patients (79%) complained of fatigue and one of them refused continuation of the chemoradiotherapy. Nine patients (21%) experienced grade 3 nausea. Liver function abnormality was another major adverse effect. Four patients (10%) showed grade 3 elevation of serum transaminase levels. Two of them discontinued the treatments after 19.8 and 21.6 Gy, respectively, due to serum ALT elevation of 10 times UNL according to the protocol criteria (maximum level: 452 and 435 IU l^{-1}), although the serum ALT levels of both recovered to the grade 1 levels 4 days after discontinuation of the treatment. We suspected that the ALT elevation in these two patients was gemcitabine-related toxicity because it was never reproduced after their treatment was switched over to chemoradiotherapy using 5-FU. One patient suffered unexpected acute abdominal pain requiring morphine 2 months after the completion of the chemoradiotherapy and was diagnosed with perforation of pancreatic pseudocyst into the duodenum. This pain disappeared completely by only medical management within 1 week. No patients experienced any symptoms considered to be late toxicity as of the time of analysis.

Table 2 Patterns of initial disease progression

Local	No. (%)
Distant metastasis	33 (94)
Peritoneum	17 (49)
Liver	15 (43)
Lymph node	1 (3)
Ovary	1 (3)
Bone	1 (3)
Local and distant metastasis	1 (3)

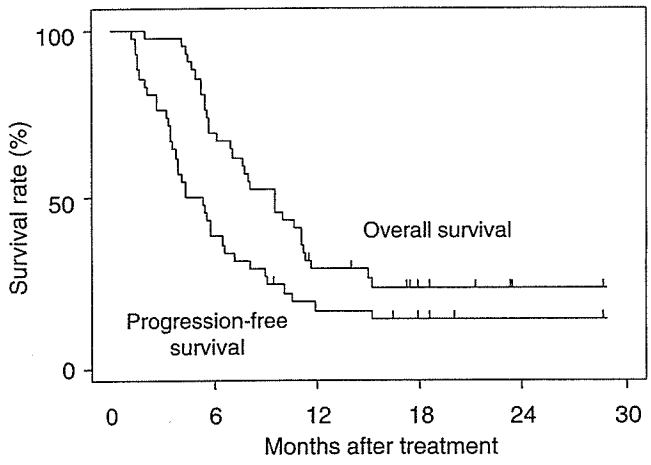

Figure 1 Progression-free survival and overall survival curves of patients with locally advanced pancreatic cancer receiving radiotherapy with gemcitabine.

Table 3 Acute toxicity

Grade	1 (%)	2 (%)	3 (%)	4 (%)
Haematological toxicity				
Leucocytopenia	3 (7)	17 (40)	21 (50)	1 (2)
Neutropenia	9 (21)	15 (36)	11 (26)	3 (7)
Thrombcoytopenia	22 (52)	2 (5)	1 (2)	0 (0)
Anaemia	21 (50)	17 (40)	0 (0)	1[a] (2)
Nonhaematological toxicity				
Total bilirubin	10 (24)	5 (12)	1 (2)	0 (0)
AST	14 (33)	5 (12)	1 (2)	0 (0)
ALT	15 (36)	11 (26)	4 (10)	0 (0)
ALP	15 (36)	5 (12)	0 (0)	0 (0)
Creatinine	0 (0)	0 (0)	0 (0)	0 (0)
Anorexia	9 (21)	5 (12)	10 (24)	14 (33)
Nausea	11 (26)	11 (26)	9 (21)	0 (0)
Vomiting	10 (24)	7 (17)	0 (0)	0 (0)
Diarrhoea	1 (2)	1 (2)	0 (0)	0 (0)
Mucositis	0 (0)	0 (0)	0 (0)	0 (0)
Duodenal ulcer	0 (0)	0 (0)	0 (0)	1[a] (2)
Fatigue	17 (40)	14 (33)	2 (5)	0 (0)
Skin rash	0 (0)	1 (2)	0 (0)	0 (0)
Infection	0 (0)	0 (0)	0 (0)	1[a] (2)

AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase. [a]One patient died of duodenal bleeding and sepsis.

DISCUSSION

Based on previous randomised trials (Moertel *et al*, 1969; Gastrointestinal Tumor Study Group, 1981; Gastrointestinal Tumor Study Group, 1988), concurrent external-beam radiotherapy and 5-FU have been generally accepted as the standard treatment for locally advanced carcinomas. To intensify the treatment efficacy, various anticancer agents and radiation schedules are being investigated in clinical trials of chemoradiotherapy (Roldan *et al*, 1988; Seydel *et al*, 1990; Wagener *et al*, 1996; Thomas *et al*, 1997; Prott *et al*, 1997; Okusaka *et al*, 2001). However, marked improvement in their survival has not been observed. In an attempt to optimise radiosensitisation, radiotherapy with protracted 5-FU infusion has been examined recently, but the median survival times were similar to those observed in previous studies (Ishii *et al*, 1997).

Gemcitabine has been expected to be an agent that improves the outcome of chemoradiotherapy for locally advanced pancreatic cancer because it is a chemotherapeutic drug having meaningful palliative and prognostic impact against advanced pancreatic cancer, and it is also a potent radiosensitiser. Several experimental studies have shown that more than one mechanism leads to the potentiation of radiation-induced cell killing by gemcitabine (Lawrence *et al*, 1996; Shewach and Lawrence, 1996; van Putten *et al*, 2001). In clinics, various phase I studies for radiotherapy with gemcitabine have been conducted (McGinn *et al*, 2001; Pipas *et al*, 2001; Wolff *et al*, 2001; Ikeda *et al*, 2002; Poggi *et al*, 2002),

although the efficacy and safety of this combination have not been fully elucidated in phase II trials. A phase I trial that was conducted in our hospital determined the recommended dose of weekly gemcitabine in the phase II chemoradiotherapy trial to be 250 mg m^{-2}, because three of the six patients give a dose of 350 mg m^{-2} of gemcitabine demonstrated dose-limiting toxicities involving neutropenia/leucopenia and elevated transaminase (Ikeda *et al*, 2002).

The toxicity associated with radiotherapy with gemcitabine was relatively severe in this phase II study. Grade 3–4 leucopenia and neutropenia were observed in 52 and 33% of the patients, respectively, although none of the patients showed neutropenic fever. Nausea and anorexia were the most serious non-haematological toxicities in this treatment; 73% of the patients experienced various degrees of nausea and 33% required intravenous hyperalimentation. In all, 78% of the patients complained of general fatigue and one patient (2%) refused continuation of the treatment because of this adverse effect. These troublesome toxicities observed in this study seem to be more frequent and more severe compared with those in 5-FU-based chemoradiotherapy (Ishii *et al*, 1997). There was one death attributed to duodenal bleeding, which was arrested by transcatheter arterial embolisation, but deterioration of the general condition and lethal sepsis were induced subsequently.

The present study, in which 42 patients with locally advanced pancreatic cancer were treated with radiotherapy and weekly gemcitabine, documented a marginal impact on patient survival; the median survival time of 9.5 months is comparable to that in patients receiving conventional chemotherapy using 5-FU. However, the incidence rate of distant metastasis at the time of disease progression was remarkably higher with this treatment (97%) as compared to that with 5-FU-based chemoradiotherapy, which was reported to be 50% in our previous study (Ishii *et al*, 1997). This suggests that gemcitabine at a dose of 250 mg m^{-2} is a potent radiosensitiser for controlling local disease, but its ability as a chemotherapeutic agent is insufficient to counteract systemic tumour spread. To improve prognosis for these patients, future investigations for treatment with more systemic effects are warranted.

In an effort to increase capacity for systemic therapy, reduction of the radiation field has been attempted. Investigators at the University of Michigan elected to radiate the primary tumour alone, without the inclusion of regional lymph nodes, and administer full-dose gemcitabine concurrently, because the use of full-dose gemcitabine requires reduction of the radiation dose, based on their prior clinical experience (McGinn *et al*, 2001; Muler *et al*, 2004). Reduction of the radiation field may be one of the strategies not only for intense systemic therapy but also for decreasing the troublesome gastrointestinal toxicity often observed in our study; our recent retrospective study showed that a larger planning target volume for irradiation was only a significant predictor of severe acute intestinal toxicity in patients treated with chemoradiotherapy using gemcitabine (Ito *et al*, 2003).

Crane *et al* (2002) retrospectively compared the toxicity and efficacy of concurrent gemcitabine-based chemoradiation with those of concurrent 5-FU-based chemoradiation in patients with unresectable pancreatic cancer treated in the University of Texas MD Anderson Cancer Center. In the study, there was a significantly higher severe toxicity rate in patients treated with gemcitabine than in those with 5-FU, although the median survival times were similar between the two arms (gemcitabine *vs* 5-FU: 11 *vs* 9 months). They concluded that concurrent gemcitabine and radiotherapy could be an extremely difficult combination to administer safely, with a very narrow therapeutic index. Recently, investigators in Taiwan reported favourable results for radiotherapy with concurrent gemcitabine administration (600 mg m^2 $week^{-1}$ for 6 weeks) in a small randomised study (Li *et al*, 2003). The gemcitabine-based chemoradiotherapy showed a significantly better median survival time (14.5 months) and a comparable toxicity profile in comparison with the 5-FU-based chemoradiotherapy (7.1 months). However, the number of enrolled patients in this study was only 16–18 in each arm. The results need further confirmation by larger multi-institutional clinical trials.

In summary, the chemoradiotherapy used in this study has a moderate activity against locally advanced pancreatic cancer and an acceptable toxicity profile, but appears to have more frequent acute toxicities compared with conventional chemoradiotherapy using 5-FU. Most patients who underwent this therapy demonstrated rapid appearance of distant metastasis. To explore innovative approaches for locally advanced pancreatic cancer, future investigations for treatment with more systemic effects and less toxicity are needed.

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Phase II study of S-1 in patients with advanced biliary tract cancer

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Clinical

The aim of this study was to investigate the efficacy and safety of an oral fluoropyrimidine derivative, S-1, in patients with advanced biliary tract cancer. Patients with pathologically confirmed advanced biliary tract cancer, a measurable lesion, and no history of radiotherapy or chemotherapy were enrolled. S-1 was administered orally (40 mg m^{-2} b.i.d.) for 28 days, followed by a 14-day rest period. A pharmacokinetic study was performed on day 1 in the initial eight patients. In all, 19 consecutive eligible patients were enrolled in the study between July 2000 and January 2002. The site of the primary tumour was the gallbladder ($n=16$), the extrahepatic bile ducts ($n=2$), and the ampulla of Vater ($n=1$). A median of two courses of treatment (range, 1–12) was administered. Four patients achieved a partial response, giving an overall response rate of 21.1%. The median time-to-progression and median overall survival period were 3.7 and 8.3 months, respectively. Although grade 3 anorexia and fatigue occurred in two patients each (10.5%), no grade 4 toxicities were observed. The pharmacokinetic parameters after a single oral administration of S-1 were similar to those of patients with other cancers. S-1 exhibits definite antitumour activity and is well tolerated in patients with advanced biliary tract cancer.

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The incidence of biliary tract cancer has been steadily increasing in Japan over the past several decades (Okusaka, 2002). Currently, biliary tract cancer is the sixth leading cause of death from cancer in Japan, with statistics from 2002 indicating a total of about 16 000 deaths from this disease. As a result of the lack of characteristic early symptoms, biliary tract cancers are often diagnosed at an advanced stage, and the prognosis of patients with advanced biliary tract cancer is dismal. Although systemic treatment is used for advanced disease, the impact of existing chemotherapy is virtually negligible. A large number of agents, including 5-fluorouracil (5-FU), mitomycin-C, and cisplatin, have been tested as single agents or in combination therapies without appreciable efficacy (Hejna *et al*, 1998; van Riel *et al*, 1999; Yee *et al*, 2002). Although recent clinical studies have suggested the potential activity of gemcitabine for the treatment of biliary tract cancer, producing response rates of 8 to 36% (Mezger *et al*, 1998; Raderer *et al*, 1999; Gallardo *et al*, 2001; Gebbia *et al*, 2001; Kubicka *et al*, 2001; Penz *et al*, 2001; Tsavaris *et al*, 2004), studies on a larger scale are needed to confirm its efficacy. In any case, to improve the prognosis of patients with biliary tract cancer, a clear need exists for new, effective chemotherapeutic agents.

S-1 is a novel orally administered drug that is a combination of tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP), and oteracil potassium (Oxo) in a 1:0.4:1 molar concentration ratio (Shirasaka *et al*, 1996a). 5-chloro-2,4-dihydroxypyridine is a competitive inhibitor of dihydropyrimidine dehydrogenase, which is involved in the degradation of 5-FU, and acts to maintain efficacious concentrations of 5-FU in plasma and tumour tissues (Tatsumi *et al*, 1987). Oteracil potassium, a competitive inhibitor of orotate phosphoribosyltransferase, inhibits the phosphorylation of 5-FU in the gastrointestinal tract, reducing the serious gastrointestinal toxicity associated with 5-FU (Shirasaka *et al*, 1993). S-1 therapy in athymic nude rats was associated with the retention of a higher and more prolonged concentration of 5-FU in plasma and tumour tissues, when compared with UFT (Shirasaka *et al*, 1996b). The antitumour effect of S-1 has been already demonstrated in a variety of solid tumours: the response rates for advanced gastric cancer (Sakata *et al*, 1998; Koizumi *et al*, 2000), colorectal cancer (Ohtsu *et al*, 2000), non-small-cell lung cancer (Kawahara *et al*, 2001), and head and neck cancer (Inuyama *et al*, 2001) in the late phase II studies conducted in Japan were 44–49, 35, 22, and 29%, respectively. In addition, a recent early phase II study for advanced pancreatic cancer demonstrated a response rate of 21% in 19 patients (Okada *et al*, 2002). The efficacy of S-1 for the treatment of gastrointestinal cancer has also been reported in European patients: the response rates for advanced gastric cancer (Chollet *et al*, 2003) and colorectal cancer (Van den Brande *et al*, 2003) were 32 and 24%, respectively. However, no previous reports have described the efficacy and safety of S-1 for the treatment of biliary tract cancer. Consequently, the present early phase II study was conducted to evaluate the efficacy and safety of S-1 in patients with advanced biliary tract cancer.

PATIENTS AND METHODS

Patients

Patients were required to meet the following eligibility criteria: histologically or cytologically confirmed advanced biliary tract cancer; at least one measurable lesion; no history of prior

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antitumour treatment except resection; a Karnofsky performance status (KPS) of 80–100 points; age of 20–74 years; an estimated life expectancy of at least 2 months; adequate organ function, defined as a white blood cell count of 4000–12 000 mm^{-3}, a platelet count $\geqslant 100\,000\ mm^{-3}$, a haemoglobin level $\geqslant 10.0$ g/dl, a normal serum creatinine level, a serum total bilirubin level $\leqslant 3$ times the upper limit of normal, an aspartate aminotransferase and alanine aminotransferase level $\leqslant 2.5$ times the upper limits of normal; and written informed consent. Patients who had obstructive jaundice were considered eligible if their bilirubin level could be reduced to within 3 times the upper limit of normal after biliary drainage. The exclusion criteria were as follows: a history of drug hypersensitivity; severe complications, such as infection, heart disease, and renal disease; symptomatic metastasis of the central nervous system; active concomitant malignancy; marked pleural effusion or ascites; watery diarrhoea; and pregnancy or lactation. This study was approved by the institutional review board at the National Cancer Center and conducted in accordance with the Good Clinical Practice guidelines of Japan.

clinical

Treatments

S-1 was administered orally at a dose of 40 mg m^{-2} twice daily after breakfast and dinner. Three initial doses were established according to the body surface area (BSA) as follows: BSA $< 1.25\ m^2$, 80 mg day^{-1}; $1.25\ m^2 \leqslant$ BSA $< 1.50\ m^2$, 100 mg day^{-1}; and $1.50\ m^2 \leqslant$ BSA, 120 mg day^{-1}. S-1 was administered at the respective dose for 28 days, followed by a 14-day rest period; this treatment course was repeated until the occurrence of disease progression, unacceptable toxicities, or the patient's refusal to continue. When a grade 3 or greater haematologic or grade 2 or greater nonhaeamatologic toxicity occurred, the temporary interruption of the S-1 administrations was allowed until the toxicity subsided to grade 1 or less. If the daily dose of S-1 was considered to be intolerable, the retreatment dose was reduced by 20 mg day^{-1} (minimum dose, 80 mg day^{-1}). If no toxicity occurred, the rest period shortened to 7 days was allowed. If a rest period of more than 28 days was required because of toxicity, the patient was withdrawn from the study. Patients were not allowed to receive concomitant radiation therapy, chemotherapy, or hormonal therapy during the study. Patients maintained a daily journal to record their intake of S-1 and any signs or symptoms that they experienced. S-1 was provided by Taiho Pharmaceutical Co. Ltd (Tokyo, Japan).

Response and toxicity evaluation

The response after each course was assessed according to the Japan Society for Cancer Therapy Criteria (Japan Society for Cancer Therapy, 1993), which is similar to the World Health Organization Criteria. Briefly, a complete response (CR) was defined as the disappearance of all clinical evidence of the tumour for a minimum of 4 weeks. A partial response (PR) was defined as a 50% or greater reduction in the sum of the products of two perpendicular diameters of all measurable lesions for a minimum of 4 weeks. No change (NC) was defined as a reduction of less than 50% or a less than 25% increase in the sum of the products of two perpendicular diameters of all lesions for a minimum of 4 weeks. Progressive disease (PD) was defined as an increase of 25% or more in the sum of the products of two perpendicular diameters of all lesions, the appearance of any new lesion, or a deterioration in the clinical status that was consistent with disease progression. Primary bile duct lesions were not considered to be measurable lesions because the dimensions of such lesions are difficult to measure accurately.

The response duration was calculated from the day of the first sign of a response until disease progression; time-to-progression (TTP) was calculated from the date of study entry until documented disease progression; and overall survival time was calculated from the date of study entry to the date of death or the last follow-up. The median probability of the survival period and the median TTP were estimated using the Kaplan–Meier method. Compliance was calculated for all treatment courses using the ratio of the total dose actually administered to the scheduled dose.

Physical examinations, complete blood cell counts, biochemistry tests, and urinalyses were performed at least biweekly. Adverse events were evaluated according to the National Cancer Institute Common Toxicity Criteria, version 2.0. Objective responses and adverse events were confirmed by an external review committee.

Analysis was to be performed when 19 patients were enrolled. In this study, the threshold rate was defined as 5% and the expected rate was set as 15%. If the lower limit of the 90% confidence interval exceeded the 5% threshold (objective response in four or more of the 19 patients), S-1 was judged to be effective and we would proceed to the next large-scale study. If the upper limit of the 90% confidence interval did not exceed the expected rate of 15% (no objective response in the 19 patients), S-1 was judged to be ineffective and the study was to be ended. If response was confirmed in 1–3 of the 19 patients, whether to proceed to the next study or not was judged based on the safety and survival data from the present study.

Pharmacokinetics

A pharmacokinetic study was performed in the first eight patients enrolled in the study. Blood (5 ml) was collected before and 1, 2, 4, 6, 8, 10, and 12 h after the administration of S-1 on day 1 of the first course. The plasma was then separated by centrifugation and stored at −20°C until analysis. Plasma concentrations of FT were quantified using high-performance liquid chromatography with UV detection, and the concentrations of 5-FU, CDHP, and Oxo were quantified using gas chromatography-negative ion chemical ionisation mass spectrometry, as reported previously (Matsushima *et al*, 1997).

Pharmacokinetic parameters, including the maximum plasma concentration (C_{max}, ng ml^{-1}), time to reach C_{max} (T_{max}, h), area under the concentration *vs* time curve for zero to infinity ($AUC_{0-\infty}$, ng h ml^{-1}), and the elimination half-life ($T_{1/2}$, h) were calculated using a noncompartment model and Win-Nonlin software, Version 3.1 (Pharsight, Apex, NC, USA).

RESULTS

Patients

Nineteen consecutive eligible patients with advanced biliary tract cancer were enrolled in the study between July 2000 and January 2002 at the National Cancer Center Hospital, Tokyo, Japan. The patient characteristics are summarised in Table 1. Before the start of the study, six patients had received surgical resection and seven patients had undergone percutaneous or endoscopic biliary drainage for obstructive jaundice. Of the 19 patients, 17 had metastatic disease at the time of their enrollment in the study, while two patients were diagnosed as having locally advanced disease. The liver was the most common site of metastases (14 patients), followed by the distant lymph nodes (11 patients) and the lungs (three patients).

Treatments

In all, 19 patients were given a total of 63 courses of chemotherapy, with a median of two courses each (range, 1–12). The initial administered dose of S-1 was 100 mg day^{-1} in seven patients and 120 mg day^{-1} in 12 patients. Dose reduction was required in one patient because of grade 2 diarrhoea after the third course of treatment. The reasons for treatment discontinuation were as follows: disease progression (16 patients), grade 3 diarrhoea and

Table 1 Patient characteristics ($n = 19$)

Characteristics		No. of patients (%)
Gender		
Male		12 (63.2)
Female		7 (36.8)
Median age (years) (range)	59 (44–71)	
Karnofsky performance status, points		
100		8 (42.1)
90		10 (52.6)
80		1 (5.3)
Median body surface area (m^2) (range)	1.56 (1.37–1.83)	
Median first dose ($mg\,m^{-2}\,day^{-1}$) (range)	72.9 (65.8–78.6)	
History of surgical resection		6 (31.6)
Primary tumour site		
Gallbladder		16 (84.2)
Extrahepatic bile ducts		2 (10.5)
Ampulla of Vater		1 (5.3)
Median CEA ($ng\,ml^{-1}$) (range)	6.8 (1–737)	
Median CA 19-9 ($U\,ml^{-1}$) (range)	103 (1–48,160)	

Table 2 Response results ($n = 19$)

	Total	CR	PR	NC	PD	NE	Response rate (%)
Overall	19	0	4	9	5	1	21.1
Primary tumour site							
Gallbladder	16	0	3	8	4	1	18.8
Extrahepatic bile ducts	2	0	0	1	1	0	0
Ampulla of Vater	1	0	1	0	0	0	100.0

CR = Complete response; PR = partial response; NC = no change; PD = progressive disease; NE = not evaluable.

grade 3 stomatitis (one patient), prolonged grade 2 nausea (one patient), and patient's request for transference to another hospital (one patient). Except for two patients, in whom treatment was abandoned because of toxicities, all the patients were treated as outpatients. The overall compliance rate was 94.3%.

Response and survival

Of the 19 patients, none of the patients showed a CR but four patients achieved a PR, giving an overall response rate of 21.1% (95% confidence interval, 6.1–45.6%) (Table 2). The median response duration was 6.7 months (range, 2.8–10.0 months). Nine patients showed NC and five patients had PD. The tumour response could not be evaluated in one patient because the patient was transferred to another hospital, for personal reasons, prior to the response evaluation. At the time of analysis, 18 of the 19 patients had died because of disease progression. The median TTP was 3.7 months, and the overall median survival time was 8.3 months, with a 1-year survival rate of 21.1% (Figure 1).

Toxicity

All 19 patients were assessed for toxicities that are listed in Table 3. Treatment was generally well tolerated throughout the study. Although haematologic and gastrointestinal toxicities were common, most of the toxicities were mild and transient. Grade 3 anorexia and fatigue occurred in two patients each (10.5%), and grade 3 anaemia, neutropenia, stomatitis, nausea, diarrhoea, and fever occurred in one patient each (5.3%). No signs of cumulative

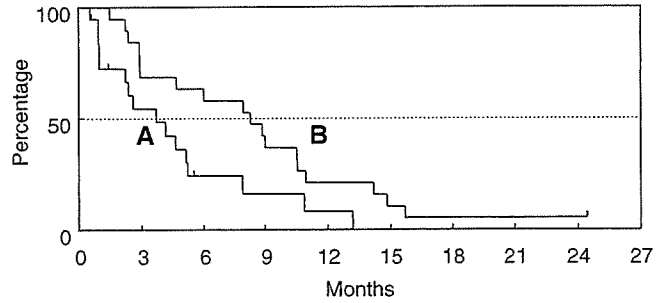

Figure 1 Time to progression (**A**) and overall survival time (**B**).

Table 3 Treatment-related adverse events ($n = 19$): worst grade reported during treatment period

	Grade[a]					
Toxicity	1	2	3	4	Grade 1–4 (%)	Grade 3–4 (%)
Haematologic						
Leukopenia	5	3	0	0	42.1	0
Neutropenia	4	2	1	0	36.8	5.3
Anaemia	3	4	1	0	42.1	5.3
Thrombocytopenia	2	0	0	0	10.5	0
Nonhaematologic						
Nausea	4	2	1	0	36.8	5.3
Vomiting	4	0	0	0	21.1	0
Anorexia	3	0	2	0	26.3	10.5
Stomatitis	3	0	1	0	21.1	5.3
Diarrhoea	2	2	1	0	26.3	5.3
Total bilirubin	1	1	0	0	10.5	0
ALT	2	4	0	0	31.6	0
AST	4	2	0	0	31.6	0
Fatigue	0	0	2	0	10.5	10.5
Fever	0	0	1	0	5.3	5.3
Rash	1	0	0	0	5.3	0
Pigmentation changes	3	0	0	0	15.8	0

AST = aspartate aminotransferase; ALT = alanine aminotransferase. [a]NCI Common Toxicity Criteria, version 2.0.

toxicity were noted. Of the 17 patients who were treated as outpatients, one patient required hospitalisation because of grade 3 nausea, anorexia, and fatigue during the middle of the first course of treatment. Although one patient died within 8 weeks of study enrollment because of rapid disease progression, no treatment-related deaths were observed.

Pharmacokinetics

Table 4 and Figure 2 show the results of the pharmacokinetic study for S-1 in the current study. The pharmacokinetic parameters for S-1 in other cancers, as reported by Hirata *et al* (1999) are also shown in Table 4 and Figure 2 for reference. Hirata *et al* investigated the pharmacokinetic parameters after the single administration of S-1 at a dose of $40\,mg\,m^{-2}$ in 12 Japanese patients with gastric, colorectal, and breast cancer. The parameters of 5-FU in both studies were similar, and no large differences in the parameters of other compounds, including CDHP, were seen.

DISCUSSION

Although most patients with biliary tract cancer have an unresectable disease at the time of diagnosis, no standard chemotherapies have been established for this disease (Hejna

Table 4 Pharmacokinetic parameters after single administration of S-1 at a dose of 40 mg m^{-2}

Compound	Parameter	Current study ($n=8$)	Hirata's study ($n=12$)
FT	C_{max} (ng ml^{-1})	1721.6±400.4	1971.0±269.0
	T_{max} (h)	3.6±1.1	2.4±1.2
	AUC (ng h ml^{-1})	24643.0±7915.0[a]	28216.9±7771.4[b]
	$T_{1/2}$ (h)	8.2±2.0	13.1±3.1
5-FU	C_{max} (ng ml^{-1})	146.9±62.1	128.5±41.5
	T_{max} (h)	4.0±0.0	3.5±1.7
	AUC (ng h ml^{-1})	799.8±285.3[a]	723.9±272.7[c]
	$T_{1/2}$ (h)	1.9±0.3	1.9±0.4
CDHP	C_{max} (ng ml^{-1})	245.3±64.9	284.6±116.6
	T_{max} (h)	3.3±1.0	2.1±1.2
	AUC (ng h ml^{-1})	1472.6±381.6[a]	1372.2±573.7[b]
	$T_{1/2}$ (h)	3.2±0.7	3.0±0.5
Oxo	C_{max} (ng ml^{-1})	55.3±48.4	78.0±58.2
	T_{max} (h)	3.3±1.0	2.3±1.1
	AUC (ng h ml^{-1})	230.6±140.2[a]	365.7±248.6[d]
	$T_{1/2}$ (h)	2.8±0.6	3.0±1.4

Parameters are represented as mean±s.d. [a]$AUC_{0-\infty}$. [b]AUC_{0-48}. [c]AUC_{0-14}. [d]AUC_{0-24}. FT = tegafur; 5-FU = 5-fluorouracil; CDHP = 5-chloro-2,4-dihydroxypyridine; Oxo = oteracil potassium.

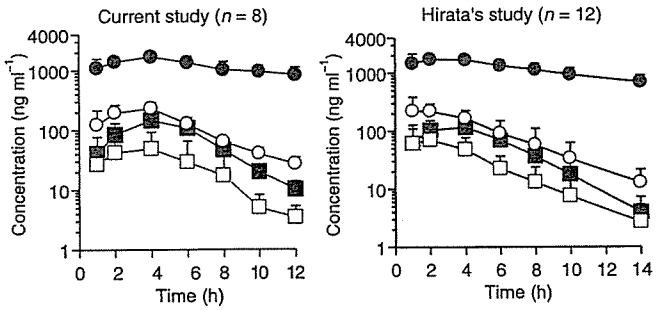

Figure 2 Plasma concentration–time profiles of FT (●), 5-FU (■), CDHP (○), and Oxo (□) after administration of S-1. The values are expressed as the mean±s.d.

et al, 1998; van Riel et al, 1999; Okusaka, 2002; Yee et al, 2002). Since biliary tract cancer is an uncommon disease, studies of chemotherapy for biliary tract cancer are relatively few, and the number of included patients is generally small. In addition, the response rates and survival times described in published studies are difficult to compare because most studies contain patients with heterogeneous tumour groups, such as intrahepatic or extrahepatic bile duct cancer and gallbladder cancer. 5-fluorouracil has been the most commonly studied drug for this disease, although the antitumour effect of single-agent 5-FU is limited, with a response rate of less than 20%. Although the combined use of 5-FU with other agents, such as leucovorin, mitomycin C, or cisplatin, often produces a response rate of over 20% (Polyzos et al, 1996; Ducreux et al, 1998; Taïeb et al, 2002), the toxicities also become greater; whether combination therapies contribute to prolonged survival remains uncertain. In recent small-scale studies, gemcitabine has shown relatively good response rates, ranging from 8 to 36%, for biliary tract cancer (Mezger et al, 1998; Raderer et al, 1999; Gallardo et al, 2001; Gebbia et al, 2001; Kubicka et al, 2001; Penz et al, 2001; Tsavaris et al, 2004), but large-scale studies are needed to confirm its efficacy. Therefore, the development of new effective chemotherapeutic agents is urgently needed to improve survival in patients with advanced biliary tract cancers.

A novel orally administered drug, S-1, has been developed based on the biochemical modulations by CDHP, a dihydropyrimidine dehydrogenase inhibitor, and Oxo, a protector against 5-FU-induced gastrointestinal toxicity; S-1 has exhibited significant antitumour effects on various solid cancers (Sakata et al, 1998; Koizumi et al, 2000; Ohtsu et al, 2000; Inuyama et al, 2001; Kawahara et al, 2001; Chollet et al, 2003; Van den Brande et al, 2003). Since the drug is available in oral form, S-1 has a potential advantage, as far as patient convenience is concerned, especially in terms of quality-of-life. This consideration is very important for biliary tract cancer patients because their remaining lifespan is generally short. Consequently, the efficacy of S-1 for the treatment of biliary tract cancer was examined.

In the current study, S-1 produced a good response rate of 21.1%, which is superior to those obtained with other single agents, including 5-FU, mitomycin C, and cisplatin (Table 5), suggesting an antitumour effect of S-1 on biliary tract cancer. In this study, patients with gallbladder cancer accounted for three of the four responders; however, the efficacy of S-1 for each primary tumour site cannot be accurately assessed because of the small number of subjects analysed.

Table 5 Recent studies of single-agent chemotherapy for biliary tract cancer

Author	Regimen	No. of patients: Total	No. of patients: Gallbladder Ca.	Response rate (%)	MST (months)
Takada et al (1994)	5-FU	18	10	0	NA
Taal et al (1993)	Mitomycin C	30	13	10	4.5
Okada et al (1994)	Cisplatin	13	6	8	5.5
Jones et al (1996)	Paclitaxel	15	4	0	NA
Pazdur et al (1999)	Docetaxel	17	0	0	NA
Papakostas et al (2001)	Docetaxel	25	16	20	8
Sanz-Altamira et al (2001)	Irinotecan	25	10	8	10
Mezger et al (1998)	Gemcitabine	13	4	8	NA
Raderer et al (1999)	Gemcitabine	19	5	16	6.5
Penz et al (2001)	Gemcitabine[a]	32	10	22	11.5
Kubicka et al (2001)	Gemcitabine	23	0	30	9.3
Gallardo et al (2001)	Gemcitabine	26	26	36	7
Gebbia et al (2001)	Gemcitabine	18	12	22	8
Tsavaris et al (2004)	Gemcitabine	30	14	30	14
Current study	S-1	19	16	21	8.3

5-FU: 5-fluorouracil; MST: median survival time; NA: not available. [a]Biweekly.

Since patients with biliary tract cancer tend to suffer various tumour-related complications, such as cholangitis and impaired liver function, enhanced chemotherapy-related toxicities, including neutropenic sepsis, are a concern. However, S-1 was well tolerated in the present study, and no grade 4 toxicities occurred. Haematological toxicities were acceptable and similar to the results of clinical studies examining S-1 for the treatment of other cancers in Japan. Gastrointestinal toxicities were also well tolerated, as in the other Japanese studies, although strong gastrointestinal toxicities, particularly severe diarrhoea, have been reported in Western countries (van Groeningen *et al*, 2000; Cohen *et al*, 2002; Chollet *et al*, 2003; Van den Brande *et al*, 2003). The difference in toxicities between the Japanese and Western studies remains unexplained, although the conversion of FT to 5-FU seems to occur more slowly in Japanese patients than in patients from other ethnic groups (Comets *et al*, 2003). A pharmacokinetic study suggested that the pharmacokinetic parameters of S-1 were similar in patients with biliary tract cancer and in patients with other cancers in Japan.

Since no serious adverse events occurred in this study, most of the patients were treated as outpatients, enabling a relatively good quality-of-life. The S-1 compliance rate of the patients was very good (94.3%), with only one patient requiring a dose reduction and only two patients discontinuing S-1 because of toxicity. In view of the favourable toxicity profile, its evaluation in combination with other agents might be of particular interest to improve therapeutic results. Combination therapy with S-1 and cisplatin has already been conducted for gastric cancer, and an excellent response rate of 76% was reported in a phase II study (Ohtsu *et al*, 2001).

In conclusion, the results of this study indicate that S-1 is a safe and active agent for the treatment of patients with biliary tract cancer. Further investigations of this agent are warranted in this population of patients with a poor prognosis.

Clinical

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Original Paper

Prognostic Factors in Patients with Advanced Biliary Tract Cancer Receiving Chemotherapy

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KEY WORDS:
Biliary tract cancer; Chemotherapy; Prognosis; Prognostic factors

ABBREVIATIONS:
Biliary Tract Cancer (BTC); World Health Organization (WHO); Eastern Cooperative Oncology Group (ECOG); Median Survival Time (MST); C-Reactive Protein (CRP); Lactate Dehydrogenase (LDH); Carcinoembryonic Antigen (CEA); Carbohydrate Antigen 19-9 (CA19-9)

ABSTRACT

Background/Aims: Prognostic factors in patients with advanced biliary tract cancer receiving chemotherapy have not been fully examined. This study investigated prognostic factors in patients with advanced biliary tract cancer receiving chemotherapy.

Methodology: Sixty-five consecutive chemo-naive patients with advanced biliary tract cancer, who received chemotherapy, were analyzed retrospectively to investigate prognostic factors.

Results: Median survival time and overall survival rates at 1 and 2 years were 180 days, 21%, and 5%, respectively. By multivariate analysis using the Cox proportional hazards model, performance status of 0, 1, serum C-reactive protein level of ≤1.0mg/dL, serum albumin level of ≥3.5g/dL, serum lactate dehydrogenase level of ≤500 U/L, and being female were independent favorable prognostic factors. A prognostic index based on the coefficients of these prognostic factors was used to classify patients into three groups with good, intermediate, and poor prognoses. The median survival times for these three groups were 246, 152, and 33 days, respectively.

Conclusions: The results may be helpful for predicting life expectancy, determining treatment strategies, and designing future clinical trials in patients with advanced biliary tract cancer.

INTRODUCTION

Biliary tract cancer (BTC) is diagnosed at an advanced stage in most patients despite the recent improvement in diagnostic techniques. Even if resection is performed, the recurrence rate is extremely high (1-5). Therefore, to improve the prognosis of BTC patients, effective non-surgical treatment is indispensable. With regard to chemotherapy for advanced BTC, numerous clinical trials have been conducted (6-10). However, at present, chemotherapy for advanced BTC has been of limited value in clinical practice, because the majority of patients do not respond well and suffer only the adverse effects of chemotherapy.

The identification of prognostic factors will be helpful for predicting life expectancy, and designing and analyzing clinical trials. However, prognostic factors in BTC patients treated with chemotherapy have not been fully examined. The current study was designed to retrospectively analyze several variables that may affect survival in patients with advanced BTC receiving chemotherapy. To our knowledge, this is the first study concerning prognostic factors and a staging system for patients with advanced BTC receiving chemotherapy.

METHODOLOGY

Patients

The study group included 65 consecutive chemo-naive patients with advanced BTC who had received chemotherapy at the National Cancer Center Hospital, Tokyo, Japan, between April, 1988 and March, 2001 **(Table 1)**. None had received any anti-cancer treatment except for surgical resection before chemotherapy. All diseases were diagnosed as advanced BTC using various imaging modalities including chest X-ray, ultrasonography, and computed tomography. Pathological confirmation of adenocarcinoma was obtained in 62 patients (95%) by a surgical procedure or by a fine-needle aspiration biopsy. Cytological examination of the peritoneal fluid was performed for patients with intraperitoneal fluid collection, and peritoneal dissemination was diagnosed by positive cytology. Patients with obstructive jaundice underwent percutaneous transhepatic or endoscopic

TABLE 1 Chemotherapeutic Regimens for Advanced Biliary Tract Cancer

Regimen	No. of patients
Fluorouracil	1
Fluorouracil + methotrexate	1
Cisplatin	8
UFT (tegafur + uracil)	2
S-1 (tegafur + gimeracil + oteracil potassium)	9
Fluorouracil + mitomycin C	1
Fluorouracil + cisplatin + epirubicin	43

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TABLE 2 Patient Characteristics

Characteristics	No. of patients (%)
Age (yrs) *	63 (28-76)
Gender	
Male	33 (51)
Female	32 (49)
Primary tumor location	
Gallbladder	53 (82)
Extrahepatic bile duct	12 (18)
Prior surgical resection (+)	16 (25)
Performance status	
0	31 (48)
1	28 (43)
2	6 (9)
Biliary drainage (+)	20 (31)
White blood cell (/mm^3) *	7,200 (3,500-25,200)
Hemoglobin (g/dL) *	11.7 (7.7-15.5)
Albumin (g/dL) *	3.6 (2.4-4.3)
Total bilirubin (mg/dL) *	0.8 (0.3-4)
LDH (IU/L) *	429 (228-5,178)
C-reactive protein (mg/dL) *	1.3 (0.0-17.1)
CEA (ng/mL) *	13.6 (1-13,680)
CA19-9 (U/mL) *	209 (1-1,480,000)

* median (range); LDH: lactic dehydrogenase; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9.

biliary drainage before chemotherapy. The tumor response was evaluated according to the criteria of the World Health Organization (WHO) every 4 weeks after the first course of chemotherapy. Survival was measured from the first day of chemotherapy until death from cancer or the last day of follow-up.

Factors Analyzed

Pretreatment clinical variables were investigated for their relation to survival by univariate analysis and multivariate analysis. The pretreatment variables were chosen by considering the possible effects on the prognosis as indicated by previous investigations (11,12) or suggested from our own clinical experience. The variables, divided into two subgroups, were as follows: age (<60 or ≥60 years), gender (male or female), prior surgical resection for BTC (presence or absence), Eastern Cooperative Oncology Group (ECOG) performance status (13) (0, 1 or 2), biliary drainage (presence or absence), white blood cell count (<7,000 or ≥7,000/mm^3), hemoglobin level (<12 or ≥12g/dL), serum albumin level (<3.5 or ≥3.5g/dL), serum total bilirubin level (<1.0 or ≥1.0mg/dL), serum lactate dehydrogenase (LDH) level (<500 or ≥500 IU/L), and serum C-reactive protein (CRP) level (<1.0 or ≥1.0mg/dL), as host-related variables; primary tumor location (extrahepatic bile duct or gallbladder), serum carcinoembryonic antigen (CEA) level (<10 or ≥10ng/mL), and serum carbohydrate antigen 19-9 (CA 19-9) level (<1,000 or ≥1,000 U/mL), as tumor-related variables.

Statistical Methods

Actuarial survival probabilities were calculated using the Kaplan-Meier method (14), and compared with the log-rank test (15). Multivariate analysis was performed following the Cox proportional hazards model (16). A prognostic index was calculated based on the regression coefficients of the variables identified from multivariate analysis. All P values presented in this report are of the two-tailed type; $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Patient Characteristics

The characteristics of the patients are shown in **Table 2**. Of the 65 patients with BTC, 33 were males and 32 females. The median age was 63 years old (range, 28-76). Performance status was 0, 1 in 59 patients (91%) and 2 in 6 patients (9%). The primary tumor location was the gallbladder in 53 (82%) and the extrahepatic bile duct in 12 patients (18%). Fifty-six patients (86%) had distant metastasis. Twenty patients (31%) underwent percutaneous or endoscopic biliary drainage before chemotherapy. Of 65 patients, 6 were evaluated as showing a partial response, twenty-eight showed no change and 29 showed progressive disease. The tumor response was not evaluated in 2 patients due to early death related to chemotherapy.

Survival

The median survival time and survival rate at 1 and 2 years in 65 patients were 180 days, 21%, and 5%, respectively **(Figure 1)**. At the time of analysis, 63 patients had died; the causes of death were cancer-related in 61 patients (97%) and chemo-related in 2 (3%).

Univariate Analysis

Table 3 lists the results of univariate analyses in relation to each variable. Patients with a performance status of 0, 1 showed better survival than those with a performance status of 2 (P=0.01); one of the 6 patients with a performance status of 2 survived 13 months, but the other 5 survived less than 4 months. Moreover, survival was significantly affected by serum albumin level (P<0.01), serum CRP level (P<0.01), and serum LDH level (P=0.01).

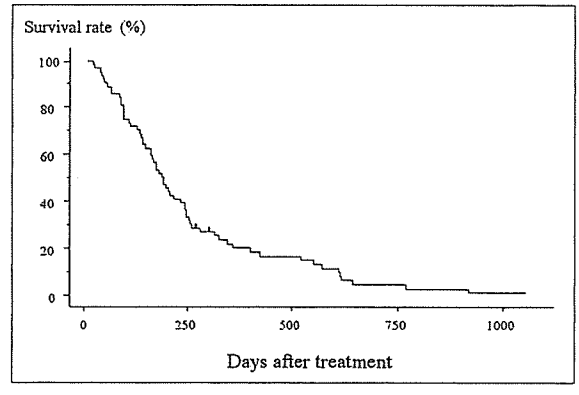

FIGURE 1 Overall survival curve for all patients with BTC receiving chemotherapy. Tick marks indicate censored cases.

Multivariate Analysis

In addition to gender and age, variables with prognostic significance in univariate analysis were subsequently included in the multivariate Cox regression model. Among them, 5 factors, performance status, serum CRP level, serum albumin level, serum LDH level, and gender were identified as independent prognostic factors **(Table 4)**.

Risk Groups Based on the Regression Model: For the clinical application of these findings, a prognostic index was calculated based on the regression coefficients derived from the five variables identified by multivariate analysis. The index equation was as follows: 1.97 (0, performance status of 0, 1; 1, performance status of 2) + 0.94 (0, CRP <1.0mg/dL; 1, CRP ≥1.0mg/dL) + 0.81 (0, albumin ≥3.5mg/dL; 1, albumin <3.5mg/dL) + 0.73 (0, LDH <500 IU/L; 1, LDH ≥500 IU/L) + 0.58 (0, female; 1, male). The individual index values for the patients ranged from 0.00 to 5.03. The patients were then classified into three groups according to the prognostic index, as follows: group A, a prognostic index <1.50 (25 patients); group B, a prognostic index from 1.50 to 2.50 (32 patients); group C, a prognostic index >2.50 (8 patients). The survival curves for these groups are shown in **Figure 2**. The median survival times in groups A, B, and C were 246, 152, 33 days, respectively. There was a significant difference among these three groups in the survival time ($P<0.01$).

TABLE 3 Univariate Analysis of Prognostic Factors Associated in Patients with Advanced Biliary Tract Cancer

Variable		No. of patients	Median survival (days)	*P* value
Age, years	<60	27	186	
	≥60	38	164	0.93
Gender	Male	33	164	
	Female	32	186	0.64
Primary tumor location	Gallbladder	53	180	
	Extrahepatic bile duct	12	138	0.25
Prior surgical resection	+	16	150	
	-	49	180	0.70
Performance status	0, 1	59	186	
	2	6	47	0.01
Biliary drainage	+	20	186	
	-	45	165	0.46
White blood cell	<7,000/mm³	35	236	
	≥7,000/mm³	30	138	0.14
Hemoglobin	<12g/dL	34	138	
	≥12g/dL	31	238	0.07
Albumin	<3.5g/dL	23	124	
	≥3.5g/dL	42	224	<0.01
Total bilirubin	<1.0mg/dL	40	181	
	≥1.0mg/dL	25	165	0.92
LDH	<500 IU/L	44	199	
	≥500 IU/L	21	152	0.01
C-reactive protein	<1.0mg/dL	28	250	
	≥1.0mg/dL	37	138	<0.01
CEA	<10ng/mL	31	206	
	≥10ng/mL	34	155	0.36
CA19-9	<1,000 U/mL	40	180	
	≥1,000 U/mL	24	172	0.82

LDH: lactic dehydrogenase; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9.

TABLE 4 Significant Prognostic Factors Identified in 65 Patients as Determined by Multivariate Analysis with Cox Proportional Hazards Model

Variable	Hazards ratio Coefficient (β)	(95% confidence interval)	*P* value
Performance status	1.97	7.14 (2.67-19.06)	<0.01
C-reactive protein	0.94	2.57 (1.46-4.53)	<0.01
Albumin	0.81	2.24 (1.23-4.09)	<0.01
LDH	0.73	2.07 (1.12-3.84)	0.02
Gender	0.58	1.79 (1.02-3.14)	0.04

LDH: lactic dehydrogenase.

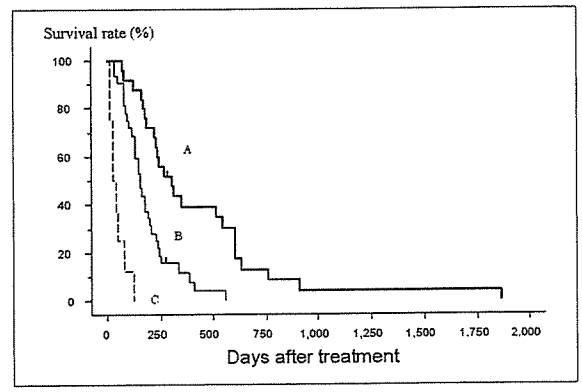

FIGURE 2 Survival curves for three groups classified by a prognostic index based on the findings of multivariate analysis. Group A, prognostic index less than 1.5 (25 patients); Group B, prognostic index from 1.5 to 2.5 (32 patients); Group C, prognostic index greater than 2.5 (8 patients). Tick marks indicated censored cases.

DISCUSSION

The prognosis of patients with advanced BTC is extremely poor, with a median survival of 4-12 months (1,4,5,8,9). To improve the prognosis of this disease, the development of effective chemotherapy is essential. However, chemotherapy for advanced BTC has been of limited value, because the majority of patients does not respond well and suffer only the adverse effects of chemotherapy. Therefore, in chemotherapy for advanced BTC, patient selection with reference to expected survival time may be important. In addition, identifying prognostic factors may be useful for the design of future trials of chemotherapy for BTC. In the present study, we investigated the prognostic factors in patients with advanced BTC receiving chemotherapy. This single institution study was undertaken using unified methods for staging the disease and identical procedures for supportive care throughout, thus enabling us to confirm important prognostic factors.

Among the 14 potential prognostic factors investigated, four factors, performance status, serum CRP level, serum albumin level, and serum LDH level, were identified as a significant predictor of survival by both univariate analysis and multivariate analysis. Moreover, in addition to these four factors, gender was

found to have independent prognostic value by multivariate analysis.

The performance status and serum albumin have been recognized as important prognostic factors in a variety of malignancies (17-21). The performance status is a simple but widely used method for evaluating the physical condition of cancer patients, and the serum albumin level also reflects the physical condition, especially the influence of nutritional status. The prognostic value of serum CRP and LDH have also been reported in a variety of neoplastic diseases (18,20,22-24). Serum CRP, which is known as a marker of the acute-phase protein response, is observed in different pathological states such as infection, inflammation, and malignancy. However, the elevated serum CRP in our patients with BTC was likely to be a consequence of the underlying malignancy, because no patients showed evidence of infection before treatment. It can be argued that the increasing bulk of the disease provides potential for greater tumor necrosis and associated inflammation, and, thus, serum CRP and LDH simply may reflect tumor burden. It was reported that females have a better prognosis than males in a large variety of malignant diseases (20,21,25-28). It is suggested that gender specific hormones may play a role in the regulation of tumor growth and should thus be taken into consideration as a possible reason for the survival advantage of females. However, the reasons for the better prognosis of females are still not fully clarified.

The prognosis of advanced BTC patients was poor in the present study; the median survival time was 180 days, and about 14% had died within 2 months after the beginning of chemotherapy. To predict patient survival more accurately, a prognostic index based on independent prognostic factors was proposed. The patients in the present study could be classified into three groups with good, intermediate, and poor prognosis. This prognostic index may therefore be useful in making an accurate prediction of survival in patients with advanced BTC and determining treatment strategies, although the validation of this model has to be tested using an independent data set in future studies. The poor prognosis group may be treated with different experimental approaches or may be offered only supportive care to maintain their quality of life.

In conclusion, performance status, serum CRP level, serum albumin level, serum LDH level, and gender were identified as significant independent prognostic factors in patients with advanced BTC receiving chemotherapy. The present findings may be helpful in predicting life expectancy, determining treatment strategies, and designing future clinical trials in patients with BTC.

ACKNOWLEDGEMENT

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Possible Detection of Pancreatic Cancer by Plasma Protein Profiling

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Abstract

The survival rate of pancreatic cancer patients is the lowest among those with common solid tumors, and early detection is one of the most feasible means of improving outcomes. We compared plasma proteomes between pancreatic cancer patients and sex- and age-matched healthy controls using surface-enhanced laser desorption/ionization coupled with hybrid quadrupole time-of-flight mass spectrometry. Proteomic spectra were generated from a total of 245 plasma samples obtained from two institutes. A discriminating proteomic pattern was extracted from a training cohort (71 pancreatic cancer patients and 71 healthy controls) using a support vector machine learning algorithm and was applied to two validation cohorts. We recognized a set of four mass peaks at 8,766, 17,272, 28,080, and 14,779 *m/z*, whose mean intensities differed significantly (Mann-Whitney *U* test, $P < 0.01$), as most accurately discriminating cancer patients from healthy controls in the training cohort [sensitivity of 97.2% (69 of 71), specificity of 94.4% (67 of 71), and area under the curve value of 0.978]. This set discriminated cancer patients in the first validation cohort with a sensitivity of 90.9% (30 of 33) and a specificity of 91.1% (41 of 45), and its discriminating capacity was further validated in an independent cohort at a second institution. When combined with CA19-9, 100% (29 of 29 patients) of pancreatic cancers, including early-stage (stages I and II) tumors, were detected. Although a multi-institutional large-scale study will be necessary to confirm clinical significance, the biomarker set identified in this study may be applicable to using plasma samples to diagnose pancreatic cancer. (Cancer Res 2005; 65(22): 10613-22)

Introduction

The 5-year survival rate of pancreatic cancer sufferers is the lowest among patients with common solid tumors. Pancreatic cancer is the fifth leading cause of cancer-related mortality in Japan and the fourth in the United States, with >19,000 estimated annual deaths in Japan and >28,000 in the United States (1–3). Pancreatic cancer is characterized by massive local invasion and early metastasis to the liver and regional lymph nodes. Because surgical resection is the only reliable curative treatment, early detection is essential to improve the outcomes of pancreatic cancer patients. However, the clinical symptoms of pancreatic cancer, except for obstructive jaundice, are often unremarkable until the advanced stages of the disease, and the anatomic location of the pancreas deep in the abdomen makes physical and ultrasonic detection of pancreatic cancer difficult. As a result, only 20% to 40% of pancreatic cancer patients undergo surgical resection (1, 4). Mass screening by computed tomography (CT), magnetic resonance imaging (MRI), or positron emission tomography (PET) may not be cost-effective because of the relatively low incidence of pancreatic cancer, and the long-term safety of these modalities has not been established (5). Thus, new diagnostic modalities allowing early detection of pancreatic cancer in a safe/noninvasive and cost-effective way are needed.

Recently, mass spectrometry (MS)–based proteomic approaches have gained considerable attention as effective modalities for identifying new biomarkers of various diseases because of their high sensitivity, but proteomic analysis of blood samples has been hampered by the marked dominance of a handful of particularly abundant proteins, including albumin, immunoglobulins, and transferrins (6). Surface-enhanced laser desorption/ionization (SELDI)-MS was developed to resolve these problems and is considered to be among the most useful tools available for the analysis of serum and plasma (7–9). Proteins are captured, concentrated, and purified on the small chemical surface of a SELDI chip, and the molecular weight (*m/z*) and relative intensity of each protein captured on the chip are measured with sensitive time-of-flight (TOF)-MS. As a result, a comprehensive proteomic profile can be created from as little as 20 μL serum/plasma samples. Combined with multivariate bioinformatical analysis, serum proteomics by SELDI-TOF-MS has been reported be successfully applied to the diagnosis of ovarian and prostate cancers (10–13).

The ProteinChip system is a sophisticated commercial platform designed for SELDI-TOF-MS. This system has been widely used because of its high-throughput automated measurements. However, relatively low resolution and poor mass accuracy have been recognized as drawbacks of the TOF-MS instrument of this system, and the reproducibility of SELDI-MS data has been controversial (14–16). Multivariate discrimination is dependent on stacks of small differences between cases and controls. Recently, Petricoin and Liotta reported the use of high-resolution performance hybrid quadrupole TOF-MS (QqTOF-MS) instruments to significantly improve the resolution and mass accuracy of SELDI-MS compared with results obtained with low-resolution instruments (17, 18).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Table 1. Clinicopathologic characteristics of the 220 cases seen at NCCH

	Training cohort			Validation cohort		
	Cancer (n = 71)	Healthy (n = 71)	P	Cancer (n = 33)	Healthy (n = 45)	P
Age (mean ± SD)	61.3 ± 9.06	62.1 ± 10.0	0.6*	62.0 ± 9.06	63.2 ± 11.7	0.6*
Gender						
Male	37	33	0.5†	18	24	0.92†
Female	34	38		15	21	
Tumor location						
Head	34			17		
Body or tail	37			10		
Unknown	0			6		
Clinical stage						
I	1			1		
II	6			4		
III	10			1		
IV	54			27		

*Student's t test.
†Fisher exact probability test.

Koopmann et al. (19) identified a set of biomarkers for pancreatic adenocarcinoma using the ProteinChip system. They increased the number of detectable peaks using stepwise anion-exchange chromatography, but only two of the six fractions were used for subsequent analyses. The two protein peaks that most effectively discriminated between pancreatic cancer patients and healthy controls reportedly achieved a sensitivity of 78% and a specificity of 97%, but this sensitivity was below the level necessary for clinical application. More importantly, diagnostic performance was not validated in an independent cohort. We reviewed and refined various aspects of SELDI-MS. In this study, we first compared the results obtained using low-resolution TOF-MS and high-resolution QqTOF-MS instruments and confirmed the high reproducibility of data obtained using the latter. Computerized machine learning may identify even a perfect multivariate classifier within a closed sample set in a nonbiological/mathematical way (16). Erroneous identification by machine learning must be eliminated by validation experiments using an independent sample set. Herein, we report the identification and validation of a set of biomarkers that can detect pancreatic cancer with high accuracy.

Materials and Methods

Patients and plasma samples. Plasma samples (n = 245) were obtained from two institutes, the National Cancer Center Hospital (NCCH; Tokyo, Japan) between August 2002 and October 2003 and the Tokyo Medical University Hospital (TMUH; Tokyo, Japan) between February 2004 and February 2005. The 220 NCCH cases included untreated pancreatic ductal adenocarcinoma patients (n = 104) and healthy controls (n = 116), whereas the 25 TMUH cases included untreated pancreatic ductal adenocarcinoma patients (n = 9), individuals with pancreatic tumors and/or cysts (n = 6), chronic pancreatitis patients (n = 5), and healthy controls (n = 5). The pancreatic tumor and/or cyst category included two pathologically unproven mucinous cystic tumors, two pathologically unproven serous

Table 2. Comparison of low-resolution and high-resolution instruments

	High-resolution QqTOF-MS		Low-resolution TOF-MS			
	Unfractionated		Unfractionated		Fractionated	
	No. unique peaks*	Correlation coefficient (r), mean ± SD	No. unique peaks*	Correlation coefficient (r), mean ± SD	No. unique peaks*	Correlation coefficient (r), mean ± SD
H50	263	0.96 ± 0.03	64	0.96 ± 0.04	214	0.76 ± 0.35
CM10 pH 4	124	0.99 ± 0.01	53	0.90 ± 0.11	219	0.73 ± 0.33
CM10 pH 7	73	0.98 ± 0.01	48	0.89 ± 0.09	168	0.61 ± 0.46
IMAC-Cu^{2+}	177	0.95 ± 0.04	61	0.87 ± 0.13	271	0.70 ± 0.44
Total	637		226		872	

*Number of unique peaks detectable in plasma samples from 24 pancreatic cancer patients and 24 healthy controls.

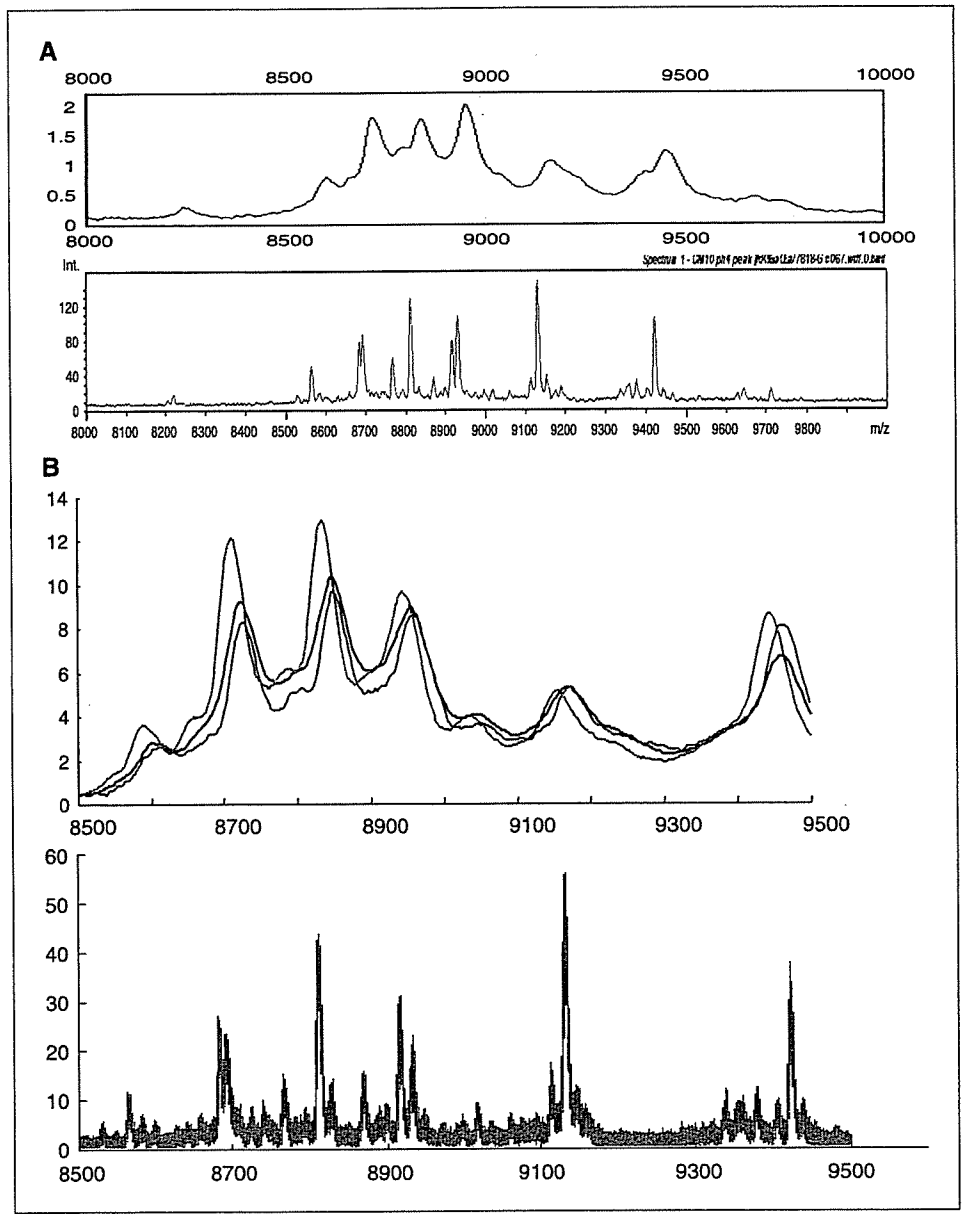

Figure 1. Comparison of low-resolution and high-resolution instruments. *A*, representative spectra of an unfractionated plasma sample in the range of 8,000 to 10,000 *m/z* obtained using a low-resolution TOF instrument (*top*) and a high-resolution QqTOF instrument (*bottom*). *B*, spectra of an unfractionated plasma sample in the range of 8,500 to 9,500 *m/z* obtained thrice every other day using a low-resolution TOF instrument (*top*) and a high-resolution QqTOF instrument (*bottom*). The spectra (*green*, *blue*, and *red lines*) were superimposed to allow visualization of the day-to-day variations. Note that only the *green line* is visible in the *bottom* because of the high reproducibility of results obtained with the QqTOF instrument.

papillary tumors, and two clinically diagnosed nonmalignant mass lesions in the pancreas. These cases are currently being followed, and a final diagnosis has not been obtained to date. The patients in the chronic pancreatitis category had no detectable mass lesions in the pancreas. Written informed consent was obtained from all of the subjects. Blood samples were collected in EDTA glass tubes. The supernatant was separated by centrifugation and cryopreserved at −80°C until analysis. All samples were processed in the same manner. The study was reviewed and approved by the ethics committees of the National Cancer Center (Tokyo, Japan; authorization nos. 16-36 and 16-71) and Tokyo Medical University (Tokyo, Japan; authorization no. 341).

The clinical characteristics of the patients are summarized in Table 1. Patients were classified as having clinical disease stage I, II, III, or IV according to the Fifth Edition of the General Rules for the Study of Pancreatic Cancer (Japanese Pancreas Society; ref. 20).

Surface-enhanced laser desorption/ionization. Ninety microliters of U9 buffer [9 mol/L urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, and 50 mmol/L Tris-HCl (pH 9)] were added to 10 μL of each plasma sample and vortexed for 20 minutes. Parts of the denatured plasma samples were fractionated using stepwise anion-exchange chromatography (pH 9 plus flow trough, pH 7, pH 5, pH 4, pH 3, and organic wash) with QHyper DF resin (Ciphergen Biosystems, Inc., Fremont, CA) using a Biomek 2000 Laboratory Automation Robot (Beckman Coulter, Fullerton, CA) according to a previously described method (12, 21).

Each sample was randomly assigned, with a 96-spot format, to 12 ProteinChip arrays (8 spots per array; Ciphergen) in duplicate using the Biomek 2000 Robot. Three types of ProteinChip arrays with different surface chemistries [i.e., immobilized metal affinity capture coupled with copper (IMAC-Cu^{2+}), weak hydrophobic (H50), or cationic (CM10) arrays] were used (21). The CM10 arrays were used under either low-stringent (pH 4) or high-stringent (pH 7) conditions as instructed by the supplier. The arrays were air-dried and applied to the matrix (50% sinapinic acid in 50% acetonitrile/0.1% trifluoroacetic acid).

Time-of-flight mass spectrometry. TOF-MS analysis was done using two types of mass spectrometers, a low-resolution TOF-MS (PBS IIc, Ciphergen) and a high-resolution QqTOF-MS [Q-star XL (Applied Biosystems, Framingham, CA) equipped with a PCI 1000 (Ciphergen)]. Peak detection for the low-resolution instrument was done using CiphergenExpress software

version 2.1 (Ciphergen). All of the spectra were compiled and normalized to the total ion currents, and the baselines were subtracted. Peaks between 3,000 and 30,000 *m/z* were autodetected using a signal-to-noise ratio of >3, and the peaks were clustered using second-pass peak selection with a signal-to-noise ratio of >2 and 0.3% mass windows. The permissible range of *m/z* drift between samples was set at 0.3% (21).

The high-resolution instrument was set to measure the range between 2,000 and 40,000 *m/z*. The laser intensity, laser frequency, and accumulation time were set to 60%, 25 Hz, and 90 seconds, respectively. The mass data obtained using the high-resolution instrument were converted to text files consisting of *m/z* and intensity after mass calibration by Analyst QS (Applied Biosystems) and were processed using newly developed in-house peak detection, normalization, and quantification software (22).

The peak data were visualized using Mass Navigator software (Mitsui Knowledge Industry, Tokyo, Japan). Mass accuracy was calibrated externally on the day of the measurements using an all-in-one-peptide molecular mass standard (Ciphergen).

Statistical analysis. Statistically significant differences were detected using the Fisher exact probability test, the Student's *t* test, and the Mann-Whitney *U* test. Receiver operator characteristics (ROC) curves were generated and the area under the curve (AUC) values were calculated using StatFlex software version 5.0 (Artech, Osaka, Japan; ref. 23).

We compiled the multivariate intensity data of the mass peaks into the distance from a support vector machine (SVM) hyperplane using the following formula (details in Supplementary Data; ref. 24):

$$dis(x_i) = \sum_{j=1}^{N} \lambda_j y_j \{k(x_j, x_i) + \alpha\}$$

where y_i is label (1 or -1), $k(x_j,x_i)$ is Gaussian kernel function, and λ_i is a value that maximizes [1] target function under [2] constrained conditions, where $L = \sum_{i=1}^{N} \lambda_i - \frac{1}{2} \sum_{i=1}^{N} \sum_{j=1}^{N} \lambda_i \lambda_j y_i y_j K(x_i, x_j)$ is the [1] target function, $0 \le \lambda_i \le C \sum_{i=1}^{N} \lambda_i y_i = 0$ are the [2] constrained conditions, and α and C are constants 0.25 and 10, respectively.

Immunoradiometric assay of CA19-9. Plasma (100 μL) was analyzed using a commercially available immunoradiometric assay kit (Fujirebio Diag-nostic, Inc., Malvern, PA) according to the manufacturer's recommendations.

Results

Comparison between low-resolution and high-resolution instruments. The reproducibility of data obtained using the low-resolution TOF-MS instrument of the ProteinChip system has been a concern. We compared the number of detectable peaks and the reproducibility of data obtained using low-resolution TOF-MS and high-resolution QqTOF-MS instruments. From unfractionated plasma samples (24 pancreatic cancer patients and 24 healthy controls), a total of 226 unique peaks were detected using the low-resolution instrument and 637 unique peaks were detected using the high-resolution instrument (Table 2). This difference seems to be attributable to the mass resolutions of the instruments (Fig. 1*A*). In addition, we noticed significant mass drifts (<0.3%) in the data obtained with the low-resolution instrument (Fig. 1*B*). In contrast, the mass deviation was <0.05% for the high-resolution instrument (Fig. 1*B*). As a result, the correlation coefficients for three independent measurements of a pooled plasma sample done every other day with the high-resolution instrument reached 0.97 to 0.99 (data not shown).

Chromatographic fractionation reduced the reproducibility of measurements. Fractionation via stepwise anion-exchange chromatography has been widely done to increase the number of detectable peaks obtained with low-resolution instruments. Actually, the total number of detectable peaks increased from 226 to 872 with fractionation of the same plasma samples (Table 2). However, the fractionation procedure seemed to compromise the reproducibility of the measurements. Forty-eight plasma samples (24 pancreatic cancer patients and 24 healthy controls) were analyzed in duplicate, and the mean correlation coefficient of all the peaks calculated between the duplicates was 0.87 to 0.96 for the unfractionated samples and 0.61 to 0.76 for the fractionated samples (Table 2). Fig. 2*A* (unfractionated) and Fig. 2*B* (fractionated) show the results of duplicate assays of a representative plasma sample.

Based on these quality-control experiments, we decided to measure unfractionated plasma samples using the high-resolution QqTOF-MS instrument. More than 90% of the duplicate

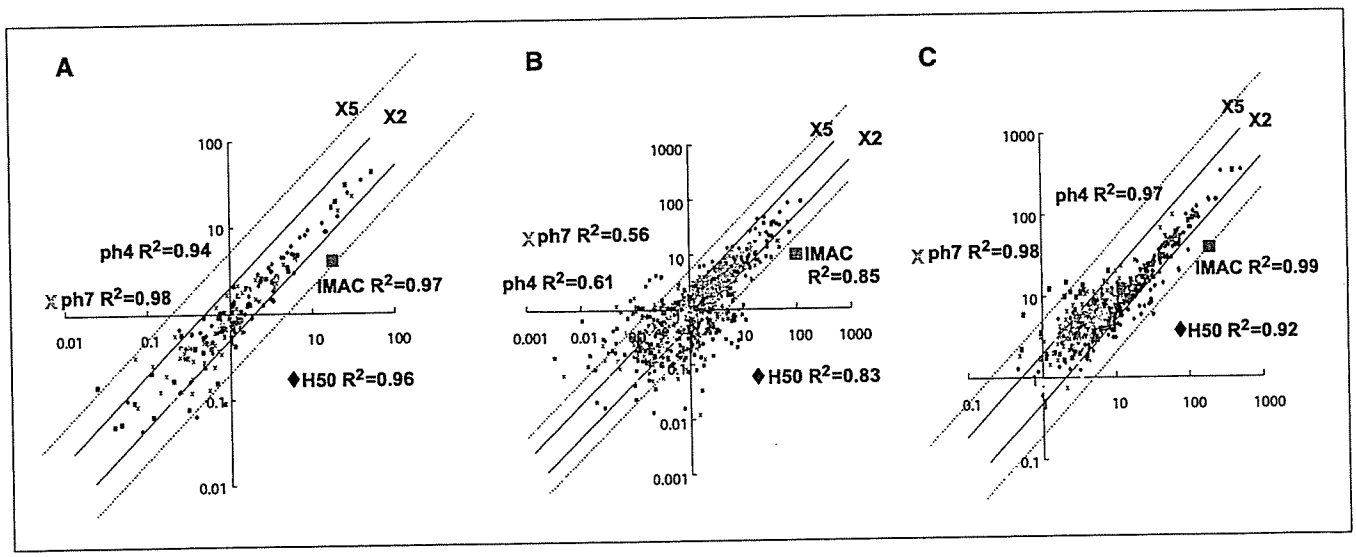

Figure 2. Reproducibility of data from the low-resolution and high-resolution instruments. Two-dimensional plot analyses of the mass intensities corresponding to the duplicated peaks that appeared in the H50 (*blue diamonds*), IMAC-Cu^{2+} (*red squares*), CM10 pH 4 (*yellow triangles*), and CM10 pH 7 (*light blue crosses*) arrays. Unfractionated (*A* and *C*) or fractionated (*B*) samples of the same plasma were measured using a low-resolution TOF instrument (*A* and *B*) and a high-resolution QqTOF instrument (*C*).

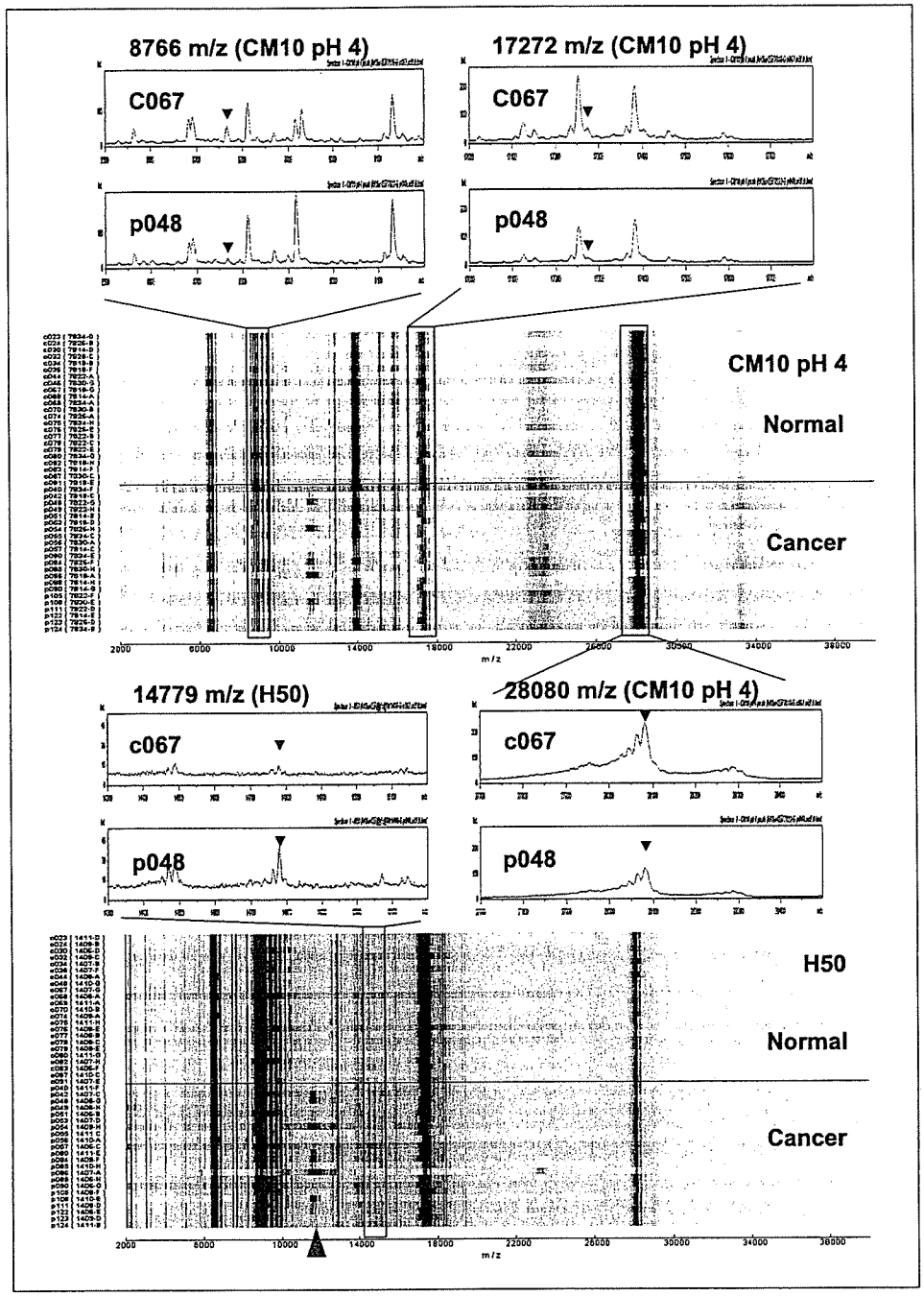

Figure 3. Representative mass spectra [a healthy control (*c067*) and a pancreatic cancer patient (*p048*)] and converted gel-like images [23 healthy controls (*c023-c091*) and 22 pancreatic cancer patients (*p040-p124*)] showing the peaks at 8,766, 17,272, 28,080 (CM10 pH 4), and 14,779 (H50) *m/z*. *Red arrowhead*, peak at 11,516 *m/z*, which was extracted using the Akaike information criterion (25).

protein peaks measured with the QqTOF-MS instrument were plotted within a 2-fold difference (Fig. 2*C*), and the mean correlation coefficient between duplicate assays was at least 0.95 (Table 2).

Identification of a candidate classifier in the training cohort by machine learning. From the total of 220 samples obtained at the NCCH, we selected 71 pancreatic cancer patients and 71 healthy controls with no statistically significant differences in age or sex distribution as a training cohort (Table 1). The remaining 78 cases served as a validation cohort. The clinicopathologic characteristics of these pancreatic cancer patients in the training and validation cohorts are summarized in Table 1.

The acquired MS peak information was stored in a large-capacity server computer, and the data set that most accurately discriminated pancreatic cancer patients from healthy controls was extracted using a rbf SVM learning algorithm (24). The set, or classifier, was composed of four protein peaks at 17,272 *m/z* (CM10 pH 4), 8,766 *m/z* (CM10 pH 4), 28,080 *m/z* (CM10 pH 4), and 14,779 *m/z* (H50). The selection of these four peaks was evaluated by leave-one-out (LOO) cross-validation. Representative spectra profiles and pseudo-gel images of the four peaks are shown in Fig. 3. Akaike information criterion procedure (25) selected another peak at 11,516 *m/z* (H50; indicated by a *red arrowhead* in Fig. 3). Although the 11,516 *m/z* peak was only

Table 3. Intensities of the 17,272, 8,766, 14,779, and 28,080 *m/z* peaks

Peaks (arrays)	Training cohort (n = 142)			Validation cohort (n = 78)		
	Cancer (n = 71)	Healthy (n = 71)	P*	Cancer (n = 33)	Healthy (n = 45)	P*
17,272 *m/z* (CM10 pH 4)	9.49 ± 2.88†	14.6 ± 2.29†	0.0000	9.74 ± 4.22†	14.5 ± 2.29†	0.0000
8,766 *m/z* (CM10 pH 4)	7.65 ± 3.53†	12.1 ± 5.55†	0.0000	7.04 ± 4.39†	13.4 ± 5.81†	0.0000
14,779 *m/z* (H50)	11.8 ± 4.43†	7.85 ± 3.68†	0.0000	10.4 ± 3.85†	6.46 ± 1.63†	0.00000
28,080 *m/z* (CM10 pH 4)	113 ± 36.7†	132 ± 33.5†	0.0022	92.4 ± 24.3†	110 ± 21.6†	0.0078

*Mann-Whitney U test.
†Mean ± SD intensities in arbitrary units.

detected in 1 of the 71 (1.4%) healthy controls, it was not included in the above discriminating data set generated by machine learning because of its low-positive rate in pancreatic cancer patients [19.7% (14 of 71)].

Statistical differences in all four peaks were recognized between the pancreatic cancer patients and the healthy controls (Mann-Whitney U test, $P < 0.0022$; Table 3). The ROC and AUC values of each peak and their combination in the 142 cases of the training cohort are shown in Fig. 4.

The intensity data of the four peaks obtained in each individual were compiled into a single value, the distance from a fixed SVM hyperplane, using the formula described in Materials and Methods and Supplementary Data. When the distance was positive, the individual was classified as having pancreatic cancer and vice versa. This classifier correctly diagnosed 97.2% (69 of 71) of the cancer patients and 94.4% (67 of 71) of the healthy controls in the training cohort (Fig. 5*A*).

Confirmation of the classifier in the first validation cohort. We next validated the discriminating performance of the classifier in a blinded manner using an independent cohort consisting of 78 individuals (NCCH) who had not been included in the training cohort (Table 1). Again, statistically significant differences in the mean intensities of every peak were observed between the 33 pancreatic cancer patients and the 45 healthy controls (Mann-Whitney U test, $P < 0.0078$; Table 3).

The SVM hyperplane determined in the training cohort was applied to the diagnosis of the 78 cases in the validation set. The same SVM hyperplane separated 90.9% (30 of 33) of the pancreatic cancer patients into the positive direction group and 91.1% (41 of 45) of the healthy controls into the negative direction group (Fig. 5*B*). The overall accuracy of the classification was 91.0% (71 of 78) in the validation cohort.

Combination of the surface-enhanced laser desorption/ionization classifier and CA19-9. Overall, the classifier was able to detect 95.2% (99 of 104) of the pancreatic cancer patients in the training and validation cohorts (Table 4). Although the number of cases was small, 83.3% (10 of 12) of stage I and II cases were detected (training and first validation cohorts). No statistically significant differences in detection rates were seen among cases with different tumor locations or different clinical stages (Table 4). To improve the detection rate, we measured plasma CA19-9 levels in all individuals whose residual samples were sufficient (29 pancreatic cancer patients and 39 healthy controls; Table 5). The sensitivity of CA19-9 (cutoff value of 37 units/mL) was 86.2% (25 of 29) and specificity was 94.9% (37 of 39). The SELDI classifier and the CA19-9 level were complementary. Combining CA19-9 and the SELDI classifier detected 100% (29 of 29) of cancer patients, but this combination yielded six false-positive cases [15.4% (6 of 39); Table 5].

Confirmation of the classifier in a second validation cohort obtained at a different institution. Finally, we did a second confirmatory experiment using samples collected prospectively at another institution. In total, 25 plasma samples from pancreatic cancer patients, individuals with other pancreatic diseases, and healthy volunteers were obtained from TMUH and analyzed in a blinded manner. Although the discovery of biomarkers useful for the differential diagnosis of pancreatic diseases was not the primary goal of this study, the classifier was able to discriminate pancreatic cancer patients and individuals with pancreatic tumors/cysts from healthy controls and pancreatitis patients (Table 4; Fig. 6). Four of the six patients with pathologically unproven pancreatic tumors/cysts were classified into the positive direction group. A close follow-up of these patients has been undertaken, because they may have premalignant or preclinical conditions. The SELDI classifier correctly identified 88.9% (8 of 9)

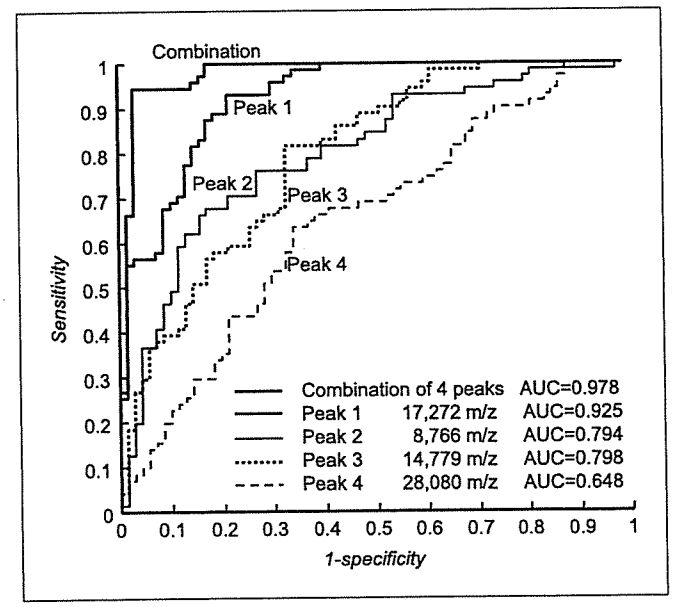

Figure 4. ROC curves and AUC values showing the discriminating capacities of the 17,272, 8,766, 28,080 (CM10 pH 4), and 14,779 (H50) *m/z* peaks individually and in combination.