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A Phase I Study of Combination Chemotherapy with Gemcitabine and Oral S-1 for Advanced Pancreatic Cancer

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Key Words
Pancreatic cancer · 5-Fluorouracil · Gemcitabine · S-1

Abstract
Objective: The aim of this study was to determine the maximum-tolerated dose and dose-limiting toxicity (DLT) of combination therapy with gemcitabine and S-1 in patients with advanced pancreatic cancer. ***Methods:*** Chemotherapy-naive patients with histologically or cytologically proven unresectable or metastatic pancreatic cancer were enrolled. The patients received gemcitabine intravenously over 30 min on days 1 and 8 and S-1 orally twice daily from days 1 to 14. Cycles were repeated every 21 days until disease progression. Patients were scheduled to receive gemcitabine (mg/m^2/week) and S-1 (mg/m^2/day) at four dose levels: 800/60 (level 1), 1,000/60 (level 2), 1,000/70 (level 3) and 1,000/80 (level 4). ***Results:*** Eighteen patients were enrolled in this study. The maximum-tolerated dose was not reached even at the highest dose level (level 4) because only 2 of the 6 patients at this level experienced DLT. The DLTs were neutropenia and rash. Six (33%) of the 18 patients achieved a partial response and median overall survival time was 7.6 months. ***Conclusions:*** Combination chemotherapy with gemcitabine and S-1 was well tolerated and showed good antitumor activity in the treatment of pancreatic cancer. We recommend a gemcitabine dose of 1,000 mg/m^2/week and an S-1 dose of 80 mg/m^2/day in further studies with this schedule.

Introduction

Pancreatic cancer is a fatal disease, with a 5-year survival rate of less than 5% [1]. Surgery remains the only curative option for patients with this disease, but the vast majority of patients unfortunately present with advanced, unresectable tumors. Effective non-surgical treatment is therefore needed to improve the outcome in patients with pancreatic cancer.

A randomized controlled study demonstrated that gemcitabine, a nucleoside analogue, is effective in palliating symptoms and prolonging survival in patients with advanced pancreatic cancer: gemcitabine showed a statistically significant advantage both in clinical benefit response (23.8 vs. 4.8%, p = 0.0022) and in median survival (5.65 vs. 4.41 months, p = 0.0025) compared with weekly bolus 5-fluorouracil (5-FU) [2]. Single-agent gemcitabine is currently accepted worldwide as first-line therapy for advanced pancreatic cancer. Nevertheless, there is substantial room for improvement in chemotherapy for pancreatic cancer, because single-agent gemcitabine pro-

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vides only limited benefit, with objective response rates of less than 15% and a median survival of less than 6 months [2–5].

S-1 is an oral fluoropyrimidine derivative that combines tegafur with two modulators of 5-FU metabolism, 5-chloro-2,4-dihydroxypyridine and potassium oxonate [6]. 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 tumor tissues [7]. Potassium oxonate, 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 [8]. The efficacy of S-1 has already been demonstrated in a variety of solid tumors: the response rates for advanced gastric cancer, colorectal cancer and non-small cell lung cancer in the phase II studies conducted in Japan were 49, 35 and 22%, respectively [9–11]. Recently, the clinical efficacy of S-1 against pancreatic cancer has also been investigated. We conducted an early phase II study of S-1 for metastatic pancreatic cancer and reported that 4 (21.1%) of 19 patients achieved a partial response, with mild toxicity [12]. Hayashi et al. [13] performed a pilot study of single-agent S-1 or S-1 plus cisplatin combination therapy in patients with advanced pancreatic cancer and reported that 3 (20.0%) of the 15 patients or 8 (57.1%) of the 14 patients showed a partial response.

Since S-1 shows a favorable toxicity profile and activity in various solid tumors, including pancreatic cancer, we decided to investigate whether combination therapy with gemcitabine and S-1 is an effective chemotherapeutic regimen for pancreatic cancer. Although many clinical studies of gemcitabine in combination with fluoropyrimidines such as 5-FU, uracil/tegafur and capecitabine have been reported [14–22], little information is available on the combination of gemcitabine and S-1. Thus, we conducted a phase I study to determine the maximum-tolerated dose (MTD) and dose-limiting toxicity (DLT) of gemcitabine and S-1 combination therapy in patients with unresectable or metastatic pancreatic cancer.

Patients and Methods

Patient Selection

Patients were considered eligible if they met the following criteria: histologically or cytologically proven pancreatic adenocarcinoma, unresectable locally advanced or metastatic disease, naive to chemotherapy, Eastern Cooperative Oncology Group performance status of 0–2, age between 20 and 74 years, life expectancy of ≥8 weeks, and adequate organ function defined as white blood cell count ≥4,000/mm^3, neutrophil count ≥2,000/mm^3, platelet count ≥100,000/mm^3, hemoglobin ≥9.0 g/dl, serum creatinine ≤the upper limit of normal, serum albumin ≥3.0 g/dl, total bilirubin ≤2.0 mg/dl, and aspartate aminotansferase and alanine aminotransferase levels ≤2.5 times the upper limit of normal or ≤5 times the upper limit of normal if liver metastases or biliary drainage were present. The exclusion criteria were severe complications, such as infection, heart disease and renal disease (in this study we did not define in detail the exclusion criteria in relation to severe complications), metastasis to the central nervous system, marked pleural effusion or ascites, and watery diarrhea. Pregnant or lactating women were also excluded. Written informed consent was obtained from all patients. This study was approved by the institutional review board at the National Cancer Center and conducted in accordance with the Declaration of Helsinki.

Treatment Plan

This was an open-label, two-center, single-arm phase I study. Gemcitabine (Eli Lilly Japan K.K., Kobe, Japan) was administered as a 30-min intravenous infusion weekly for 2 weeks followed by a 1-week rest. S-1 (Taiho Pharmaceutical Co., Ltd., Tokyo, Japan) was administered orally twice daily from day 1 to day 14 followed by a 1-week rest. The treatment cycles were repeated every 3 weeks until disease progression or unacceptable toxicity occurred. If patients experienced leucopenia <2,000/mm^3, neutropenia <1,000/mm^3, thrombocytopenia <70,000/mm^3, total bilirubin >2.0 mg/dl or aspartate aminotansferase and alanine aminotransferase levels >5 times the upper limit of normal, both gemcitabine and S-1 were withheld until recovery. If patients experienced DLT, the dose of gemcitabine was reduced by 200 mg/m^2/week and the dose of S-1 was reduced by 10 mg/m^2/day in the subsequent cycle. If a rest period of more than 3 weeks was required because of toxicity, the patient was withdrawn from the study.

Patients were scheduled to receive gemcitabine and S-1 at four dose levels (table 1). At the first dose level (level 1), gemcitabine was administered at a dose of 800 mg/m^2/week and S-1 was administered at 60 mg/m^2/day. At the next dose level (level 2), gemcitabine was increased to 1,000 mg/m^2/week with S-1 kept at the same dose. At each of dose levels 3 and 4, S-1 was increased by 10 mg/m^2/day with gemcitabine kept at 1,000 mg/m^2/week. At least 3 patients were enrolled at each dose level. If DLT was observed in the initial 3 patients, a maximum of 3 additional patients was entered into the same dose level. The MTD was defined as the highest dose level that did not cause DLT in 3 of the 3 or ≥3 of the 6 patients treated at that level during the first two cycles of treatment. DLT was defined as grade 4 leucopenia or neutropenia, febrile neutropenia, grade 4 thrombocytopenia, grade 3 thrombocytopenia requiring transfusion, ≥grade 3 non-hematological toxicity excluding nausea, vomiting, anorexia and fatigue, or any toxicity that necessitated a treatment delay of more than 3 weeks. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 2.0.

Patient Evaluation

Physical examinations, complete blood cell counts, biochemistry tests and urinalyses were performed at least once weekly. Tumor assessment with computed tomographic scan or magnetic resonance imaging and measuring of tumor marker CA 19-9 was performed every two cycles, and tumor response was evaluated by the

Table 1. Dose escalation scheme and DLT

Dose level	Gemcitabine mg/m²/week	S-1 mg/m²/day	Patients	DLT events	DLT
1	800	60	3	0	
2	1,000	60	3	0	
3	1,000	70	6	1	grade 4 neutropenia
4	1,000	80	6	2	grade 4 neutropenia grade 3 rash and grade 4 neutropenia

criteria of the Japan Society for Cancer Therapy [23], which are similar to those of the World Health Organization. Briefly, a complete response was defined as the disappearance of all clinical evidence of the tumor for a minimum of 4 weeks. A partial response was defined as a 50% or greater reduction in the sum of the products of two perpendicular diameters of all measurable lesions for 4 weeks or longer without any evidence of new lesions. No change 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 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 deterioration in clinical status that was consistent with disease progression. The response duration was calculated from the day of the first sign of a response until disease progression; progression-free survival was calculated from the date of the initiation of treatment until documented disease progression or death due to any cause (whichever occurred first); overall survival time was calculated from the date of treatment initiation to the date of death or the last follow-up. The median probabilities of the progression-free or overall survival periods were estimated by the Kaplan-Meier method.

Results

Patient Characteristics

Between September 2003 and July 2004, 18 patients were enrolled in this study. All of them received at least two cycles of chemotherapy and were evaluable for toxicity and response. Patient characteristics are listed in table 2. All patients had good performance status (0 and 1). Two patients had locally advanced unresectable disease and the remaining 16 had metastatic disease. Before the start of the study, 1 patient had received surgical resection and 3 had undergone biliary drainage for obstructive jaundice. Twelve patients had abdominal and/or back pain at study entry. A total of 125 cycles of chemotherapy was administered, with a median of 6 treatment cycles per patient (range 2–22). It was possible to treat all patients as outpatients after one or two cycles of observation in hospital.

Table 2. Patient characteristics

Characteristics		Patients
Patients enrolled		18
Sex		
Male		13
Female		5
Age, years		
Median	61	
Range	43–72	
ECOG performance status		
0		10
1		8
Body surface area, m²		
Median	1.58	
Range	1.46–1.97	
Disease stage		
Locally advanced		2
Metastatic		16
Sites of metastatic disease		
Liver		13
Lung		2
Distant lymph nodes		5
Pleura		1

ECOG = Eastern Cooperative Oncology Group.

DLT and Recommended Dose

No DLT was observed at dose levels 1 or 2 (table 1). At dose level 3, 1 patient developed grade 4 neutropenia, which was considered DLT, but the remaining 5 did not develop DLT. At dose level 4, the highest dose level, 2 of the 6 patients exhibited DLTs: 1 had grade 4 neutropenia and the other had grade 3 rash concomitant with grade 4 neutropenia. All DLTs occurred in the first cycle of treatment. The MTD was not reached because only 2 of the 6 patients experienced DLT at dose level 4. Therefore, dose level 4 (gemcitabine dose of 1,000 mg/m²/week and S-1

Table 3. Toxicities across first two cycles by dose level (patient number)

Toxicity	Dose level 1 (n = 3)				Dose level 2 (n = 3)				Dose level 3 (n = 6)				Dose level 4 (n = 6)			
Grade:	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Leucopenia	1	2	0	0	0	2	1	0	1	4	1	0	1	2	3	0
Neutropenia	1	1	0	0	0	1	2	0	0	5	0	1	0	3	1	2
Anemia	2	0	0	0	3	0	0	0	4	1	0	0	4	2	0	0
Thrombocytopenia	2	0	0	0	1	2	0	0	4	1	0	0	3	0	0	0
Nausea	2	0	0	0	1	0	1	0	2	2	0	0	2	0	0	0
Vomiting	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
Anorexia	1	0	0	0	0	0	1	0	2	1	0	0	2	1	0	0
Diarrhea	1	0	0	0	1	0	0	0	0	0	0	0	2	0	0	0
Stomatitis	2	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
Rash	0	1	0	0	1	2	0	0	2	0	0	0	3	1	1	0
ALT elevation	1	0	0	0	2	0	0	0	3	2	0	0	1	0	0	0
Creatinine elevation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fever	0	0	0	0	0	1	0	0	0	0	0	0	3	0	0	0
Fatigue	1	0	0	0	1	0	0	0	1	1	0	0	2	1	0	0

Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 2.0. ALT = Alanine aminotransferase.

dose of 80 mg/m^2/day) was considered the recommended dose in further studies with this schedule.

Toxicity

All 18 patients were assessable for toxicity. The major toxicities observed during the first two cycles are summarized in table 3. Hematological toxicity, particularly neutropenia, was the most pronounced toxicity of gemcitabine and S-1 with this schedule of administration. Although 3 patients experienced grade 4 neutropenia during the first two cycles of treatment, all of them recovered quickly without any severe complications. The neutrophil nadir typically occurred on day 15, and neutrophil counts recovered to baseline values by day 22. The non-hematological toxicities commonly observed with our regimen were gastrointestinal toxicities, such as nausea (≥grade 1; 55.6%) and anorexia (≥grade 1; 44.4%), although most of them were mild and transient. Although 1 patient at dose level 2 experienced grade 3 anorexia and grade 3 nausea in the first cycle, he recovered from the toxicities with the use of antiemetic agents and could continue treatment without reducing the doses of gemcitabine and S-1. Skin rash was also frequently seen in the current study (≥grade 1; 61.1%). The rash typically appeared on the arms and legs and spread to the trunk within 10 days of the initiation of chemotherapy. Most rashes were mild and resolved promptly with appropriate medical treatment such as antihistamines and steroids, although 1 patient at dose level 4 exhibited grade 3 rash that required temporary treatment discontinuation and dose reduction in the next cycle. Although 125 cycles of chemotherapy have been administered, there was no indication of cumulative toxicity.

Table 4. Objective tumor response

Dose level	Patients	Response				Response rate, %
		CR	PR	NC	PD	
1	3	0	2	1	0	66.7
2	3	0	0	1	2	0
3	6	0	3	3	0	50
4	6	0	1	4	1	16.7
Total	18	0	6	9	3	33.3

CR = Complete response; PR = partial response; NC = no change; PD = progressive disease.

Efficacy

The objective tumor responses at each dose level are shown in table 4. A partial response was seen even at the lowest dose level, and across all dose levels, 6 of the 18 patients achieved a partial response, resulting in an over-

all response rate of 33.3 (95% confidence interval, 13.3–59.0%). No change was noted in 9 patients (50%) and progressive disease in 3 patients (16.7%). The mean response duration was 4.8 months (range 2.8–15.9). The serum CA 19-9 level was reduced to less than half from baseline values in 8 (61.5%) of the 13 patients who had a pretreatment level greater than the upper limit of normal (37 U/ml). At the time of analysis, 9 patients had died because of disease progression. The median progression-free and the median overall survival times were 5.0 and 7.6 months, respectively.

Discussion

To improve the prognosis of patients with advanced pancreatic cancer, gemcitabine-based combination chemotherapy has been actively investigated, although many phase III trials have failed to demonstrate any survival benefit of combination chemotherapy in comparison with gemcitabine as a single agent. 5-FU has been selected as a candidate to be investigated in combination with gemcitabine in patients with pancreatic cancer because of its favorable toxicity profile and modest but substantial activity in this disease. Gemcitabine is considered to enhance the effect of the 5-FU metabolite 5-FdUMP by reducing the concentration of its physiological competitor via inhibition of ribonucleotide reductase [24]. Preclinical studies have demonstrated synergy between gemcitabine and 5-FU in tumor cell lines, including pancreatic cancer cells [25, 26]. Clinical studies have reported activity of gemcitabine in pancreatic cancer patients with refractoriness to 5-FU [27], suggesting the lack of cross-resistance between the two agents. Several phase I and II studies of combination therapy with gemcitabine and 5-FU for advanced pancreatic cancer have demonstrated relatively good response rates of around 20% with acceptable toxicity profiles [14–18]. A phase III study comparing gemcitabine alone with gemcitabine plus weekly bolus 5-FU showed that median progression-free survival was significantly longer in the combination arm compared with gemcitabine alone (3.4 vs. 2.2 months, $p = 0.022$); however, median overall survival was not significantly prolonged (6.7 vs. 5.4 months, $p = 0.09$) [5].

The novel oral anticancer agent S-1 was developed to improve the tumor-selective toxicity of 5-FU and has shown efficacy in a variety of solid tumors, including pancreatic cancer [9–13]. With the aim of developing a more effective chemotherapeutic regimen for pancreatic cancer, we decided to conduct a clinical study of combination therapy with gemcitabine and S-1. Since this combination has not previously been investigated, a phase I study was carried out to determine MTD and DLT.

In the present study, MTD was not reached because only 2 of the 6 patients experienced DLT at the highest dose, level 4. Although the 6 patients at level 4 have received a total of 34 cycles of treatment (average 5.7, range 2–12), there was no indication of cumulative toxicity. Therefore, dose level 4 (gemcitabine 1,000 mg/m^2/week, S-1 80 mg/m^2/day) was considered the recommended dose in further studies of this combination regimen. Because 2 of the 6 patients experienced DLT at this level, it goes without saying that more large-scale studies will be necessary to confirm the safety of our recommended dose. The overall toxicity of this regimen was mild, and neither unexpected nor life-threatening toxicities were observed during the study, indicating that S-1, like other fluoropyrimidines, can be safely combined with gemcitabine.

Neutropenia was the major DLT of this combination regimen: 1 of the 6 patients at dose level 3, and 2 of the 6 patients at dose level 4, experienced grade 4 neutropenia. Neutropenia as the DLT was to be expected because myelosuppression, especially neutropenia, is one of the most common toxicities of each individual drug. The neutrophil nadir typically occurred on day 15, but in most cases, the neutrophil count spontaneously recovered to baseline values within a week. Furthermore, no febrile neutropenia was observed during any of the 125 cycles of treatment, suggesting that the myelosuppression caused by this combination regimen is manageable on an outpatient basis.

The non-hematological toxicities commonly observed with our regimen were gastrointestinal toxicities such as nausea and anorexia. Although 1 patient at dose level 2 experienced transient grade 3 nausea and grade 3 anorexia, no DLTs associated with gastrointestinal toxicities were observed. Diarrhea was also mild and rare in the current study, similar to previous reports from Japanese studies of single-agent S-1; however, relatively severe diarrhea induced by S-1 has been reported in studies from Europe and the United States [28–30]. For example, Hoff et al. [28] reported that severe diarrhea occurred in all of the 3 patients who received S-1 at a dose of 40 mg/m^2 b.i.d. It is not clear why the toxicity profile and MTD of S-1 in Western studies differ from those in studies with Japanese populations, although a pharmacokinetic study suggested that the conversion of tegafur to 5-FU may occur more slowly in Japanese patients than in patients from other ethnic groups [31]. In any event, it may be dangerous to apply the results of our study directly to

treatment of Western patients, particularly from the viewpoint of gastrointestinal toxicity.

In the present study, 11 (61.1%) of the 18 patients experienced grade 1 or greater rash. This toxicity was mild and manageable, although 1 patient at dose level 4 developed grade 3 rash, requiring temporary treatment discontinuation. The reason for the enhanced cutaneous toxicity during combination therapy with gemcitabine and S-1 is unknown, although cutaneous toxicity has already been reported in patients receiving gemcitabine and 5-FU combination regimens. Hidalgo et al. [14] reported grade 1 or greater cutaneous toxicity in 11 (42.3%) of the 26 patients in a phase I–II study with gemcitabine and 5-FU. One of these patients developed a severe cutaneous reaction, manifested as generalized exfoliative dermatitis, after the first cycle of chemotherapy.

Combination therapy with gemcitabine and S-1 was associated with promising activity in advanced pancreatic cancer. Six (33.3%) of the 18 patients achieved an objective response. Of the 13 patients who had a pretreatment serum CA 19-9 level greater than 37 U/ml, the CA 19-9 level decreased more than 50% in 8 patients (61.5%). In addition, the median progression-free survival time of 5.0 months and the median overall survival time of 7.6 months are encouraging. These efficacy data in this study, which compare favorably with those reported for single-agent gemcitabine, support further studies of this regimen.

In conclusion, our combination regimen of gemcitabine and S-1 was well tolerated up to dose level 4. The major toxicities were myelosuppression, gastrointestinal toxicity and skin rash, although most of these toxicities were mild and reversible. Six of the 18 patients showed a partial response, suggesting a promising antitumor activity of this regimen against pancreatic cancer. A multicenter phase II study of this regimen, 1,000 mg/m^2/week gemcitabine on days 1 and 8 and 80 mg/m^2/day S-1 from days 1 to 14 every 3 weeks, is under way in patients with metastatic pancreatic cancer.

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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 (/mm³) *	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/mm³), 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).

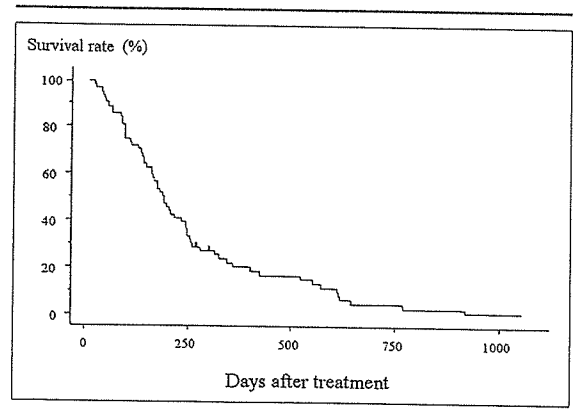

FIGURE 1 Overall survival curve for all patients with BTC receiving chemotherapy. Tick marks indicate censored cases.

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.

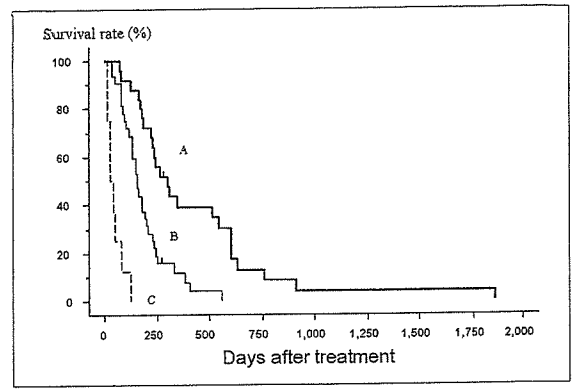

FIGURE 2 Survival curves for three groups classified by a prognostic index based on the findings of multivariate analysis.
Group A, prognostic index less than 1.5 (25 patients); Group B, prognostic index from 1.5 to 2.5 (32 patients); Group C, prognostic index greater than 2.5 (8 patients). Tick marks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

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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.

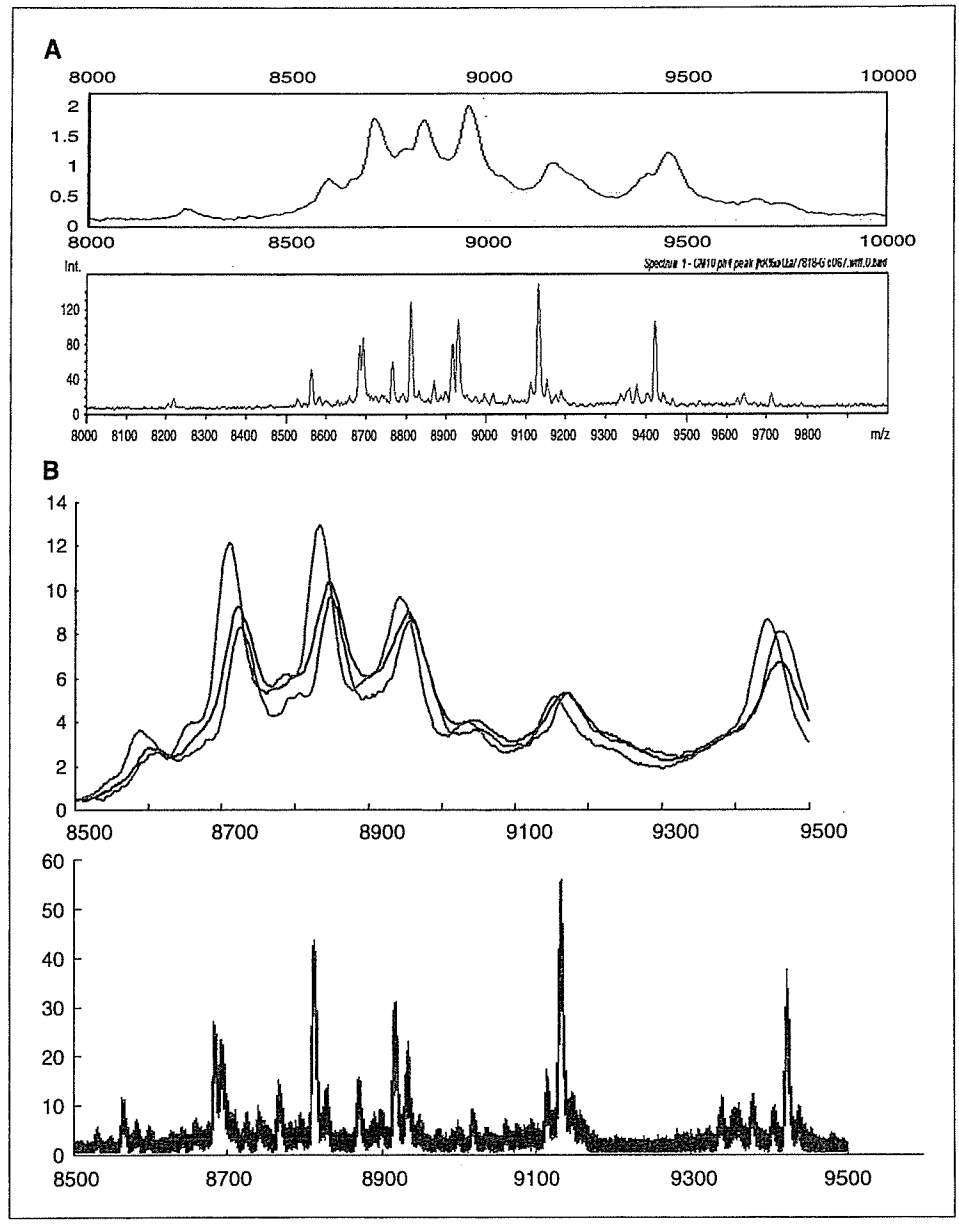

Figure 1. Comparison of low-resolution and high-resolution instruments. *A*, representative spectra of an unfractionated plasma sample in the range of 8,000 to 10,000 *m/z* obtained using a low-resolution TOF instrument (*top*) and a high-resolution QqTOF instrument (*bottom*). *B*, spectra of an unfractionated 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

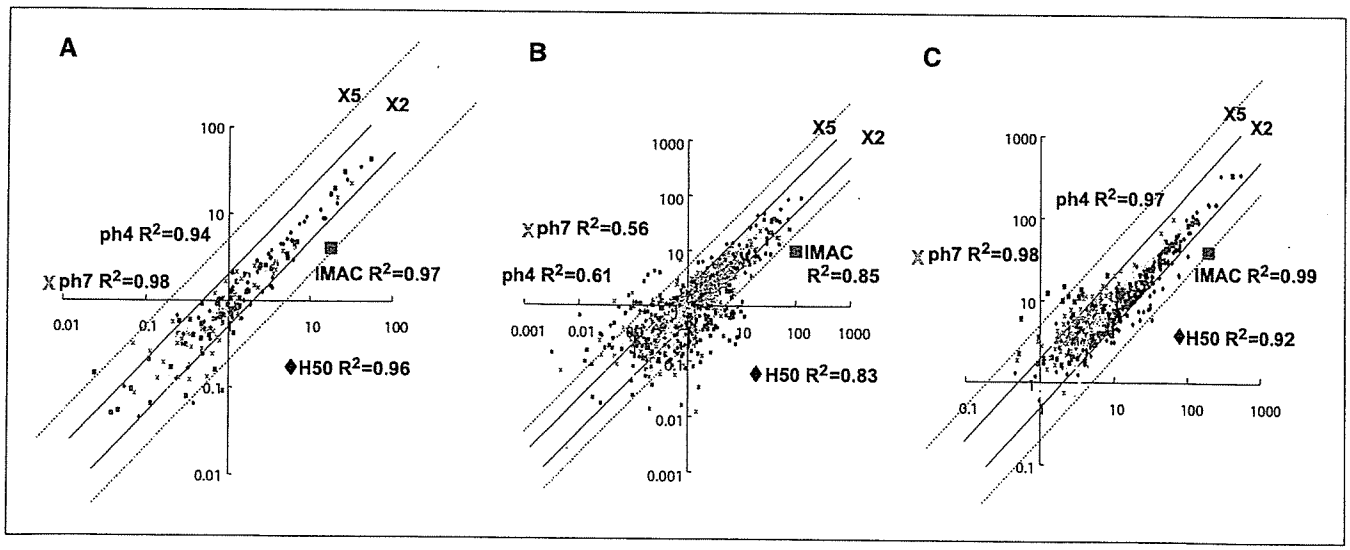

Figure 2. Reproducibility of data from the low-resolution and high-resolution instruments. Two-dimensional plot analyses of the mass intensities corresponding to the duplicated peaks that appeared in the H50 (*blue diamonds*), IMAC-Cu^{2+} (*red squares*), CM10 pH 4 (*yellow triangles*), and CM10 pH 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*).

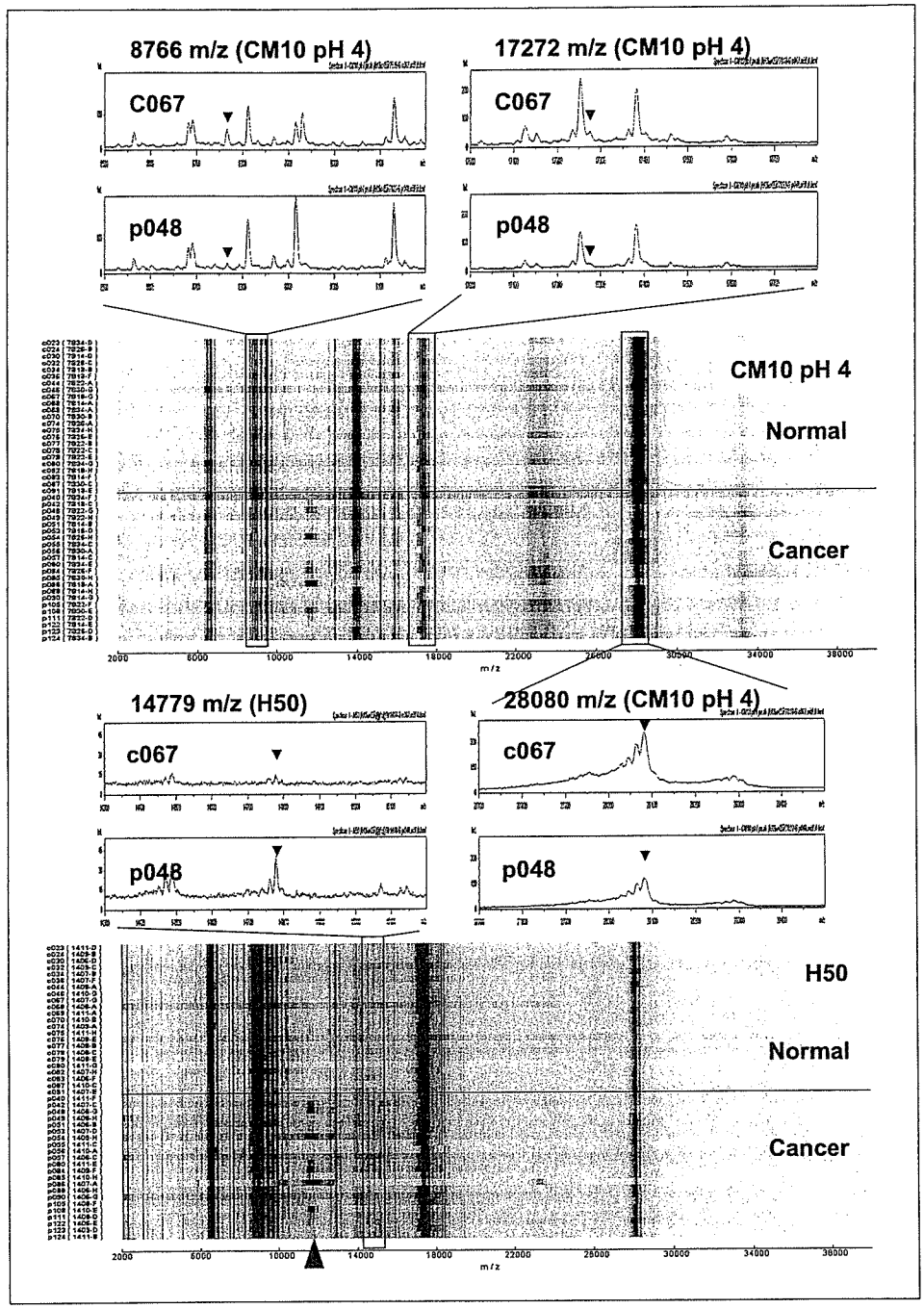

Figure 3. Representative mass spectra [a healthy control (*c067*) and a pancreatic cancer patient (*p048*)] and converted gel-like images [23 healthy controls (*c023-c091*) and 22 pancreatic cancer patients (*p040-p124*)] showing the peaks at 8,766, 17,272, 28,080 (CM10 pH 4), and 14,779 (H50) *m/z*.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

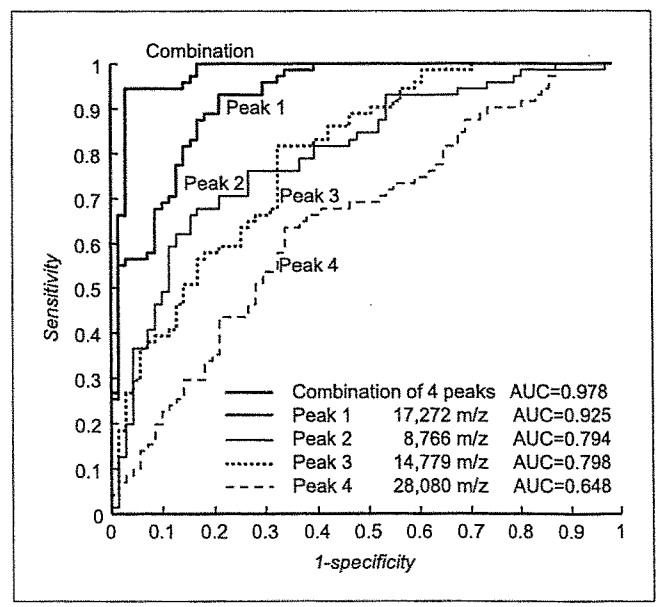

Figure 4. ROC curves and AUC values showing the discriminating capacities of the 17,272, 8,766, 28,080 (CM10 pH 4), and 14,779 (H50) *m/z* peaks individually and in combination.

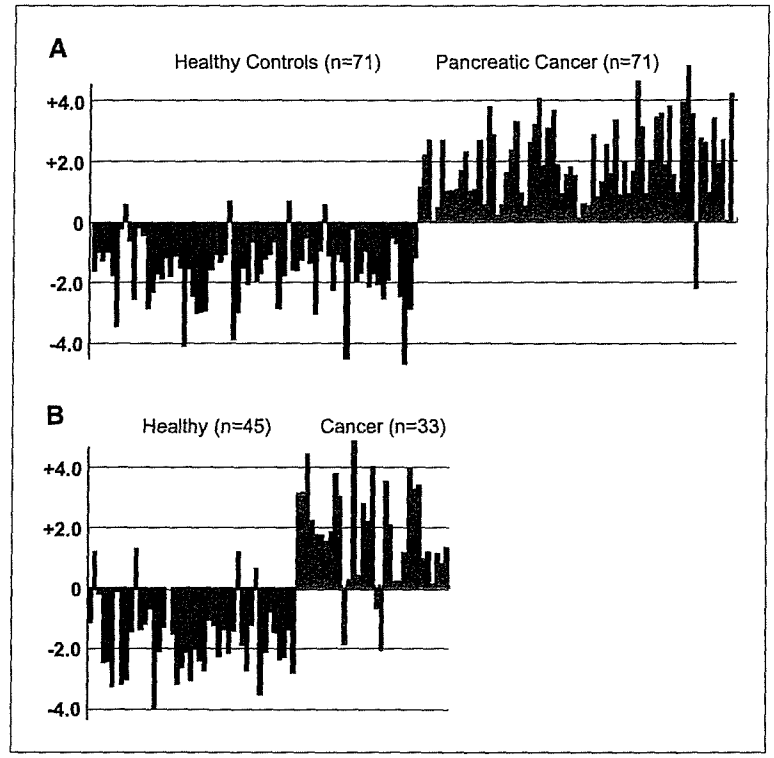

Figure 5. Calculated SVM distances of healthy controls (*black columns*) and pancreatic cancer patients (*gray columns*) in the training (*A*) and first validation (*B*) cohorts. Cases separated into the positive direction from the SVM hyperplane were classified as having "cancer" and those separated into the negative direction were classified as being "healthy."

of the pancreatic cancer patients and 80% (4 of 5) of the healthy controls, whereas the CA19-9 level correctly identified 66.7% (6 of 9) of the pancreatic cancer patients and 100% (5 of 5) of the healthy controls (Fig. 6). Again, in all the pancreatic cancer patients (9 of 9), the SELDI classifier and the CA19-9 level provided complementary results, even in this second validation cohort.

Discussion

Comparative proteomic profiling coupled with a computerized machine learning approach may revolutionize medical practice and cancer diagnosis. We compared the plasma protein profiles of a large number of pancreatic cancer patients and healthy controls with identical age and gender distributions (Table 1) to identify a biomarker for detecting pancreatic cancer patients in a large

Table 4. Diagnostic accuracy of the SELDI classifier

	Training cohort		Validation cohort (NCCH)		Validation cohort (TMUH)	
	No. cases	No. correctly classified samples* (%)	No. cases	No. correctly classified samples* (%)	No. cases	No. correctly classified samples* (%)
Healthy	71	67 (94.4)	45	41 (91.1)	5	4 (80)
Pancreatitis					5	4 (80)
Tumor/cyst†					6	4 (66.6)
Cancer	71	69 (97.2)	33	30 (90.9)	9	8 (88.9)
Cancer location						
Head	34	33 (97.1)	17	14 (82.4)	7	7 (100)
Body or tail	37	36 (97.3)	10	10 (100)	2	1 (50)
Unknown	0	0	6	10 (100)		
Clinical stage						
I	1	0 (0)	1	1 (100)	0	0
II	6	6 (100)	4	3 (75)	0	0
III	10	9 (90)	1	1 (100)	3	3 (100)
IV	54	54 (100)	27	25 (92.6)	6	5 (83.3)

*Number of healthy and chronic pancreatitis cases, considered to be "healthy," and number of pancreatic tumor/cyst and cancer cases given a diagnosis of "cancer."

†Pathologically unproven pancreatic tumor and/or cyst.