

is an orally administered molecular-targeted drug that targets tumor cell proliferation and tumor angiogenesis by inhibiting the serine–threonine kinases Raf-1 and B-Raf and the receptor tyrosine kinase activity of vascular endothelial growth factor receptors 1, 2 and 3 and platelet-derived growth factor receptor  $\beta$ . This drug was reported to confer an overall survival advantage, with manageable toxicity, in comparison with placebo in a Phase III trial, and it has been accepted worldwide as the first-line chemotherapy for advanced HCC (8). But the advantage is modest. There is urgent need to develop more effective regimens.

5-Fluorouracil (5-FU) has been widely used for the treatment of various gastrointestinal malignancies, including advanced HCC (9,10). A high level of efficacy can be expected when the drug is given as a continuous intravenous infusion (11). However, this would necessitate a permanent intravenous access. Uracil/tegafur (UFT) is an orally administered drug which is a mixture of uracil and tegafur at a molar ratio of 4:1. Tegafur is a prodrug of 5-FU that is hydroxylated and converted to 5-FU by hepatic microsomal enzymes, and uracil prevents the degradation of 5-FU by inhibiting the enzyme dihydropyrimidine dehydrogenase, which results in an increased level of 5-FU in the plasma and tumor tissues (12,13). UFT has been reported to be as effective as intravenous 5-FU for the treatment of malignancies (14,15) and to be effective for the treatment of advanced HCC (16,17).

The therapeutic usefulness of doxorubicin in patients with advanced HCC has also been widely explored since the 1970s. A randomized trial in which doxorubicin was compared with supportive care alone for advanced HCC showed a significant survival benefit in the doxorubicin arm. However, treatment with this drug has not been accepted as a standard chemotherapy because of the high rate of fatal complications reported (18). Mitoxantrone, another anthracycline, has shown similar antitumor activity to that of doxorubicin in both human tumor cell lines and animal models of leukemia and has fewer myelotoxic and cardiotoxic effects than doxorubicin (19). Clinical trials of mitoxantrone have also demonstrated moderate activity against HCC, with a low incidence rate of adverse effects (20,21).

Combination chemotherapeutic regimens composed of a fluoropyrimidine and an anthracycline antibiotic have been reported to show moderate efficacy against HCC with tolerable toxicity (22–24), but combined chemotherapy with UFT and mitoxantrone has not yet been examined. We conducted Phase I/II studies to determine the recommended dosage of the combination of UFT with mitoxantrone (UFM regimen) and to clarify the efficacy and safety when administered at the recommended dose in patients with advanced HCC.

## PATIENTS AND METHODS

### ELIGIBILITY CRITERIA

The eligibility criteria for study enrolment were: (i) patients with histologically confirmed HCC, who were (ii) unsuitable

for surgical resection, local ablation therapy or TACE, (iii) were  $\geq 20$  years old, (iv) had an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2, (v) had adequate bone marrow function (white blood cell  $\geq 3000$  cells/mm<sup>3</sup>, absolute neutrophil count  $\geq 1500$  cells/mm<sup>3</sup>, platelet count  $\geq 70\,000$  cells/mm<sup>3</sup> and hemoglobin  $\geq 8.0$  g/dl), renal function [serum creatinine concentration  $\leq$  upper limit of normal (ULN)] and hepatic function [serum albumin level  $\geq 3.0$  mg/dl, total bilirubin level  $\leq 3.0$  mg/dl, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels  $\leq 5.0 \times$  ULN], (vi) had a life expectancy of at least 12 weeks and (vii) provided written informed consent from each patient.

The exclusion criteria were: clinically evident congestive heart failure, serious cardiac arrhythmia, active or symptomatic coronary artery disease or ischemia, clinically serious infection, seizure disorder requiring medication, prior malignancy (any cancer treated curatively was permitted), clinically evident brain or meningeal metastasis, and pregnant/lactating women. This protocol was approved by the Institutional Review Board for clinical investigation of the National Cancer Center, in conformity with the provisions of the Declaration of Helsinki, Good Clinical Practice guidelines, and local laws and regulations.

### STUDY TREATMENT

UFT was administered orally at the dose of 300 mg/m<sup>2</sup> per day in two divided doses for 21 consecutive days, followed by a rest period of 7 days (400 mg/body per day in patients with a body surface area of  $< 1.50$  m<sup>2</sup> and 500 mg/body/day in patients with a body surface area of  $\geq 1.50$  m<sup>2</sup>). Mitoxantrone was given as a 60 min intravenous infusion on day 1. This cycle was repeated every 28 days. Patients continued to receive additional courses of this regimen until a cumulative dose of mitoxantrone of 100 mg/m<sup>2</sup>, evidence of disease progression or the appearance of unacceptable toxicity.

### PHASE I PART

The objectives of the Phase I study were to investigate the frequency of dose-limiting toxicity (DLT) and to determine the recommended dose of mitoxantrone and UFT. The criteria of DLT included: Grade 4 leukopenia or neutropenia, Grade 3 neutropenia accompanied by fever ( $\geq 38^\circ\text{C}$ ) or infection (clinically or biologically confirmed), thrombocytopenia  $< 25\,000/\text{mm}^3$  or necessity of transfusion, Grade 3 or 4 non-hematological toxicity (except nausea/vomiting, anorexia, fatigue and hyperglycemia), AST and ALT  $> 10$  times the ULN, suspension of UFT administration for over 3 successive weeks, or an over 6-week delay in the commencement of the next treatment cycle.

Three possible dosage levels of mitoxantrone (Level 1: 6 mg/m<sup>2</sup>/day, Level 2: 8 mg/m<sup>2</sup>/day and Level 3: 10 mg/m<sup>2</sup>/day) were assigned for the Phase I part (Table 1). The first

**Table 1.** Dose-escalation schedules of mitoxantrone and uracil/tegafur

Dose level	Mitoxantrone (mg/m <sup>2</sup> )	UFT (mg/m <sup>2</sup> )	Number of patients enrolled
1	6	300	3
2	8	300	6
3	10	300	3

UFT, uracil/tegafur.

patient to enter the study was started at Level 1. At least three patients were treated at this level and observed for DLT. Dose escalation was continued until at least one-third of the patients in a given cohort showed DLT. If none of the first three treated patients developed DLT during the first cycle at a specific dose level, the dose escalation was continued. If one of the first three treated patients developed DLT at any dose level, three additional patients were entered at the same dose level; if only one or two of six patients at a given level experienced a DLT, the dose escalation was continued. The maximum tolerated dose (MTD) was defined as the dose level at which one-third or more of the patients experienced a DLT. The recommended dose for the Phase II study was defined as the dose level preceding the attainment of the MTD.

*PHASE II PART*

The primary endpoint of the Phase II part was the objective response rate. The secondary endpoints were the overall survival, progression-free survival and the frequency and severity of adverse events. The Phase II part was begun after determination of the recommended dosage from the Phase I part.

ASSESSMENT OF THE RESPONSE AND TOXICITY

Physical examination including cardiac symptoms, complete blood cell counts, serum chemistries and urinalysis was performed at the baseline and at least once every 2 weeks after the start of the treatment. Dynamic computed tomography or magnetic resonance imaging was undertaken to evaluate the response at 4- to 6-week intervals after the start of treatment. Tumor response was assessed using the Response Evaluation Criteria in Solid Tumors (25). Toxicity was graded according to the National Cancer Institute common toxicity criteria, version 2.0. Progression-free survival was calculated from the first day of treatment to the appearance of evidence of tumor progression, clinical progression or last date of follow-up. The overall survival was calculated from the first day of treatment until death due to any cause or date of last follow-up. Survival data were analyzed using the Kaplan–Meier method.

STATISTICAL ANALYSIS

In the Phase II part, the primary endpoint was the response rate, and data from at least 19 patients were accrued. The threshold response rate was set at 5% and the expected response rate at 15%. If no responses were observed in the 19 patients and the upper limit of the 90% confidence interval (CI) did not exceed the expected rate of 15%, the UFM regimen was judged to have no activity against HCC. If response was confirmed in one or more of the 19 patients, the decision of whether or not to proceed to a further study using the UFM regimen was taken on the basis of other factors, such as the safety and rate of response, overall survival and time to progression in this study.

RESULTS

PATIENTS

From April 2004 to April 2007, 25 patients were registered for the present study: 12 patients completed the Phase I part (Level 1: 3 patients, Level 2: 6 patients and Level 3: 3 patients). Nineteen patients who received the recommended dose (6 patients received this dose during the Phase I part) were analyzed during the Phase II part. Table 2 shows the baseline characteristics of the patients in the Phase I and Phase II parts of the study of the UFM regimen. There were 19 males and 6 females with a median age of 67 years. All the patients had a good ECOG PS score of 0–1. There were 21 (84%) and 4 (16%) patients with the Child–Pugh Stages A and B, respectively. Thirteen (68%) patients had extrahepatic metastasis, and the major sites of metastasis were lymph node [*n* = 7 (28%)] and lung [*n* = 6 (24%)].

TREATMENTS

In the Phase I part, there was no occurrence of DLT at the Level 1 and Level 2 doses, but all of the three patients who received the Level 3 dose experienced DLT; two of these patients developed Grade 4 neutropenia and one patient developed Grade 3 creatinine elevation. The additional three patients at the Level 2 dose did not experience any DLT. Therefore, Level 3 was considered as the MTD and Level 2 (UFT 300 mg/m<sup>2</sup> and mitoxantrone 8 mg/m<sup>2</sup>) as the recommended dose for the Phase II part.

At the recommended dosage level, a total of 69 courses of the UFM regimen were administered with a median of three courses to each patient (range, 1–8 courses). The dose intensity was 98.9% of the planned dosage for mitoxantrone and 97.9% for UFT.

The reasons for treatment discontinuation in the Phase I and Phase II parts were disease progression in 19 patients, liver dysfunction in 1 patient, DLT according to this protocol in 3 patients during the Phase I part and an over 6-week delay in the start of the next course because of the development of leukopenia in 2 patients. After abandoning the UFM

**Table 2.** Profile of hepatocellular carcinoma patients population

	Phase I	Phase II
No. of patients	12	19
Gender		
Male	9	14
Female	3	5
Age (years)		
Median	63	67
Range	56–78	56–77
Performance status		
0	11	7
1	1	12
Viral marker		
Hepatitis C antibody+	7	7
Hepatitis B antigen+	2	5
Previous treatment		
Surgical resection	4	10
Percutaneous ablation therapy	3	3
Transcatheter arterial chemoembolization	5	8
Transcatheter arterial infusion	3	5
Radiation therapy	1	2
None	3	3
Child–Pugh classification		
A	8	17
B	4	2
UICC tumor stage <sup>a</sup>		
III	4	6
IVa	3	1
IVb	5	12
Portal vein tumor thrombosis		
(+)	5	4
Extrahepatic metastasis		
Lymph node	5	7
Lung	0	6
Bone	0	3
Adrenal gland	0	1
Peritoneum	0	1
None	7	6

<sup>a</sup>The International Union Against Cancer, 6th edition.

regimen, 10 patients received the second-line treatment. Five patients received systemic chemotherapy, one patient received UFT alone and four patients received a combined chemotherapy with UFT and doxorubicin. Two patients received transcatheter arterial infusion with cisplatin, one patient received salvage TACE because of HCC rupture during the follow-up period, one patient received salvage

**Table 3.** Toxicity

Toxicity grade	Phase I part						Phase II part					
	Level 1 (n = 3)			Level 2 (n = 6)			Level 3 (n = 3)			Level 2 (n = 19)		
	1–2	3	4	1–2	3	4	1–2	3	4	1–2	3	4
<b>Hematological toxicity</b>												
Leukopenia	2	1	0	0	2	0	0	1	1	4	9	3
Neutropenia	0	1	0	0	2	0	0	0	2	4	11	2
Thrombocytopenia	1	1	0	0	0	0	1	0	0	4	1	0
Anemia	0	0	0	1	0	0	0	0	0	1	0	0
<b>Non-hematological toxicity</b>												
Nausea	3	0	0	0	0	0	2	0	0	3	0	0
Anorexia	0	0	0	2	0	0	1	0	0	3	0	0
Elevated bilirubin	2	0	0	0	1	0	1	0	0	6	0	0
Hypoalbuminemia	1	0	0	0	0	0	0	0	0	1	0	0
Fatigue	0	0	0	0	0	0	1	0	0	1	0	0
Hyperpigmentation	0	0	0	0	0	0	0	0	0	1	0	0
Constipation	0	0	0	0	0	0	0	0	0	1	0	0
Elevated creatinine	0	0	0	0	0	0	0	1	0	0	0	0
Elevated AST	0	0	0	1	0	0	0	0	0	2	1	1 <sup>a</sup>
Elevated ALT	0	0	0	1	0	0	0	0	0	1	2	1 <sup>a</sup>
Liver dysfunction	0	0	0	0	0	0	0	0	0	0	0	1 <sup>a</sup>

AST, aspartate aminotransferase; ALT, alanine aminotransferase.  
<sup>a</sup>Death related to adverse event.

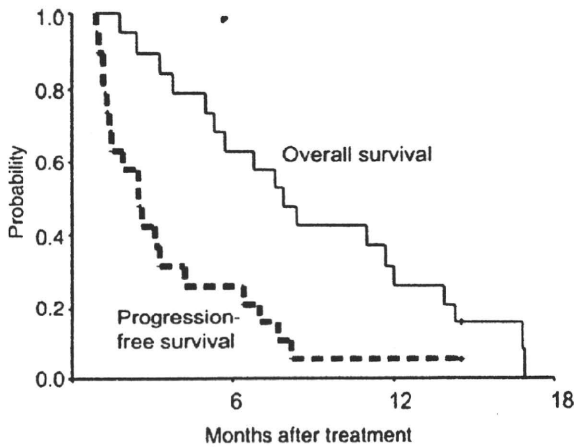
radiofrequency ablation because of rapid growth of HCC that needed control and one patient received immunotherapy.

**TOXICITY**

Table 3 summarizes the toxicities observed in the patients. At the recommended dose (level 2), the major Grade 3–4 hematological toxicities were leukopenia (63.2%) and neutropenia (68.4%). The most common non-hematological toxicities were elevated serum total bilirubin level (31.6%), elevated AST level (26.3%), elevated ALP level (26.3%) and anorexia (21.1%); however, no Grade 3–4 non-hematological toxicities were observed. One patient died of hepatic failure due to hepatitis B virus (HBV) reactivation.

**EFFICACY**

Of the 19 patients who were administered the recommended dosage, 18 died during the follow-up period. All of the 19 patients administered the recommended dosage were evaluable for tumor response; of these, 1 patient achieved partial response (PR), with an overall response rate of 5.3% (95% CI, 0.0–26.0%). Eight patients (42.1%) had stable disease



**Figure 1.** Overall survival and progression-free survival in 19 patients at the recommended dose. Tick marks indicate censored cases.

and 10 patients (52.6%) had progressive disease. The 1-year survival rate, median overall survival, median progression-free survival and time to progression were 26.3%, 8.4 months (95% CI, 5.4–11.4) and 2.5 months (95% CI, 1.5–3.5), respectively (Fig. 1).

**DISCUSSION**

Systemic chemotherapy for unresectable HCC is recognized as an important treatment modality, because some patients who have recurrent or very advanced disease are not suitable candidates for effective local treatments such as surgical resection, liver transplantation, local ablation therapy and TACE. Many patients with HCC have underlying chronic liver disease and impaired hepatic function, increasing the toxicity of standard doses of many chemotherapeutic agents and causing difficulty in delivering combination chemotherapies. The results, in terms of the therapeutic efficacy, of investigation of cytotoxic agents for advanced HCC have been disappointing, with few agents have yielded response rates of over 20%, and no cytotoxic agents have produced convincing survival benefits in the Phase III setting (26–28).

In Japan, only five anticancer agents, UFT, adriamycin, cytarabine, mitomycin and 5-FU, had been approved for the systemic chemotherapy of HCC by the Ministry of Health, Labor and Welfare of Japan before sorafenib has been approved. Among these drugs, the results of multiagent regimens containing both a fluoropyrimidine and an anthracycline antibiotic have shown favorable results for advanced HCC (22–24). Thus, it was expected that the combination of mitoxantrone and UFT (UFM regimen) would have effective anticancer activity, and we conducted a Phase I/II study to evaluate this regimen.

In the Phase I part, we determined the recommended dose of mitoxantrone as 8 mg/m<sup>2</sup> on day 1 and of UFT as 300 mg/m<sup>2</sup> from days 1 to 21 of a 28-day cycle. The DLTs observed at Level 3 were Grade 4 neutropenia (two patients) and Grade 3 creatinine elevation (one patient).

Patients with HCC tend to experience more severe myelosuppression and hepatic toxicity than those with other malignant diseases, because most have underlying cirrhosis, which is usually associated with compromised hepatic function, leukopenia and thrombocytopenia (24). In 19 patients treated at the recommended dose level, the most frequently encountered toxicities were leukopenia and neutropenia, which are well-known toxicities of the two drugs. When compared with that in trial of mitoxantrone or UFT for other malignancies, Grade 3 or 4 hematological toxicities occurred more frequently (29–31). However, these toxicities were reversible and generally well tolerated in patients with advanced HCC, except for one case of treatment-related death; this patient developed hepatic failure due to HBV reactivation, because no antiviral drug for HBV infection, such as lamivudine or entecavir, was given. This is a well-recognized complication in patients with HBV infection who received immunosuppressive therapy or chemotherapeutic agents (32,33). Thus, patients with HBV infection should receive prophylactic antiviral treatment before chemotherapy.

In the current study, 1 of the 19 patients showed a PR (response rate, 5.3%). However, the rate of progressive disease was 52.6%. In addition, the result of median time to progression was only 2.5 months. Those results were unfavorable when compared with those reported from other clinical trials (8,21–23). Therefore, this regimen is considered to be ineffective and cannot be recommended for use in clinical practice. There were several reasons for this negative result. One of the reasons was the number of anticancer drugs in the regimen. A regimen containing two drugs may have little activity, and three or more drugs may be needed to obtain activity against HCC, because many of the regimens that have been shown to exert anticancer effect against HCC contain three or more drugs. The other reason was the recommended doses of the drugs in this regimen. We set the criteria of DLT which had included Grade 4 neutropenia or leukopenia. Two patients experienced DLT based on these criteria. However, both recovered soon, with only observation. Therefore, the criteria may be too strict, although the two drugs have been used at these recommended doses for other malignancies. It may be possible to set higher dose levels to obtain higher antitumor effect.

Recently, increasing knowledge of the molecular pathogenesis of HCC as well as the introduction of molecular-targeted therapies has created an encouraging trend in the management of HCC. Combination regimens consisting of molecular-targeted agents such as sorafenib and cytotoxic agents have been reported as promising regimens for patients with advanced HCC and other malignancies (34–37). The UFM regimen itself has little antitumor activity, but the result may be useful in the setting of future clinical trials of cytotoxic agents used in combination with molecular-targeted agents.

In conclusion, the recommended dose was mitoxantrone at 8 mg/m<sup>2</sup> and UFT at 300 mg/m<sup>2</sup>/day. A combined chemotherapy with mitoxantrone and UFT appeared to show

little activity in patients with advanced HCC, although this regimen was generally well tolerated. These findings do argue against the use of this regimen in clinical practice.

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## Conflict of interest statement

None declared.

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## Increased circulating cell signalling phosphoproteins in sera are useful for the detection of pancreatic cancer

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**BACKGROUND:** Intracellular phosphoprotein activation significantly regulates cancer progression. However, the significance of circulating phosphoproteins in the blood remains unknown. We investigated the serum phosphoprotein profile involved in pancreatic cancer (PaCa) by a novel approach that comprehensively measured serum phosphoproteins levels, and clinically applied this method to the detection of PaCa.

**METHODS:** We analysed the serum phosphoproteins that comprised cancer cellular signal pathways by comparing sera from PaCa patients and benign controls including healthy volunteers (HVs) and pancreatitis patients.

**RESULTS:** Hierarchical clustering analysis between PaCa patients and HVs revealed differential pathway-specific profiles. In particular, the components of the extracellular signal-regulated kinase (ERK) signalling pathway were significantly increased in the sera of PaCa patients compared with HVs. The positive rate of p-ERK1/2 (82%) was found to be superior to that of CA19-9 (53%) for early stage PaCa. For the combination of these serum levels, the area under the receiver-operator characteristics curves was showing significant ability to distinguish between the two populations in independent validation set, and between cancer and non-cancer populations in another validation set.

**CONCLUSION:** The comprehensive measurement of serum cell signal phosphoproteins is useful for the detection of PaCa. Further investigations will lead to the implementation of tailor-made molecular-targeted therapeutics.

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**Keywords:** serum marker; pancreatic cancer; phospho-extracellular signal-regulated kinase; early diagnosis; cell signalling

Pancreatic cancer (PaCa) is an exceptionally devastating disease with a 5-year survival rate of only 5% (Jemal *et al*, 2009). One of the most crucial reasons for the poor prognosis is the lack of early diagnostic markers for PaCa. To overcome pancreatic malignancy, there is an urgent need to discover highly sensitive markers for early detection. The widely used serum-circulating marker for PaCa, carbohydrate tumour-associated antigen 19-9 (CA19-9), is not sufficiently accurate to be used as a diagnostic marker. CA19-9 is elevated in only approximately 65% of individuals with a resectable PaCa, and is also frequently elevated in patients with various benign pancreaticobiliary disorders; notably cholestasis and chronic pancreatitis (PT) (Goggins, 2005). Thus, CA19-9 is not recommended for diagnostic purposes (Locker *et al*, 2006).

In the past decade, various approaches have been used to discover new cancer serum biomarkers, and have identified some attractive molecular targets as diagnostic or prognostic markers for PaCa (Gold *et al*, 2006; Takano *et al*, 2008). Despite the identification of candidate proteins that have high diagnostic

sensitivity and specificity in validation tests, translating these research findings to useful and reliable clinical tests still remains difficult (Zhang *et al*, 2004; Petricoin *et al*, 2002).

Protein phosphorylation is one of the most prominent, and intensively studied post-translational modifications in biological systems. Specifically, better understanding of the defective or hyperactive signalling pathways in cancer cells has been the major focus of mechanistic studies of cancer progression and differentiation, as well as in the identification of candidate markers for diagnosis and therapeutic targets (Petricoin *et al*, 2005). Ultimately, the signalling pathways promote tumourigenesis through the coordinated phosphorylation of proteins that directly regulate protein synthesis, cell-cycle progression and of transcription factors that regulate the expression of genes involved in these processes. Although these intracellular signalling pathways and its components (activated or inactivated forms) that are closely associated with cancer progression are among the most thoroughly studied in molecular cancer research, there has been little understanding of the dynamic nature of these circulating proteins in the bloodstream.

Because blood continuously perfuse the tissues of the body, it is thought to contain most human proteins (at least in fragment

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forms), thereby supplying the richest and most detailed source of information about the physiological state of the body (Anderson *et al*, 2004). Thus, blood has a pivotal role in early disease detection. The turnover of proteins in cells requires calculated degradation processes to secrete intact or fragment forms into blood and to also remove proteins that are no longer necessary or those that have lost functional capabilities. Meanwhile, it is thought that a large part of those human serum-circulating proteins that promote signal transduction in cells may be cleaved by degradation of endogenous substrates and proteases (Villanueva *et al*, 2006a,b; Schilling and Knapp, 2008). Taken together, it is worthwhile for the early cancer detection to elucidate the molecular networks in cancerous cells and the microenvironments, and to investigate the dynamic nature of those cancer-related phosphorylated proteins including the fragments in circulating blood.

The Bio-Plex suspension array using specific antibodies and based on the principle of flow cytometry is a high-throughput technology that can measure multiple proteins in low sample volumes. Immunoaffinity approaches, particularly phospho-specific antibodies that recognise low abundance phosphotyrosine, -serine and -threonine residues in the specific epitopes, have been used to assess phosphoprotein enrichment. In further developing this technology, it is possible to detect the phospho-specific sites of the parent molecule and its degraded fragments in serum as early diagnostic markers by comprehensive analysis in cancer patients. This technology has overcome a critical problem for the translational application of proteomics by developing a procedure that is convenient with high sensitivity, specificity and reproducibility. Moreover, this may also provide important information regarding the activation state of kinase-driven signalling networks in each cancer patient for therapeutic target selection with the advantage that the screen is less invasive.

In this study, we analysed the circulating cell signalling phosphoproteins in sera using this proteomic approach and investigated whether these protein levels are useful for detection, as early diagnostic serum markers for PaCa, in combination with comprehensive and hierarchical cluster analyses. Our study results indicate that the use of this new approach will lead to new insights in proteomic cancer biology and in the potential development of patient-tailored combination molecular targeting therapy, through elucidation of the phosphoprotein networks in serum from cancer patients.

## MATERIALS AND METHODS

### Patient samples

We selected four populations of patients with PaCa, PT and healthy control volunteer (HV). Blood samples were obtained from all patients who were diagnosed with PaCa and PT in the Chiba University Hospital, Chiba, Japan, from November 2002 to March 2009, and the samples were also obtained from HVs in the Chiba University Hospital and the Kashiwado Hospital, Chiba, Japan. Sera were collected from 26 patients with PaCa and 25 HVs for the training set, from 80 patients with PaCa and 68 HVs for validation set 1, furthermore, to assess the diagnostic ability of discriminating between cancer and non-cancer populations, sera of 35 patients with PaCa as a cancer group, and 40 patients with PT as well as 48 HVs as a non-cancer group were selected to match for age for validation set 2 (Table 1). All patients were histologically confirmed as PaCa. The characteristics of 141 patients with PaCa are summarised in Table 2. All blood samples were processed according to a standardised protocol, and serum sample aliquots were frozen until the subsequent analysis. None of the patients received any therapeutic treatments, such as radiation, chemotherapy or operation, until serum samples were collected.

**Table 1** Summary of all participants

Experimental groups (number of patients)	Sex (M/F)	Age (mean $\pm$ s.d.)
<i>Training set (n = 51)</i>		
Pancreatic cancer (n = 26)	17/9	65.2 $\pm$ 8.0
Healthy volunteer (n = 25)	17/8	52.0 $\pm$ 10.6
<i>Validation set 1 (n = 148)</i>		
Pancreatic cancer (n = 80)	48/32	62.9 $\pm$ 10.8
Healthy volunteer (n = 68)	41/27	54.1 $\pm$ 6.8
<i>Validation set 2 (n = 123)</i>		
Pancreatic cancer (n = 35)	21/14	63.6 $\pm$ 8.8
Pancreatitis (n = 40)	38/2	61.7 $\pm$ 8.8
Healthy volunteer (n = 48)	32/16	62.4 $\pm$ 7.3

Abbreviations: F = female; M = male.

**Table 2** Characteristics of patients with pancreatic cancer

Variables	Training set (n = 26)	Validation set 1 (n = 80)	Validation set 2 (n = 35)
<i>Tumor stage</i>			
T1	0	3	8
T2	2	2	4
T3	21	47	12
T4	2	10	2
TX	1	18	9
<i>Nodal status</i>			
N0	7	16	16
N1	17	41	10
NX	2	23	9
<i>Metastasis</i>			
M0	24	53	30
M1	2	27	5
<i>UICC stage</i>			
IA	0	2	8
IB	1	2	4
IIA	6	11	3
IIB	16	32	10
III	1	6	5
IV	2	27	5
<i>Resection status</i>			
R0	12	38	21
R1	7	9	5
R2	4	6	0
RX	3	27	9

Abbreviations: RX = unresectable case; UICC = Union Internationale Contre le Cancer.

The ethics committee for each institute approved the protocol. Written informed consent was obtained from all patients and HVs.

### Bio-Plex phosphoprotein suspension assay

Phosphorylated proteins in serum were detected with a Bio-Rad phosphoprotein immunoassay kit using the Bio-Plex 200 suspension array system (Bio-Rad Laboratories, Hercules, CA, USA). The human serum diluent buffer was added up to 50  $\mu$ l to the eight-fold diluted samples, 50  $\mu$ l aliquots of each of the diluted samples were plated in the 96-well filter plate, coated with anti-phosphoprotein antibody-coupled beads, and incubated for 16 h on a platform shaker at 300 r.p.m. at room temperature. The wells were vacuum

filtered and washed, 1  $\mu$ l of detection antibodies ( $25\times$ ) was added, vortexed and incubated for 30 min. After additional vacuum filtration and washing of the wells, 0.5  $\mu$ l streptavidin-PE ( $100\times$ ) was added to each well and allowed to incubate for 10 min. The wells were again vacuum filtered and washed, 125  $\mu$ l of re-suspension buffer was added and incubated for 30 s. Data acquisition and analysis were performed using Bio-Plex Manager software version 5.0. The data of measurement by the Bio-Plex 200 suspension array system are presented in the Supplementary Information (Supplementary Figure S1; Supplementary Table S1).

For the training set, 18 targeted phosphorylated (p-) proteins were measured using Bio-Plex 200 suspension array system in the comprehensive phosphoprotein analysis. Focusing on the more promising candidate proteins, phospho-mitogen-regulated kinase 1 (p-MEK1), phospho-extracellular signal-regulated kinase 1/2 (p-ERK1/2) and those total proteins; we measured (t-) for the further validation sets.

### Immunohistochemistry

Paraffin-embedded tissues were cut into 4  $\mu$ m-thick serial sections and were de-paraffinised. Serial section slides were placed in citric acid buffer (10 mmol l<sup>-1</sup>, pH 6.0) with 0.2% Tween 20 and boiled in a microwave oven (2  $\times$  6 min) to retrieve the antigen. The slides were then rinsed and blocked in a 3% H<sub>2</sub>O<sub>2</sub> solution with methanol for 10 min, before being incubated overnight at 4°C with the primary antibodies; rabbit anti-phospho-MEK1/2 monoclonal antibody (Cell Signaling Technology, Beverly, MA, USA) and rabbit anti-phospho-ERK1/2 (p44/42 MAPK) monoclonal antibody (Cell Signaling Technology) (1:50 and 1:200 dilution respectively). SignalStain Antibody Diluent (Cell Signaling Technology) was used as the dilution buffer. They were then rinsed in PBS, and incubated for 60 min with secondary antibody labelled with streptavidin-biotin-peroxidase (DAKO LSAB + kit; DakoCytomation, Glostrup, Denmark). The bound complex was visualised using diaminobenzidine liquid chromogen and counterstained with hematoxylin.

### Comprehensive and hierarchical clustering analyses of the training and validation sets

To investigate the similarity of expression patterns, we performed hierarchical clustering analysis using R statistical software (version 2.8.0). Before analysis, the expression levels were standardised using Z-transformation (mean=0 and variance=1) for each protein. We then used Euclidian distance of expression patterns for calculation of distance matrices (i.e., one for proteins and the other for samples) between each variable, as well as the average linkage method for clustering analysis.

### Multivariate logistic regression using selected proteins

To assess the diagnostic ability for PaCa patients and controls, we performed univariate and multivariate logistic regression analyses using p-ERK1/2, CA19-9 and the combination of p-ERK1/2 and CA19-9 models. Receiver-operating characteristic (ROC) curves and area under the curve (AUC) based on the prediction results of the obtained regression models were calculated by the R statistical software.

### Statistical analysis

Statistical analyses were performed using the appropriate tests as indicated. *P*-values <0.05 were considered statistically significant. To compare the positive rate for detecting early stage pancreatic malignancies, we determined the positive levels of p-ERK1/2 in disease patients by the reference values in each of the respective three sets (Solberg, 1987). The reference values in the three sets were calculated using reference limits corresponding to

0.95 fraction of the distribution, that is the upper limit of the 95% confidence interval (CI), in the three respective healthy control groups. For CA19-9, a cut-off value of 37 IU ml<sup>-1</sup> was used, according to the manufacturer's specifications for the reference range of CA19-9.

## RESULTS

### Circulating phosphoproteins levels are increased in sera from patients with pancreatic cancer

To detect new biomarkers characteristic of the PaCa patients, we comprehensively first measured the 18 major targeted cell signalling phosphoproteins levels in sera of the training set using the Bio-Plex suspension array. Many of the target phosphoproteins levels were increased significantly in sera from PaCa patients compared with the HVs (see detail of the experimental data in Supplementary Table S2). Hierarchical clustering analysis showed that the relative differential expressions of circulating phosphoproteins clearly distinguished PaCa patients from HVs (Figure 1A). Six candidate phosphoproteins (p-ERK1/2: *P*<0.00001, p-MEK1: *P*<0.0005, phospho-p90 ribosomal S6 kinase (p-p90RSK): *P*<0.0001, phospho-cAMP response element binding protein (p-CREB): *P*<0.00001, p-Akt: *P*<0.00005 and p-I $\kappa$ B- $\alpha$ : *P*<0.0001; Mann-Whitney *U*-test) were significantly increased in sera from patients with PaCa compared with the HVs.

As shown in the lower panel of Figure 1A, similar cluster structures were obtained in the clustering analysis of these six phosphoproteins. Subclass analysis separated the PaCa patients into two groups, based on hierarchical clustering of the six candidate marker levels, and revealed that each of the two groups correlated well with the groups that had favourable and unfavourable prognoses (*P*=0.07; log-rank test; Figure 1B). These were also closely correlated with each protein belonging to the phosphatidylinositol-3-OH kinase/Akt, NF- $\kappa$ B and ERK signalling pathways that are crucial for cancer survival (Figure 1C).

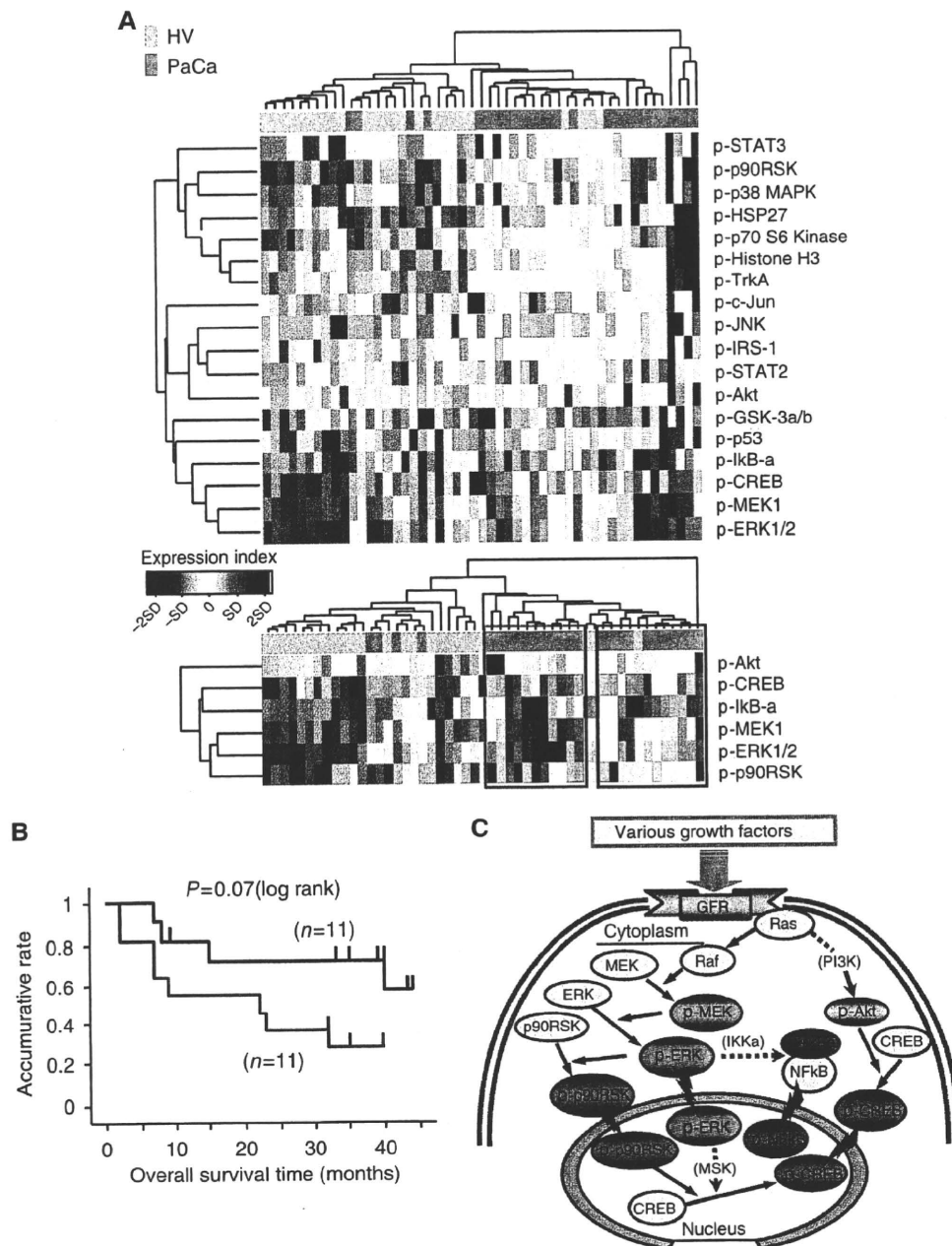
Two of these proteins, p-ERK1/2 and p-MEK1, are shown in Figure 2A. Of particular interest, four of the six phosphoproteins were proteins directly associated with the most popular pathway of pancreatic carcinogenesis, the Ras/Raf/MEK/ERK signalling cascade to two proteins (p-p90RSK and p-CREB) that are directly or indirectly phosphorylated by ERK. Surprisingly, the results indicate that p-ERK1/2 levels in serum showed a significantly positive correlation with p-MEK1 levels (*r*=0.57, *P*<0.00002; Pearson's correlation coefficient test) as well as p-ERK, which would theoretically be dependant on the activity of MEK and is in turn promoted by an entire series of upstream events (Figure 2B). Therefore, we mainly selected two key molecules, ERK and MEK, to investigate the expression of those phosphorylated and total proteins in sera for further validation analyses by Bio-Plex assay.

### Confirmation of target phosphoprotein serum levels by western blot analysis

To confirm the results obtained from Bio-Plex assay, we assessed the expression of phosphoproteins both sera from patients with PaCa and HVs by immunoprecipitation assay and western blot analysis. Corresponding with Bio-Plex data, increased p-ERK1/2 expression levels were confirmed by western blot analysis in sera from three PaCa patients and one HV (see detail of the methods and experimental data in Supplementary Figure S2).

### Activated p-ERK and p-MEK are expressed in pancreatic cancer cells

To examine the potential source of the activated ERK and MEK in serum, we performed immunohistochemical staining for these phosphoproteins in resected PaCa tissues. As shown in Figure 2C,

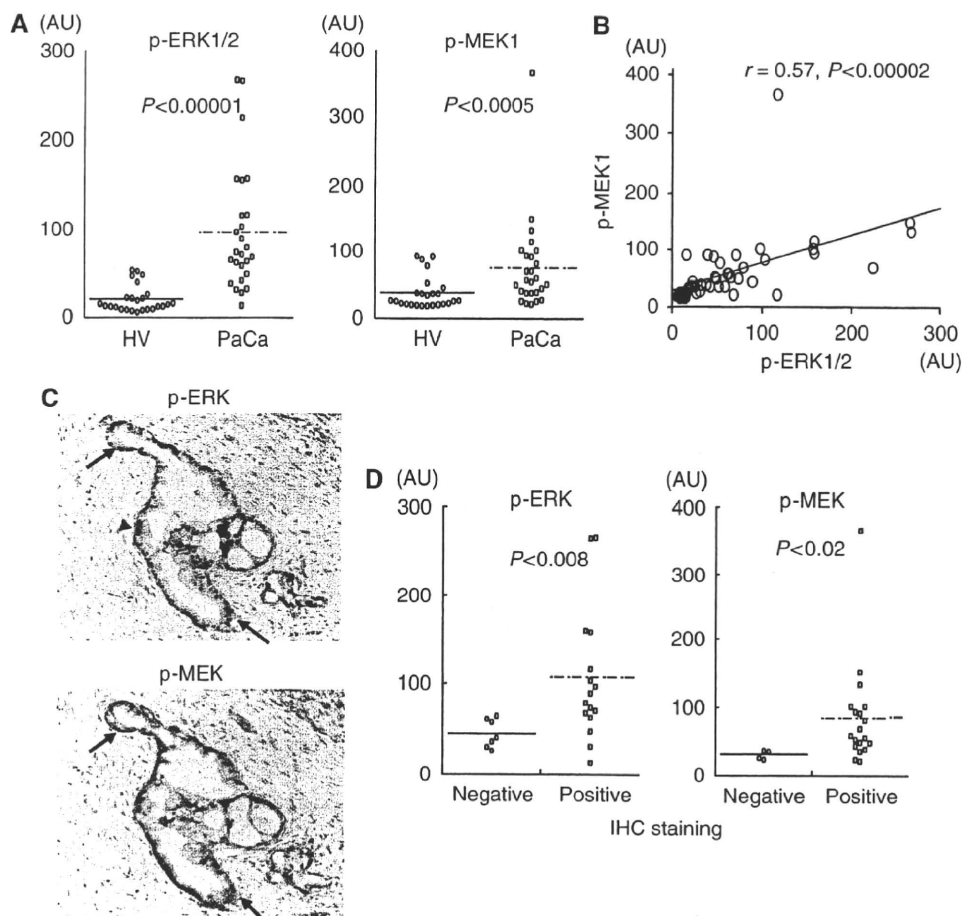


**Figure 1** Hierarchical clustering of phosphoprotein expression profiles in the training set. **(A)** Healthy volunteers (HVs) are denoted in light blue in the vertical side bar, and pancreatic cancer (PaCa) patients are denoted in orange. Dendrograms show the classification determined by hierarchical clustering analysis of 18 targeted phosphoproteins. As shown in Expression index, red and blue in the matrix indicate relative overexpression and underexpression respectively (s.d.; standard deviation). The hierarchical clustering analysis clearly distinguished the two groups; the majority of PaCa patients are found in the right side, whereas HVs are mainly located in the left side of the heat map (upper panel). The profiles of six candidate circulating phosphoproteins associated with PaCa are revealed by hierarchical clustering analysis (lower panel). Subclass analysis separated the PaCa patients into two groups based on the candidate phosphoproteins. The distinction of the two groups is indicated by the blue and purple lined boxes. **(B)** Kaplan–Meier analysis revealed that each of the two groups (blue and purple lines) distinguished by the hierarchical clustering analysis was well matched with a favourable and unfavourable prognosis of PaCa patient groups. **(C)** The schema of the correlation between candidate phosphoproteins by cell signal transduction in the intracellular environment. The crucial interaction among these molecules allows the cancer to proliferate and differentiate through representative cell signalling pathways.

both activated p-ERK and p-MEK expression levels were clearly positive in pancreatic ductal carcinoma cells. The p-ERK and p-MEK expression was localised to the neoplastic epithelial cells and some stromal cells especially surrounding the ductal carcinoma cells. Activated p-ERK was found in both the nucleus and cytoplasm of cancer cells. Conversely, p-MEK expression was not found in the nucleus of cancer cells, but stained intensely in the cytoplasm of cancer cells.

### Both p-ERK and p-MEK levels in sera were significantly correlated with the positive staining of pancreatic cancer tissues

Next, we investigated whether the two phosphoproteins levels in sera correlated with the positive staining in PaCa tissues. Among 26 PaCa patients in training set, 23 patients (R0, R1 and R2; all resectable cases) were analysed by immunohistochemical staining



**Figure 2** Serum phosphoproteins increased in PaCa patients compared to HVs in the training set. **(A)** Circulating p-ERK1/2 and p-MEK1 levels in sera are significantly greater in PaCa patients than HVs. **(B)** Linear regression with p-ERK1/2 and p-MEK1 levels reveals a significant, positive correlation in the training set. **(C)** Immunostaining for p-ERK and p-MEK in PaCa tissues (original magnification,  $\times 200$ ). Note that expression of the two phosphoproteins is evident in the cancerous cytoplasm (arrows in p-ERK and p-MEK) and nucleus (arrowhead in p-ERK), and is also found in stromal cells surrounding the ductal carcinoma cells. **(D)** Both p-ERK and p-MEK levels in sera were significantly correlated with the positive staining of PaCa tissues (p-ERK:  $P < 0.008$ , p-MEK:  $P < 0.02$ ; Mann–Whitney *U*-test).

of the PaCa tissues. The 23 patients were divided into two groups based on positive or negative staining in PaCa cells, 16 (69.6%) of 23 cases were p-ERK-positive staining, and 19 (82.6%) cases were p-MEK-positive staining. Notably, as shown in Figure 2D, both p-ERK and p-MEK levels in sera were significantly correlated with the positive staining of PaCa tissues respectively (p-ERK:  $P < 0.008$ , p-MEK:  $P < 0.02$ ; Mann–Whitney *U*-test).

### Both phospho- and total-ERK1/2 simultaneously increase with a positive correlation in sera of patients with pancreatic cancer

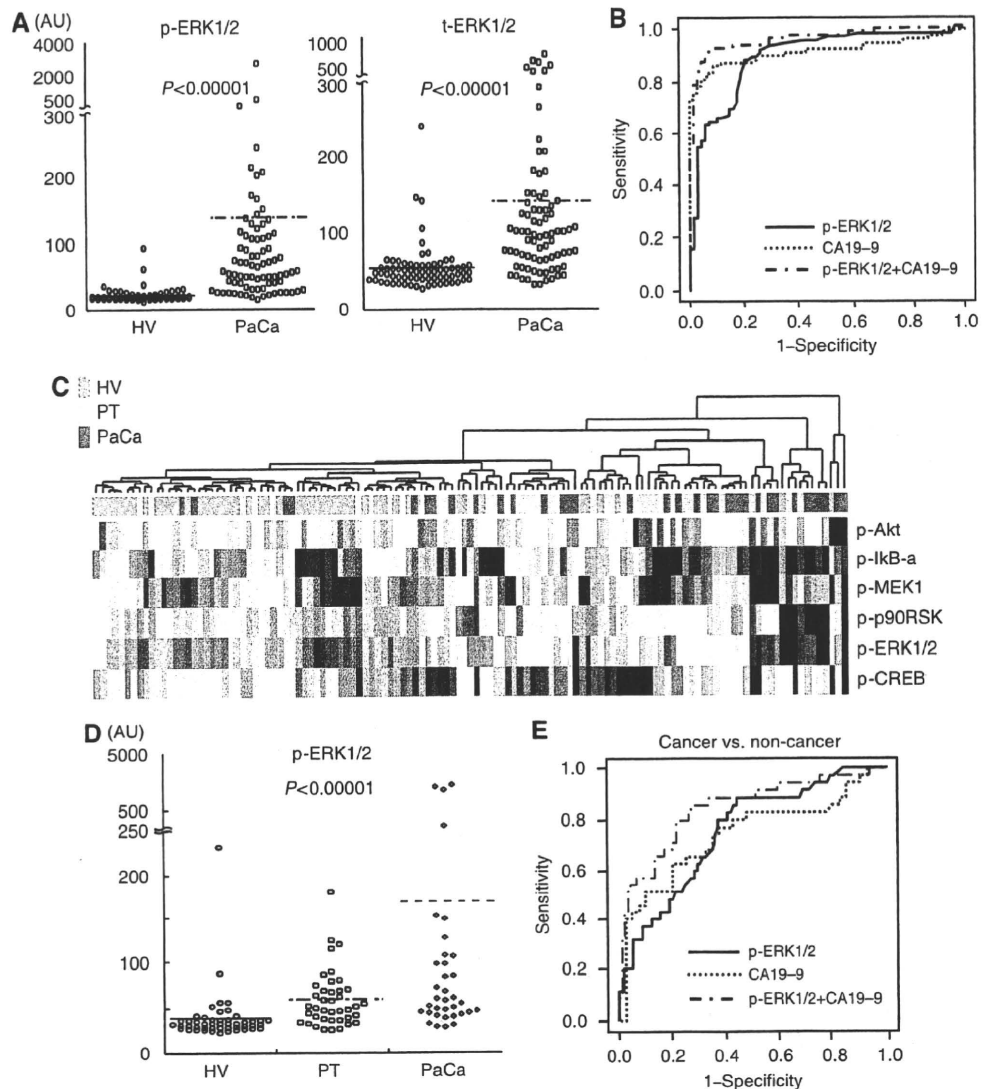
To confirm the results obtained from the training set, we measured and analysed both ERK and MEK serum levels with an increased sample size in validation set 1. Similar results were obtained, that both p-ERK1/2 and p-MEK1 levels were significantly increased in sera from PaCa patients compared with that of HVs for validation set 1 (p-ERK1/2;  $P < 0.00001$ , p-MEK1;  $P < 0.00001$ ; Mann–Whitney *U*-test; Figure 3A). In addition, t-ERK1/2 levels were also significantly more upregulated in sera from PaCa patients compared with that of HVs in validation set 1 ( $P < 0.00001$ ; Mann–Whitney *U*-test). Of particular interest, both p- and t-ERK1/2 levels increased simultaneously with a positive

correlation in sera from PaCa patients ( $r = 0.38$ ,  $P < 0.0004$ ; Pearson's correlation coefficient test) (figure not shown).

### Phospho-ERK1/2 level in serum excels in the detection of pancreatic cancer

To estimate the cell signalling phosphoprotein, p-ERK1/2, as a novel serum biomarker to detect PaCa patients, we calculated the ROC curves, which correlate the true- and false-positive rates (sensitivity and 1 specificity) between PaCa patients and HVs. The area under the ROC curve (AUC) was 0.94 for p-ERK1/2, and concerning with other phosphoproteins, the AUCs were 0.79 for p-MEK1, 0.81 for p-p90RSK, 0.86 for p-CREB and 0.83 for p-Akt in the training set. To validate and compare the abilities of serum markers for the diagnosis of PaCa, we constructed ROC curves for p-ERK1/2, CA19-9 and the combination of two serum levels in validation set 1. The respective AUC was 0.88 for p-ERK1/2, 0.91 for CA19-9 and 0.96 for the combination p-ERK1/2 and CA19-9 (Figure 3B).

The positive rate of serum p-ERK1/2 in the disease groups was calculated using the reference values determined according to the upper limit of 95% CI in the three respective healthy control groups. In all three sets of this study, only five patients showed negative levels for both p-ERK1/2 and CA19-9. For CA19-9,



**Figure 3** Confirmation of the results obtained from the training set for validation set 1. **(A)** The similar result of the training set shows that both circulating p-ERK1/2 and t-ERK1/2 levels were significantly increased in sera from PaCa patients compared with that of HVs for validation set 1. **(B)** The ROC analyses were performed for the serum levels of p-ERK1/2, CA19-9 and a combination of p-ERK1/2 and CA19-9 between PaCa patients and HVs. The respective AUCs were 0.88 for p-ERK1/2 level, 0.91 for CA19-9 level and 0.96 for the combination of p-ERK1/2 and CA19-9 levels. Comparing among three populations in validation set 2. **(C)** Serum levels of six candidate phosphoproteins were able to distinguish among the three populations (HV, PT and PaCa) by hierarchical clustering analysis. The analysis distinguished the three groups; the majority of PaCa patients are found on the right side, whereas PT are located diffusely in the approximate centre and HVs are mainly located on the left side of the heat map. **(D)** Circulating p-ERK1/2 levels in sera were significantly differentiated among three populations (PaCa, PT and HVs) ( $P < 0.00001$ ; Kruskal–Wallis test). **(E)** The ROC analyses were performed for the serum levels of p-ERK1/2 and CA19-9 between cancer (PaCa) and non-cancer (HV and PT) populations. The respective AUCs were 0.75 for p-ERK1/2 level and 0.70 for CA19-9 level and 0.84 for the combination of p-ERK1/2 and CA19-9 levels.

39 false-negative patients were mostly picked up as p-ERK1/2-positive (87.2%) patients with PaCa (Table 3). These results indicate that the combination of p-ERK1/2 and CA19-9 achieved sufficiently high sensitivity and specificity to diagnose PaCa accurately by supplementing the low sensitivity of CA19-9 that was caused by deficiency of Lewis antigens and so on.

#### Combination of p-ERK1/2 and CA19-9 levels is superior discriminatory power between cancer and non-cancer populations

In validation set 2, we compared and analysed the differential protein expression of six candidate phosphoproteins levels in sera from PaCa, PT patients and HVs. Hierarchical clustering analysis

indicated that the populations of PT patients were located diffusely but approximately in between the HVs and PaCa patient groups (Figure 3C).

Furthermore, to assess the discriminatory power of serum p-ERK1/2 levels, we measured to compare p-ERK1/2 and CA19-9 levels in sera of PaCa patients and age-matched benign controls including PT patients. Phospho-ERK1/2 levels were significantly increased in sera among three populations ( $P < 0.00001$ ; Kruskal–Wallis test; Figure 3D), and between cancer and non-cancer populations ( $P < 0.00002$ ; Mann–Whitney *U*-test). To discriminate cancer from non-cancer groups, we performed multivariate logistic regression analysis using p-ERK1/2 and CA19-9. As a result, both p-ERK1/2 (odds ratio: 13.4, 95% CI: 2.14–83.6,  $P = 0.0056$ ; Wald test) and CA19-9 (odds ratio: 3.67, 95% CI: 1.86–7.22,  $P = 0.0002$ ;

**Table 3** p-ERK1/2-positive rate in CA19-9 false-negative patients with pancreatic cancer

	CA19-9 false negative (%)	p-ERK1/2-positive in CA19-9 false negative (%)
Training set	6/26 (23.1)	6/6 (100.0)
Validation set 1	22/80 (27.5)	20/22 (90.9)
Validation set 2	11/35 (31.4)	8/11 (72.7)
Total	39/141 (27.7)	34/39 (87.2)

Abbreviations: CA19-9 = carbohydrate tumour-associated antigen 19-9; p-ERK1/2 = phospho-extracellular signal-regulated kinases 1/2.

Wald test) were identified as significant variables for the detection of PaCa. For distinguishing between cancer and non-cancer groups, the respective AUC was 0.75 for p-ERK1/2 and 0.70 for CA19-9, and the AUC was 0.84, showing high ability to distinguish between cancer and non-cancer groups, for the combination of the two serum levels (Figure 3E). These results suggest the combination of p-ERK1/2 and CA19-9 levels is better discriminatory power compare to CA19-9 alone between cancer and non-cancer populations.

### Circulating p-ERK1/2 is a potential novel marker for early stage of pancreatic cancer

To emphasise the diagnosis of early stage of patients with pancreatic malignancy, we found that the sensitivity of serum p-ERK1/2 levels for predicting stage I PaCa in our study population was 82% (14 out of 17 patients with stage IA or IB cancers had elevated p-ERK1/2), whereas only 9 out of 17 (53%) patients showed elevated CA19-9. These results suggest that the measurement of serum p-ERK1/2 levels could be particularly helpful in the detection of early stage PaCa.

## DISCUSSION

The results reported herein show that the measurement of circulating signal transduction proteins in serum led to the detection of PaCa. To elucidate molecules related to PaCa progression, we used a new strategy based on the multiplexed cell signalling of phosphoproteins in serum by hierarchical clustering analysis. To detect pre-malignant tumour or early stage malignancies, it is necessary to be able to assess very low abundant substances that are likely produced by tumour itself (i.e., fragments of cellular components, endo- or exogenous protease and secretion derived from tumour) (Villanueva *et al*, 2006a,b), the microenvironment of the tumour–host interface (Iacobuzio-Donahue *et al*, 2002) and the host immune response to tumour (Koomen *et al*, 2005). Recently, it was reported that both lymphatic vessel compression with resultant functional abnormalities and elevated interstitial fluid pressure occur during the early stages of carcinogenesis (Hagendoorn *et al*, 2006). These insights have formed the theoretical foundation for the detection of early stages of cancer.

Biological fluids, such as serum, are a readily obtainable source of potential cancer biomarkers that are shed or secreted by cancer cells, and are produced as a consequence of humoral immunity (Lu *et al*, 2008). Serum immerses most tissues in the body and is therefore likely to contain cell-derived proteins that can provide dynamic information about various biological processes. In addition, it is thought that cellular or tissue protein might likely present as a full-length form or the cleavage fragments that

freely enter circulation by diffusion or convection (Liotta and Petricoin, 2006).

Concerning proteins as indicators, it has been recognised that blood protein biomarkers are amplified in the circulatory system because they accumulate on the high concentration of resident proteins, such as albumin, and then acquire the longer half-life of albumin, thereby protecting the bound species from renal clearance (Lowenthal *et al*, 2005; Araujo *et al*, 2008). Lowenthal *et al* also indicated that among many individual sequences that were predicted from albumin-associated proteins in serum from patients with three stages of ovarian cancer, the predicted sequences were largely fragments derived from proteins with diverse biological functions, including crucial cellular signal transduction factors. Interestingly, the kinds of signal transduction factors were more numerous in sera from patients with early stage than in advanced stage of cancer among the identified proteins. In an recent study, the enrichment of serum phosphopeptides using the modified particles was successful to identify phosphorylated peptides that were related to cancer. The profiling of these degraded fragments has been found to be able to distinguish between hepatocellular carcinoma patients and healthy individuals (Hu *et al*, 2009). Our current study results are consistent with these theories of protein amplification and actual identification in the circulatory system.

The activation of epidermal growth factor receptor (EGFR) and the various downstream targets, such as Ras, Raf, MEK and ERK, are deeply implicated in the pathogenesis of PaCa with malignant transformation and enhanced tumour aggressiveness. In addition, the signalling cascade is likely crucial for PaCa progression because K-Ras gene mutations have been found in many populations of human PaCa specimens. The efficacy of molecular targeting therapies for PaCa, such as an inhibitor of EGFR tyrosine kinase, small-molecule inhibitor of Raf kinase and that of the dual specificity kinase MEK1/2, have recently being evaluated in some clinical trials, however, the results have not been impressive (Rinehart *et al*, 2004; Siu *et al*, 2006; Moore *et al*, 2007). The major reason is considered that the dysregulation or hyperactivity in the network of intracellular and extracellular signalling pathways is so complicated with multiplicity that each individual may have a differential profile even among similar malignancies. It is reasonable to surmise that to obtain maximum efficacy of molecular-targeted therapies it is necessary to investigate which pathway is more highly activated for each cancer patient (Jimeno *et al*, 2008). In the near future, our new insights may resolve this problem with a minimally invasive approach.

This is the first study to show circulating cell signalling phosphoproteins in blood of PaCa patients. In our experiments, comprehensive and hierarchical clustering analyses of serum phosphoproteins between PaCa patients and HVs revealed pathway-specific profiles, in particular components of the ERK signalling pathway, and a new method to classify serum phosphoproteins possibly derived from tumour itself, based on intracellular signalling portraits. As mentioned above, overcoming the issue of specificity as well as discovering highly sensitive markers for early detection are undoubtedly important. We confirmed that this signature could be used to discriminate not only between cancer and healthy controls in an independent validation set but also between cancer and non-cancer populations in an age-matched sample as another validation set. We also found that these circulating molecules were potentially useful for the diagnosis of early stage PaCa. These results suggest that the level of circulating p-ERK may be associated with early stage of pancreas carcinogenesis.

Immunohistochemistry of PaCa tissues showed that two target phosphoproteins, p-ERK and p-MEK, were simultaneously well expressed during the early stage neoplasms, even in the cancer cells of non-invasive or minimally invasive ductal carcinoma, as well as in the advanced stage patients with PaCa. Furthermore,

both p-ERK and p-MEK levels in sera of PaCa patients were in good correlation with the positive staining of their PaCa tissues. Taken together, we consider that the major source for the elevation of cell signal phosphoproteins levels in serum may be cancer cells that are showing augmented cell signalling.

Subclasses distinguished by hierarchical clustering analysis of six candidate markers indicated good correlation with the prediction of the prognosis of PaCa patients in this study. Further investigation of subclass analysis by hierarchical clustering will provide fruitful information regarding which factors of cell signalling phosphoproteins in serum are associated with the malignant behaviour of PaCa.

PaCa develops as a result of the stimulation and activation of various growth factor receptors. The continuous stimulation of these signal transduction pathways leads to increases in both the activated and inactivated forms of the cell signalling molecules in the intracellular environments of cancerous cells. The downstream activation transmits information through post-translational protein modifications with reversible protein phosphorylation. Increasing signal molecules that accumulate in the cell trigger changes in the penetration of cell membrane, which causes the release of both phosphoproteins and the degraded fragments to extracellular environments by cellular apoptosis. Once released from the intracellular environments, those proteins likely lose their original function, and are then carried to nearby blood vessels, and circulate freely or with binding to high-affinity transfer proteins in

blood circulatory system. However, further research is needed to elucidate the sequence of this pathway.

In conclusion, we found cancer-associated cell signal phosphoproteins in serum using multiplexed cell signalling analysis. The measurement of circulating phosphoproteins in serum was able to discriminate between cancer patients and benign controls, and this new approach was helpful in the early diagnosis of patients with PaCa. This method shows the feasibility of this analysis, with a less invasive approach. The next step is to elucidate the profiling of cell signal activation by these comprehensive and hierarchical clustering analyses may discriminate subclasses into clinically significant groups. In the near future, investigations determining the footprints of circulating phosphoproteins will lead to the clinical application of this method that will be used for targeted tailor-made therapeutics.

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Supplementary Information accompanies the paper on British Journal of Cancer website (<http://www.nature.com/bjc>)

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# Detection of oesophageal cancer biomarkers by plasma proteomic profiling of human cell line xenografts in response to chemotherapy

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**BACKGROUND:** The incidence of oesophageal adenocarcinoma is increasing worldwide but survival remains poor. Neoadjuvant chemotherapy may improve survival, but targeting treatment to patients who respond to chemotherapy could be improved by the availability of markers of response. This study sought proteomic markers of therapeutic response using an adenocarcinoma xenograft model.

**METHODS:** Epirubicin, cisplatin or 5-fluorouracil was administered to severe combined immune-deficient mice bearing OE19 oesophageal adenocarcinoma xenografts. Murine plasma samples from treated and untreated xenografts were analysed by surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry, and panels of peaks were found using class prediction models that distinguished treatment groups. Proteins in these peaks were identified by mass spectrometry in tryptic digests of purified fractions. Five paired samples from oesophageal cancer patients before and after chemotherapy were analysed using the same methodology.

**RESULTS:** Plasma protein peaks were identified that differed significantly ( $P < 0.05$ , ANOVA) between the treated xenograft and control groups. Marker panels predicted treated vs untreated xenografts with sensitivities of 100%, specificities of 86–100% and test efficiencies of 89–100%. Three of the proteins identified in these panels, apolipoprotein A-I, serum amyloid A and transthyretin were confirmed in the clinical samples.

**CONCLUSION:** Plasma protein markers can be detected in response to chemotherapy in oesophageal adenocarcinoma xenografts and in clinical samples, and have the potential to monitor response and guide chemotherapy in oesophageal adenocarcinoma.

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**Keywords:** oesophageal adenocarcinoma; response to chemotherapy; proteomics; biomarkers

The incidence of oesophageal cancer, particularly adenocarcinoma in western populations, is increasing worldwide (Botterweck *et al*, 2000; Park, 2002; Lagergren, 2005) and carries a poor prognosis, even in the minority with resectable disease (Gilbert *et al*, 2002; Munro, 2004) for whom 5-year survival ranges from 10 to 35% (Hulscher *et al*, 2002; Thompson *et al*, 2007). Trials of neoadjuvant chemotherapy and neoadjuvant chemoradiotherapy have reported mixed results ranging from no difference in curative resection or overall survival (Kelsen *et al*, 2007) to improved resection rates and survival (MRC Oesophageal Working Party, 2002; Geh *et al*, 2006). A systematic review of 11 randomised controlled trials showed an increase in overall survival with the use of chemotherapy, but statistical significance was only achieved after 5 years (Maltaner and Fenlon, 2003). Palliative chemotherapy for advanced oesophageal cancer results in control of symptoms in 70–80% of patients with 40–50% objective response rates but only 30–40% surviving for 1 year (Gilbert *et al*, 2002).

The ability to determine, at an early stage, which patients are most likely to respond to chemotherapy could prevent patients undergoing ineffective and potentially toxic treatments and allow direction of treatment to those most likely to benefit. Imaging techniques such as computerised tomography, magnetic resonance imaging, endoscopic ultrasound and positron emission tomography range in their effectiveness to predict response to chemotherapy (Westerterp *et al*, 2005). Pathological criteria for assessment of the degree of tumour regression in the resected oesophagus using tumour regression grades may be a significant predictor of disease-free survival (Mandard *et al*, 1994) but is not an independent prognostic indicator for oesophageal adenocarcinomas (Dunne *et al*, 2001). Pathological response using modified staging criteria has been shown to predict survival following chemoradiotherapy (Swisher *et al*, 2005). In addition, pathological response to pre-operative chemotherapy has been shown to improve overall survival (Kelsen *et al*, 2007). However, neither imaging techniques nor resectional pathology have to date provided robust guidance of response during chemotherapy.

There has been growing interest in the use of proteomic methods on peripheral blood plasma to rapidly profile protein

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## Widespread and multifocal carcinomas in situ (CISs) through almost the entire pancreas: report of a case with preoperative cytological diagnosis

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### Abstract

**Purpose** It is imperative for prognostic improvement of pancreatic cancer that we try to diagnose carcinoma in situ (CIS) of lesions, i.e., precursors of invasive ductal carcinomas (IDCs) at an early stage, because results of treatment of patients with IDCs themselves continue to be rather unsatisfactory.

**Materials and results** We report here a case of a patient who received subtotal pancreatectomy for widespread and multifocal CISs of the pancreas after preoperative brushing cytology from the epithelium of dilated main pancreatic duct proved cancer-positive preoperatively.

**Conclusions** From our experience, we conclude that examination for CIS of the pancreas must be recommended whenever dilatation of relatively large pancreatic ducts is found by ultrasound or computed tomography. We should therefore advance to magnetic resonance cholangiopancreatography or endoscopic retrograde cholangiopancreatography and then cytological and/or pathological assessment of the pancreatic duct whenever non-continuous narrowing, localized dilatation, or other irregularities are encountered.

**Keywords** Carcinoma in situ (CIS) of the pancreas · Diagnosis · Early detection · Pancreatic intraepithelial neoplasia (PanIN)

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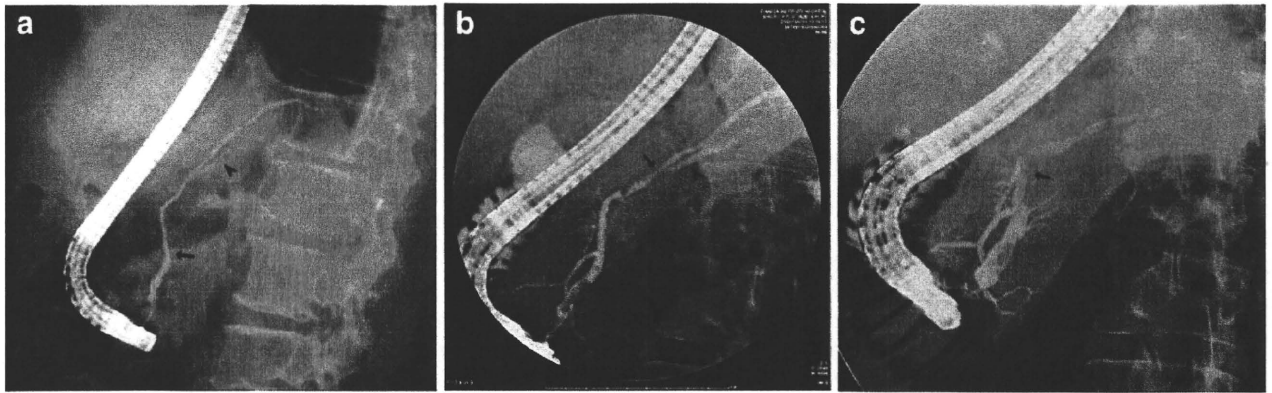
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### Introduction

Although the rates for discovery of small pancreatic cancers have increased with advances in clinical imaging and great efforts of numerous clinicians to improve the prognosis, many lesions continue to be too advanced for curative therapy at the time of detection. Consequently, it is essential for prognostic improvement of pancreatic cancer that we focus on carcinoma in situ (CIS), a precursor of the invasive ductal carcinoma (IDC).

In general, it is rather difficult for CIS to be found by clinical imaging and thus for preoperative cytologic or histologic diagnosis to be feasible. We report here a case of a patient who underwent subtotal pancreatectomy for widespread and multifocal CISs of the pancreas after a cancer-positive result from transpapillary brushing cytology of samples from the epithelium of the dilated main pancreatic duct. In the present case report, we discuss problems of preoperative diagnosis and management of pancreatic CIS.



**Fig. 1** **a** ERCP shows an irregular dilatation, 3 mm in diameter, extensively involving the main pancreatic duct (MPD, *arrow*) and an inferior branch of the body (IBB, *arrowhead*). **b** A subsequent ERCP, 2 years after the first, reveals more increased dilatation, 6 mm in diameter, of the MPD and IBB (*arrowhead*). Brushing cytology was obtained from a MPD narrowing in the body (*arrow*) because

sampling from the IBB was technically impossible. However, the specimen was negative for cancer and follow-up was continued. **c** The MPD diameter is further increased to 10 mm on ERCP 2.5 years after the first ERCP. Brushing cytology obtained from dilated and irregular MPD of the head (*arrow*) was positive for cancer

### Case report

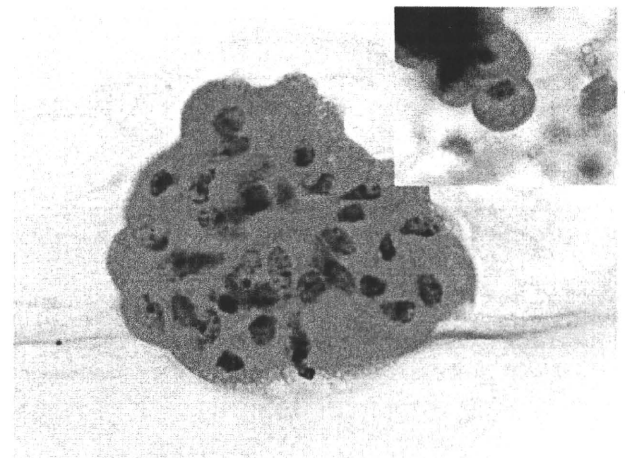
The patient was a man aged 65 years, suffering from dull backache for a year. Abdominal ultrasonography (US) and computed tomography (CT) disclosed no space-occupying lesion except for a small cystic lesion in the uncinata process, but irregular dilatations were apparent in the main pancreatic duct (MPD) and a tributary branch in the body. As endoscopic retrograde cholangiopancreatography (ERCP) confirmed extensive irregular dilatation (Fig. 1a), he was diagnosed as suffering from chronic pancreatitis with suspicion of CIS.

During subsequent follow-up, at evaluation by alternating ERCP and magnetic resonance cholangiopancreatography (MRCP) at half-year intervals, the dilatation gradually became more prominent. The maximum diameter had increased to 6 mm as assessed by ERCP 2 years later, and at that time cytology of a brushing specimen obtained from a MPD narrowing in the body of the pancreas (Fig. 1b, *arrow*), was negative for cancer. An irregular branch duct could not be sampled because of technical difficulties (Fig. 1b, *arrowhead*). However, the MPD diameter further increased to 7 mm on MRCP, and then 10 mm on ERCP, conducted 6 and 8 months later, respectively. Brushing cytology samples obtained from the dilated and irregular MPD (Fig. 1c, *arrow*) eventually proved positive for cancer (Fig. 2).

Under the diagnosis of CIS of MPD in the head of the pancreas and chronic pancreatitis or questionable CIS of an inferior tributary branch in the body, pancreatoduodenectomy was performed 2.5 years later from the initial visit at our institute, resulting in subtotal proximal pancreatectomy after two additional resections, because the first and second frozen sections both were positive for CIS. As indicated in

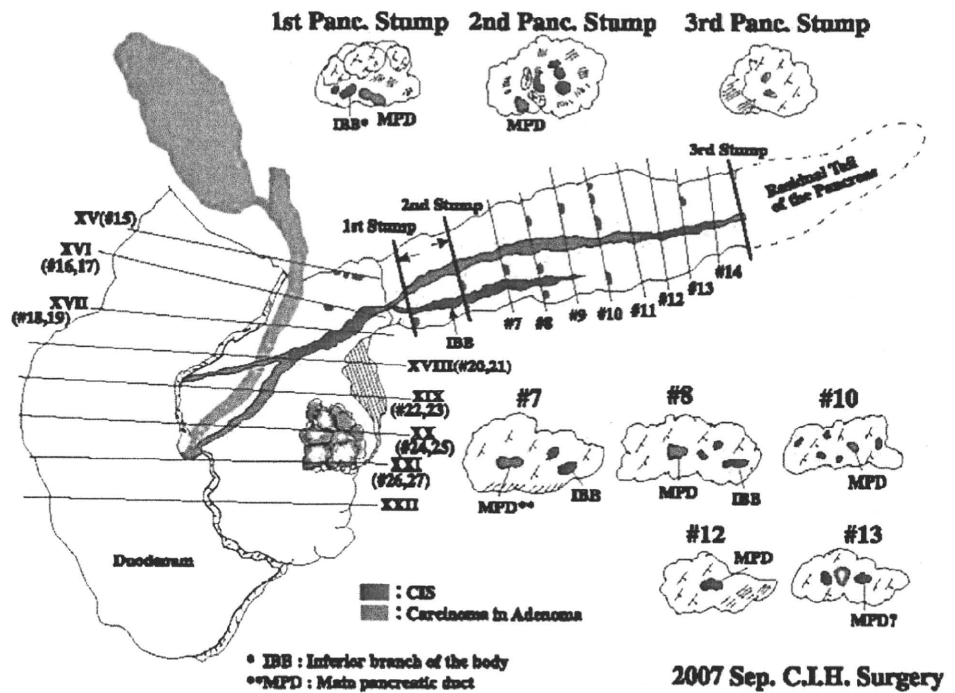
Fig. 3, CISs were sporadically observed in an inferior tributary and surrounding smaller branches of the body both in the first (Fig. 4a) and second pancreatic stump. Accordingly, a second additional pancreatic resection, extending for about 7 cm and including the whole tributary branch of the body with CIS, and a third frozen section was PanIN-1B, i.e., negative for CIS. The residual pancreas was about 4 cm in length (Fig. 3).

As illustrated in Fig. 3, the resected material was sliced serially at about 5-mm intervals and examined histologically, widespread multifocal CISs being observed



**Fig. 2** Transpapillary brushing cytology obtained from the MPD reveals a papillary cluster of atypical columnar cells. Note granular cytoplasm, eccentrically located and hyperchromatic nuclei and prominent nucleoli (Papanicolaou stain,  $\times 1,000$ ). *Inset* shows atypical oval-to-round cells with a large amount of cytoplasm containing mucin (Papanicolaou stain,  $\times 1,000$ )

**Fig. 3** Schematic illustration of the resected specimen, which was sliced serially at about 5-mm intervals and examined histologically. Widespread multifocal CISs were revealed in both larger pancreatic ducts, i.e., the main pancreatic duct (MPD) and an inferior branch in the body of the pancreas (IBB) sporadically and in smaller branching ducts throughout almost the whole pancreas. Additionally, an intraductal papillary–mucinous neoplasm (IPMN) was observed at the uncinate process



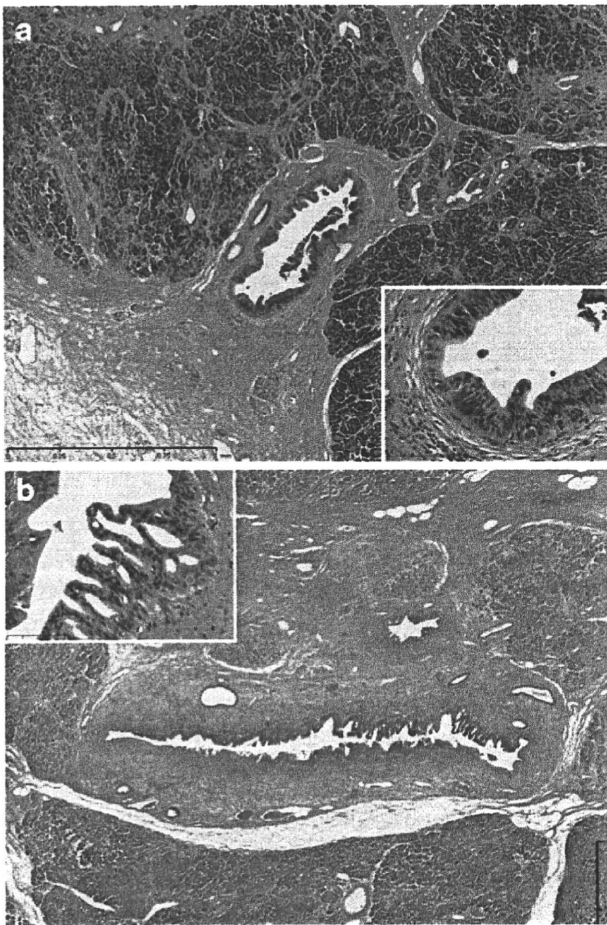
through almost the whole pancreas. While high-grade atypical lesions, i.e., CIS or grade 3 of the pancreatic intraepithelial neoplasia classification (PanIN-3) showing that intraductal low papillary growth were extensive in the largest of pancreatic ducts, i.e., the MPD of the head (Fig. 4b) and tail and an inferior branch of the body separately; they were also sporadically apparent in smaller branching ducts (Fig. 4a), so that a multifocal origin was speculated. Additionally, an intraductal papillary–mucinous carcinoma in adenoma, branch type, 15 mm in size, was revealed at the uncinate process of the pancreas (Fig. 3), separate from the CIS of the MPD. The postoperative course was uneventful, and the patient is doing well without disease recurrence after 3 years following surgery.

**Discussion**

CIS of the pancreas is now attracting a great deal of attention as a precursor for the IDC, bearing in mind the unsatisfactory treatment of patients with pancreatic malignancies by either surgery or chemotherapy. It is imperative for prognostic improvement that we can more readily diagnose CIS of the pancreas. When dilatation of a larger pancreatic duct is found by US or CT, we need to advance to MRCP or ERCP in order to ascertain whether the lesion is localized. Singh and Maitra [1] emphasized the importance of early detection of non-invasive precursors of pancreatic cancer radiologically as cysts and/or dilatations of the major pancreatic duct. It was stressed by Seki et al.

[2] that irregularity, non-continuous narrowing, granular defects, and dilatation are most important pancreatographic findings in ERCP and highly suggestive of CIS of the pancreas. Castellano-Sanchez et al. [3] also pointed out that a “beaded pattern” of alternating stenosis and dilatation of the main pancreatic duct, mimicking non-continuous narrowing mentioned by Seki et al. [2], is a characteristic ERCP feature. Once CIS is suspected on the basis of MRCP or ERCP, aspiration or brushing cytology of pancreatic juice is necessary for diagnosis. Biopsy of the pancreatic duct during ERCP is recommended, if possible, anatomically. As mentioned above, cytological confirmation of the diagnosis of CIS was obtained with our patient. It is extremely rare that CIS of the pancreas can be cytologically [4] or histologically diagnosed preoperatively. However, we are convinced that this chain of modalities is the best way for diagnosis of CIS of the pancreas at present.

An intraductal papillary–mucinous neoplasm (IPMN), branch type was here found at the uncinate process of the pancreas, coincident with the CISs present throughout almost the entire pancreas in this patient. It is by no means uncommon that duct cell carcinoma of the pancreas is accompanied by IPMNs [5]. We consider that this case thus featured two different pathways of neoplasia, involving PanINs and IPMNs [6]. It is also interesting to speculate whether the multifocal CISs originated separately or were the result of spread throughout the pancreas. Although the former is more likely given the range in histological findings, it is conceivable that they are genetically continuous. It



**Fig. 4** **a** As illustrated in Fig. 3, first and second pancreatic stumps were positive for CIS at the IBB and smaller branches around it. Low-power view of a small branch near the IBB at the first stump demonstrates epithelial proliferation with severe atypia in the branch. The *insert at the bottom right* is high-power view of the branch showing marked pleomorphism and lack of polarity of nuclei (HE). **b** Low-power view of the #17 cross-section in Fig. 3 demonstrates epithelial proliferation with severe atypia in the MPD and its side branch in the head. The *high-power view of the MPD inserted at the top left* discloses intraluminal bridging and piling up of nuclei with loss of polarity (HE)

should be noted that Pan IN-1 or Pan IN-2 lesions could be sporadically seen among the Pan IN-3. In this case, we left about 4-cm-tail of the pancreas mostly because we considered that the patient would have a better quality of life, i.e., a relatively easier control of his blood sugar levels after the operation. Retrospectively, we have thought that brush cytology to check the remnant pancreas at risk should have been performed, when the third frozen section left, about

4 cm pancreatic gland, was revealed to be PanIN-1B (adenomatous hyperplasia), i.e., negative for cancer. It is lucky for us that there is eventually no sign of recurrence 3 years after the operation.

Although there are a number of options for treatment of CIS of the pancreas, we consider that surgical resection is best. Preoperative complete diagnosis of CIS is indispensable for appropriate surgery because lesions are usually impalpable and are also difficult to detect even by intraoperative US during an operation. We experience great problems in deciding exactly where the most appropriate transection line might be because we could not establish precise spread preoperatively. Clearly, we should avoid over-surgery and try to perform resection after precise definition of lesion locations.

In conclusion, it is highly recommended in order to detect CIS of the pancreas that we advance to MRCP or ERCP when a dilatation of relatively larger pancreatic duct is found by US or CT. Cytological and/or pathologic examinations of the pancreatic duct should then be conducted whenever non-continuous narrowing, localized dilatation, or any irregularity is seen by MRCP or ERCP.

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Clinical Science

## Intrahepatic cholangiocarcinoma: analysis of 44 consecutive resected cases including 5 cases with repeat resections

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### KEYWORDS:

Intrahepatic  
cholangiocarcinoma;  
Liver resection;  
Pulmonary resection

### Abstract

**BACKGROUND:** Prognosis after resection for intrahepatic cholangiocarcinoma (ICC) remains unsatisfactory. There remains no effective therapy after recurrent ICC.

**OBJECTIVE:** The current study sought to evaluate risk factors associated with recurrent ICC and possible therapies after resection.

**METHOD:** A review of data from patients who underwent potentially curative resection for ICC was performed.

**RESULTS:** A total of 44 potentially curative resections were performed from 1995 to 2008. Mortality was 0% and morbidity was 35%. The 5-year overall and recurrence-free survival rates were 43% and 39%, respectively. Multivariate analysis identified the presence of multiple nodules and poor histologic grade as independent negative prognostic factors for overall and recurrent-free survival. Postoperative recurrence occurred in 25 patients (57%). Solitary recurrence occurred in 5 patients (liver, n = 4; lung, n = 1), all of who had undergone surgical resection. Three of the 5 patients survived for more than 5 years after 2 resections.

**CONCLUSION:** Prognosis after curative resection of solitary ICC appears favorable. In selected patients with sequential single hepatic or pulmonary recurrence, repeat resection may prolong survival.  
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Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer, accounting for 10% of primary hepatic cancers.<sup>1</sup> In recent years, the incidence of ICC has been increasing worldwide, and the tumor is gain-

ing attention.<sup>1–4</sup> In contrast to hepatocellular carcinoma, no strong high-risk groups have been identified for ICC. Therapeutic outcomes for patients with ICC are poor due to the highly malignant nature of the cancer. Surgical resection is currently the only curative option, but 5-year survival rates following curative resection range from 23% to 40%, with a median survival time of 18 to 37 months.<sup>5–9</sup> Furthermore, prognosis for patients with unresectable ICC is extremely poor, at less than 1 year.<sup>4</sup> No treatments have been established for unresectable or recurrent ICC. Although local chemotherapy, systemic chemotherapy, or a second surgical

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