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ORIGINAL ARTICLE

Clinical utility of a newly developed microfluidic device for detecting circulating tumor cells in the blood of patients with pancreatico-biliary malignancies

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Abstract

Background: The development of an optimal screening method is required to improve the prognosis of pancreatico-biliary (PB) cancers. A recently developed microfluidic device achieved a high diagnostic yield by detecting circulating tumor cells (CTCs) in the blood of cancer patients. We conducted this study to investigate the clinical utility of measuring CTCs in peripheral venous blood to diagnose PB cancer.

Methods: Sixty-three subjects were enrolled in this study (29 with pancreatic cancer [PC], 19 with biliary cancer [BC] and 16 non-tumor controls). Using a microfluidic chip device and image analyzer, circulating blood cells were selected based on their size and immunocytochemistry staining pattern. The primary endpoint was the diagnostic accuracy of CTCs with regard to distinguishing between PB cancer patients and controls. We divided all cases into the training set (n = 32) and validation set (n = 31). The diagnostic accuracy of CTCs, carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9) were analyzed.

Results: In both the training set and validation set, CTCs showed the highest diagnostic accuracy (training set: CTCs 90.6%, CA19-9 90.6%, CEA 65.6%, validation set: CTCs 87.5%, CA19-9 78.1%, CEA 81.2). Regarding non-metastatic PC (cStage I-III, n = 11), CTCs also had the highest diagnostic accuracy among the three markers tested (CTCs: 84.6%, CA19-9:80.7%, CEA 73.0%).

Conclusions: A newly developed microfluidic device could diagnose PB cancers by detecting CTCs. This trial was registered with the UMIN Clinical Trials Registry, no. UMIN000029808.

KEYWORDS

biliary cancer, cancer screening, circulating tumor cell, liquid biopsy, pancreatic cancer

1 | BACKGROUND

Pancreatico-biliary (PB) cancers are the most lethal malignancies in the world. Despite advanced treatment, the prognosis of metastatic PB cancers is very poor because they are highly resistant to anti-tumor agents.[1,2] Accordingly, the detection of PB cancers at a non-metastatic stage is necessary to improve patient prognosis.[3,4]

Currently available evidence recommends cancer screening for colorectal,[5] breast,[6,7] lung[8] and cervical cancer[9] in

the general population; however, little evidence suggests the potential benefit of cancer screening for PB cancers, mainly due to the invasiveness of imaging tests (eg, computed tomography, magnetic resonance imaging and endoscopic ultrasound) and the lack of reliable diagnostic biomarkers.[10,11] To address this challenging situation, we need non-invasive and reliable diagnostic tests for PB cancer screening.

Although carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9) are widely utilized as clinical biomarkers for PB cancer, they have an insufficient diagnostic yield, particularly in the early stages. Thus far, liquid biopsies with tests for circulating tumor cells (CTCs), cell-free DNA, and microRNA have been evaluated.[12,13] Among these, CTCs have attracted the attention of researchers because they can be diagnostic as well as prognostic markers in various cancers.[14,15] Regarding PB cancers, several studies have investigated the clinical utility of CTCs as prognostic markers, but few studies have used CTCs as diagnostic markers, presumably due to their low sensitivity.[16–18]

In this study, we used a newly developed microfluidic device to capture and characterize CTCs based on cell size and cell-surface markers with the aim of clarifying the role of CTCs in the detection of PB cancer at a non-metastatic stage.[14,19]

2 | METHODS

2.1 | Patients and treatments

We prospectively enrolled 29 pancreatic cancer (PC) patients, 18 biliary cancer (BC) patients and 16 non-tumor controls in our study between November 2017 and August 2020 (Table 1). The median age of the PC patients was 68.0 years old (range: 54.0-84.0), and 16 patients were male (55.2%). Eleven PC patients had clinical stage (cStage) I-III disease. The median tumor size was 30.0 mm (10.0-88.0). The median age of the BC patients was 69.0 years old (58.0-80.0), and 12 patients were male (63.2%). Ten patients had cStage I-IVA disease. All cancer patients underwent biopsy to confirm the diagnosis of adenocarcinoma. The BCs identified were 17 extrahepatic cholangiocarcinomas and one gallbladder cancer.

Disease stage was determined according to the American Joint Committee on Cancer (AJCC)/Union for International Cancer (UICC) staging system, version 8.[20,21] Regarding unresectable PC patients, the chemotherapy regimen (combination therapy: FOLFIRINOX or gemcitabine plus nab-paclitaxel, monotherapy: gemcitabine or S-1) was decided by the physician. All clinico-pathological data were obtained prior to treatment. Overall survival (OS) for cancer patients treated with gemcitabine-based chemotherapy was calculated from the date of diagnosis to the date of death from any cause or the date of the last follow-up examination. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of Fukushima Medical University (IRB #29334). All participants provided written informed consent. This trial was registered with the UMIN Clinical Trials Registry (no. UMIN000029808).

Patient clinical data, including age, sex, and serum levels of CEA and CA19-9, were obtained from the electronic medical records. The pathological characteristics of the tumors (eg, tumor size and disease stage) were also retrieved. To obtain the serum samples, 8 mL of blood was collected and incubated at room temperature for at least 60 minutes to allow clotting. Samples were then centrifuged at 1000 *g* for 10 minutes. The serum was collected and stored in aliquots at −80°C. CTCs were collected before initiation of systemic chemotherapy or other types of treatments against cancers.

TABLE 1 Clinical background of controls and patients with PB cancer

Pancreatic cancer (n = 29)	
Age (years old), median (range)	68.0 (54.0-84.0)
Sex, M, n (%)	16 (55.2)
cStage I-III/IV (AJCC/UICC ver. 8)	11/18
Background disease	Adenocarcinoma (29)
Tumor size (mm), median (range)	30.0 (10.0-88.0)
CEA (ng/mL), median (range)	4.4 (1.9-539.1)
CA19-9 (U/L), median (range)	1853.0 (119.1-160310.0)
CTC (cells/2 mL), median (range)	2.0 (0.0-15.5)
Biliary cancer (n = 18)	
Age (years old), median (range)	69.0 (58.0-80.0)
Sex, M, n (%)	12 (66.7)
cStage I-IVA/IVB (AJCC/UICC ver. 8)	10/8
Background disease	Extrahepatic biliary cancer (17) Gallbladder cancer (1)
Tumor size (mm), median (range)	25.0 (10.0-53.0)
CEA (ng/mL), median (range)	3.7 (1.9-329.0)
CA19-9 (U/L), median (range)	263.6 (9.1-10810.0)
CTC (cells/2 mL), median (range)	2.0 (0-196.5)
Control (n = 16)	
Age (years old), median (range)	66.0 (23.0-85.0)
Sex, M, n (%)	13 (72.2)
Background disease	Healthy volunteer (8) Bile duct stone (5) Pancreatitis (3)
CEA (ng/mL), median (range)	1.5 (1.1-65.9)
CA19-9 (U/L), median (range)	6.8 (2.0-1096.0)
CTC (cells/2 mL), median (range)	0.0 (0.0-1.0)

Abbreviations: AJCC/UICC, American Joint Committee on Cancer/Union for International Cancer; CA19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen; cStage, clinical stage; CTC, circulating tumor cell; M, male.

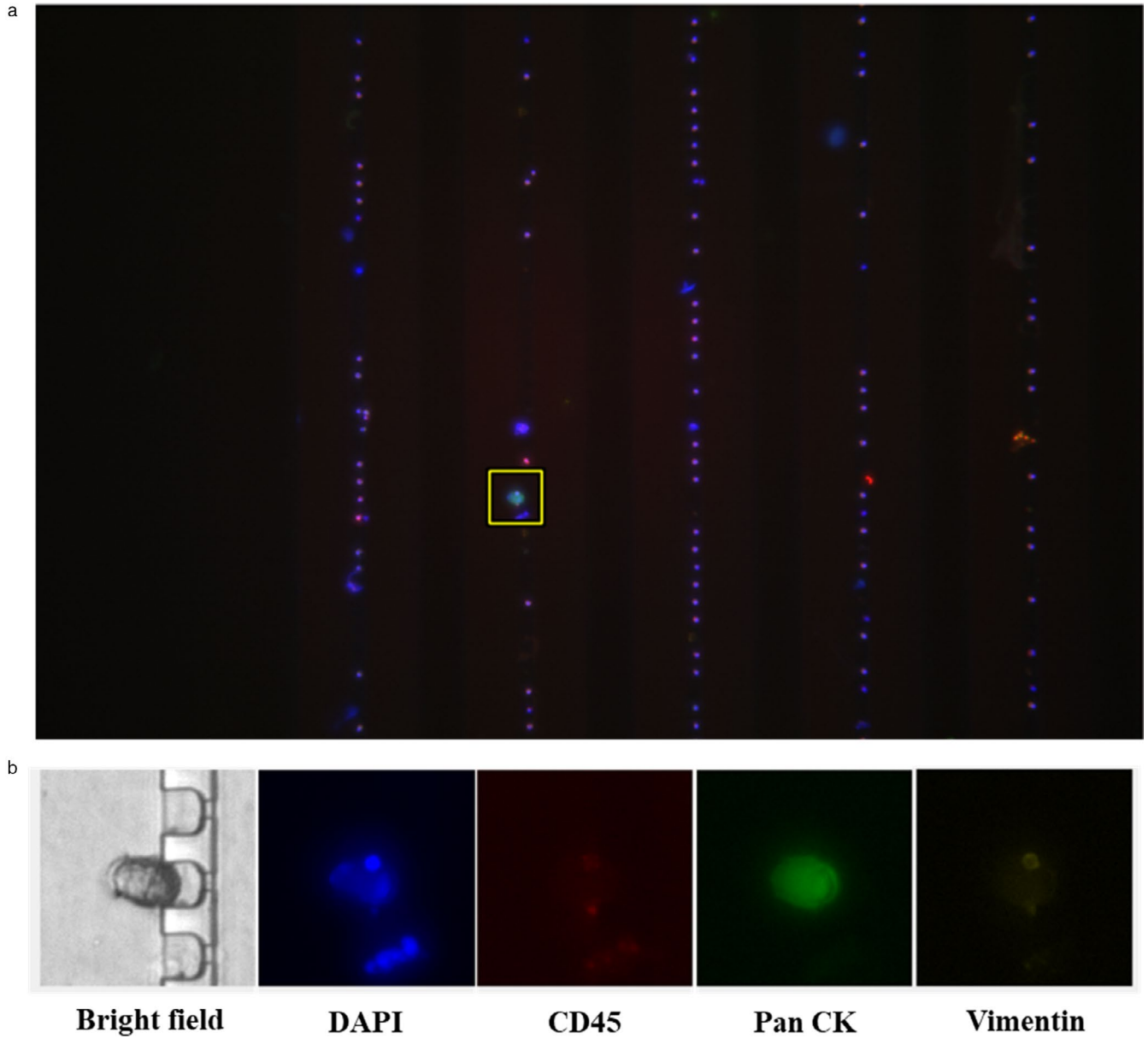


FIGURE 1 Representative images of circulating tumor cell (CTC) detection. A, CTCs captured and isolated from blood samples were stained and scanned automatically. B, CTCs were negative for the white blood cell marker CD45 and positive for pan-cytokeratin

2.2 | Circulating tumor cell analysis

We used a newly developed microfluidic system consisting of two instruments: the Celsee PREP™ 400 sample processing system and Celsee Analyzer™ imaging station (Celsee Inc). The Celsee PREP™ 400 system captured and isolated CTCs from 2-mL blood samples using a microfluidic slide with multiple cell trapping chambers (20 × 20 × 30 μm) with individual pore channels (7.5 μm in width × 8 μm in depth × 10 μm in length) based on deformability differences between CTCs and blood cells. Fluorescence images of cells were obtained using the Celsee Analyzer™ imaging station.[14,19] CTCs were negative for the white blood cell marker CD45 and positive for either pan-cytokeratin or vimentin (Figure 1a,b). The total number of CTCs in 2 mL of blood was used in the analysis. CTCs were measured using a microfluidic chip device at Nihon Gene Research Laboratories.

2.3 | Statistics

Continuous variables are reported as the medians (ranges). For categorical data, the chi-square test or Fisher's exact test was performed, as appropriate. To compare continuous variables, the Mann–Whitney *U* test was performed. The median OS after initial chemotherapy was calculated using the

Kaplan-Meier method and compared with the log-rank test. Receiver operating characteristic (ROC) analysis was performed, and the area under the curve (AUC) was calculated. The optimal cut-off values were determined at the value maximizing the Youden index (sensitivity + specificity-1). All statistical analyses were performed with GraphPad Prism 7.0 (GraphPad). $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Diagnostic yield of CTCs and other tumor markers

To confirm the diagnostic yield of the three biomarkers (CEA, CA19-9 and CTCs) for distinguishing PB cancer patients and non-tumor controls, we randomly divided all cases into the training set (n = 32) and validation set (n = 31) using Microsoft Excel software. There were no significant differences between the two sets in clinical background, including age and background disease (Table 2). Additionally, there were no significant differences between the non-tumor controls and PB cancer patients in age and gender proportions in both the training and validation sets (training set: age 48.0 vs 68.5 years, $P = .17$, proportion of males 75% vs 54.1%, $P = .42$. validation set: age 49.5 vs 68.5 years, $P = .08$, proportion of males 62.5% vs 60.8%, $P > .99$) In comparisons between PB cancer patients with any stage disease and non-tumor controls, the median values of all three biomarkers were significantly higher in the PB cancer patients than in the controls in the training (PB cancer vs control. CEA: 3.6 vs 1.5 ng/mL, $P = .0008$, CA19-9: 370.8 vs 15.3 U/mL, $P = .0004$, CTCs: 3.0 vs 0.0 cells/2 mL, $P = .0001$, respectively) and validation sets (PB cancer vs control. CEA: 6.8 vs 1.5 ng/mL, $P = .004$, CA19-9: 882.6 vs 4.8 U/mL, $P = .0007$, CTCs: 1.5 vs 0.0 cells/2 mL, $P = .0001$, respectively; Figure 2a,b). Among the three biomarkers, CTCs showed the highest diagnostic performance (CTCs: AUC 0.92, cut-off 0.25 cells/2 mL, accuracy 90.6%, sensitivity 91.3% and specificity 87.5%), while the other markers showed modest diagnostic results (CA19-9: AUC 0.88, cut-off 8.5 U/mL, accuracy 90.6%, sensitivity 95.6%, specificity 75.0%. CEA: AUC 0.83, cut-off 3.15 ng/mL, accuracy 65.6%, sensitivity 82.6%, specificity 87.5%). Similar results were obtained for the validation set (CTCs: AUC 0.92, cut-off 0.25 cells/2 mL, accuracy 87.5%, sensitivity 91.7%, specificity 75.0%. CA19-9: AUC 0.89, cut-off 8.5 U/mL, accuracy 78.1%, sensitivity 95.8%, specificity 37.5%. CEA: AUC 0.87, cut-off 3.15 ng/mL, accuracy 81.2%, sensitivity 58.3%, specificity 87.5%; Figure 2c,d).

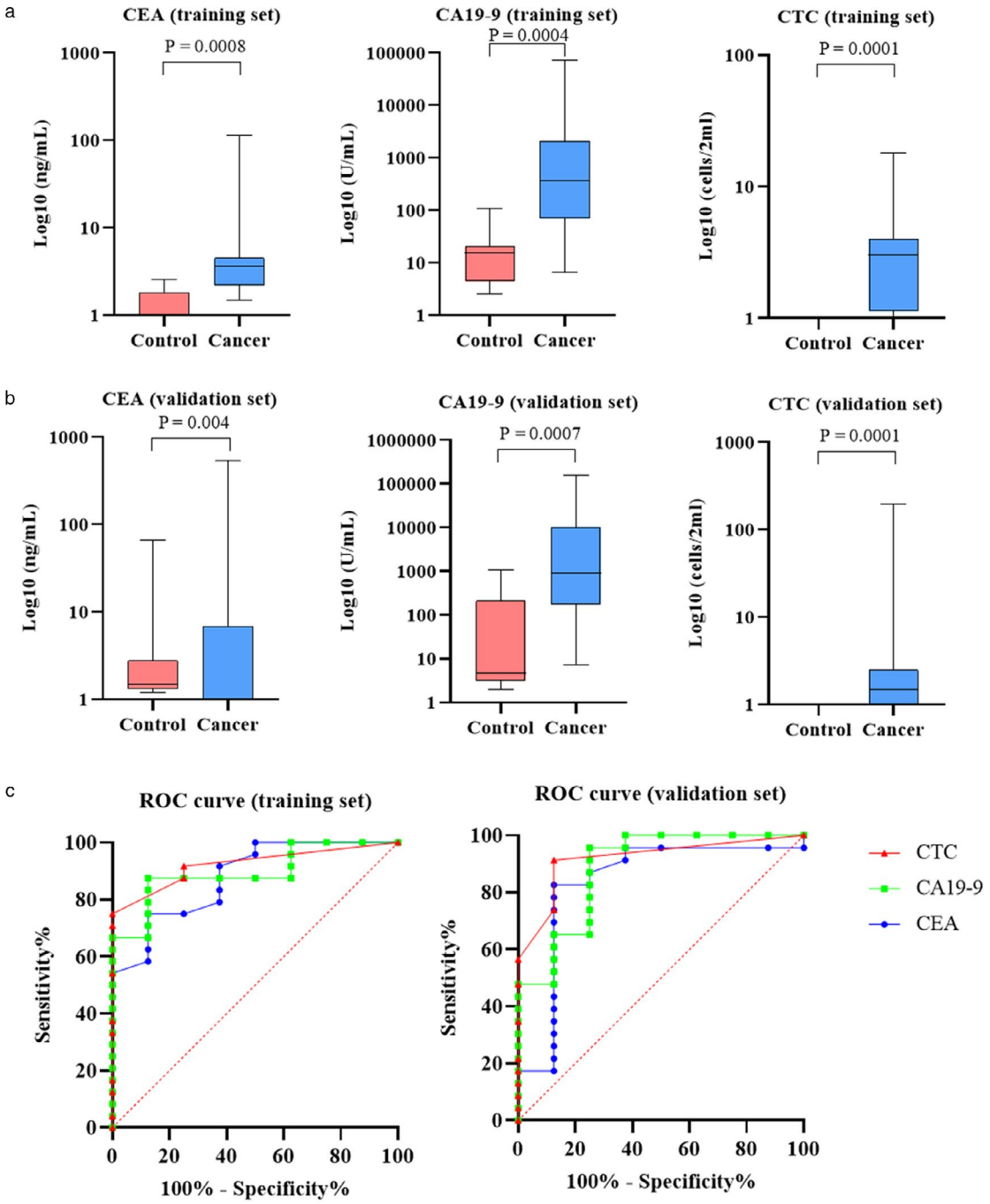
3.2 | CTCs can discriminate patients with non-metastatic PB cancer from controls

To clarify whether CTCs could discriminate between the controls and patients with non-metastatic PB cancer, we investigated the diagnostic yield of CTCs and two tumor markers. We included 10 controls, 11 patients with cStage I-III PC and 10 patients with cStage I-IVA BC. The median values of all three biomarkers were higher in patients with PC than in the controls (PC vs control. CEA: 3.2 vs 1.7 ng/mL, $P = .05$, CA19-9:120.5 vs 6.9 U/mL, $P = .003$. CTC: 2.0 vs 0.0 cells/2 mL, $P < .0001$, respectively; Figure 3a). Among the three biomarkers, CTCs had the highest diagnostic performance (CTCs: AUC 0.90, cut-off 0.25 cells/2 mL, sensitivity 90.9%, specificity 81.3% and accuracy 84.6%), while other markers showed modest diagnostic performance (CA19-9: AUC 0.84, cut-off 43.7 U/mL, sensitivity 81.8%, specificity 81.3% and accuracy 80.7%. CEA: AUC 0.69, cut-off 2.3 ng/mL, sensitivity 64.3%, specificity 78.9% and accuracy 73.0%; Figure 3b,c). The median values of CA19-9 and CTCs were higher in patients with BC than in the controls (BC vs control. CA19-9: 96.2 vs 6.8 U/mL, $P = .003$. CTC: 2.0 vs 0.0 cells/2 mL, $P < .0001$, respectively), while the serum level of CEA was not significantly different between the two groups (2.4 vs 1.7 ng/mL, $P = .11$; Figure 4a). Among the three

TABLE 2 Clinical background of controls and patients with PB cancer

	Training set			Validation set		
	Control (n = 8)	Cancer (n = 24)	*P*-value	Control (n = 8)	Cancer (n = 23)	*P*-value
Age (years old), median (range)	48.0 (23.0-85.0)	68.5 (56.0-84.0)	.17	49.5 (30.0-77.0)	70.0 (54.0-75.0)	.09
Sex, M, n (%)	6 (75.0)	13 (54.2)	.42	5 (62.5)	14 (60.9)	.99
Background disease (n)		PC (15), BC (9)			PC (14), BC (9)	
cStage IV/IVB (AJCC/UICC ver. 8), n (%)		10 (32.3)			16 (51.6)	

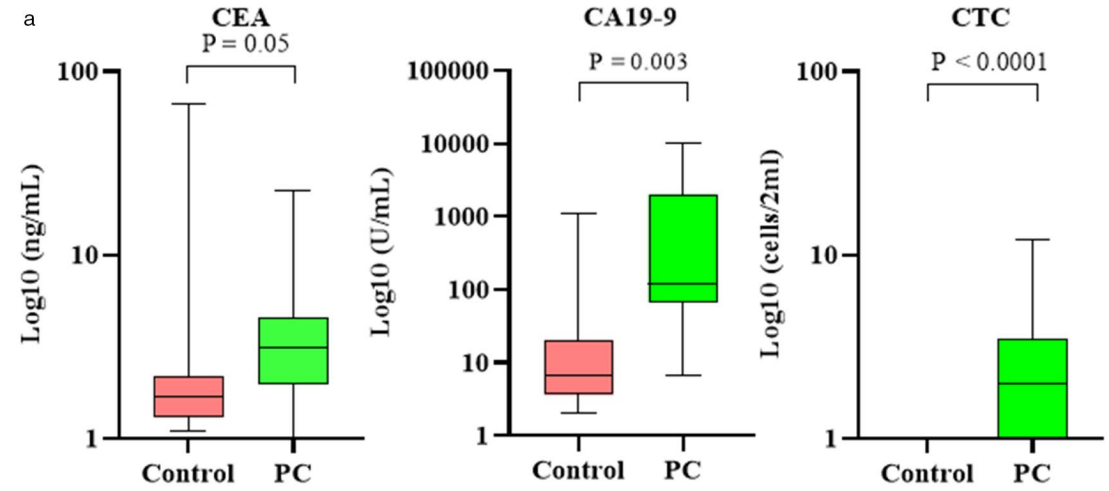
Abbreviations: AJCC/UICC, American Joint Committee on Cancer/Union for International Cancer; BC, biliary cancer; cStage, clinical stage; M, male; PC, pancreatic cancer.

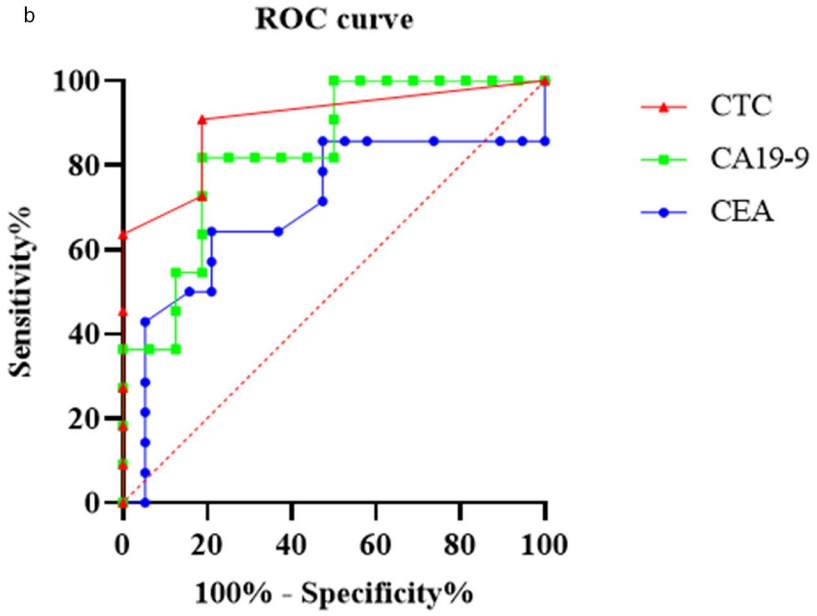


d

Markers	Cut-off value	Training set (n = 32)				Validation set (n = 31)			
		AUC	Accuracy (%)	Sensitivity (%)	Specificity (%)	AUC	Accuracy (%)	Sensitivity (%)	Specificity (%)
CTC (cells/2mL)	0.25	0.92	90.6	91.3	87.5	0.92	87.5	91.7	75.0
CA19-9 (U/mL)	8.5	0.88	90.6	95.6	75.0	0.89	78.1	95.8	37.5
CEA (ng/mL)	3.15	0.83	65.6	82.6	87.5	0.87	81.2	58.3	87.5

FIGURE 2 Diagnostic performance of biomarkers to distinguish non-tumor controls and patients with PB cancer. A, B, Comparison of three biomarkers between the training set (n = 32) and validation set (n = 31). C, D, Diagnostic performance of CEA, CA19-9 and CTC with regard to distinguishing between controls and patients with PB cancer. AUC, area under ROC curve; CEA, carcinoembryonic antigen; CA19-9, cancer antigen 19-9; CTC, circulating tumor cell; PB, pancreatico-biliary cancer; ROC, receiver operating characteristic





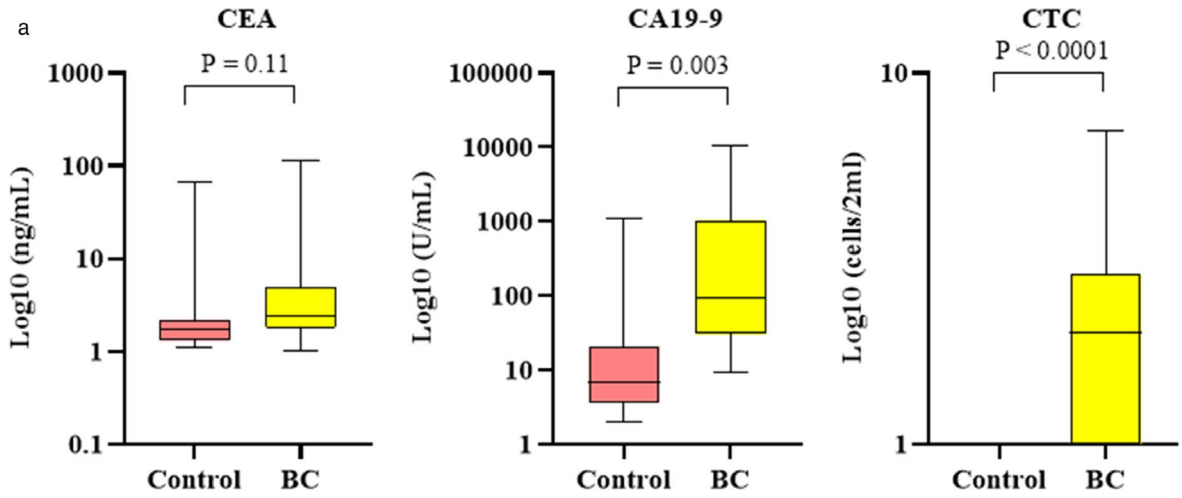
c

Markers	Cut-off value	AUC	Accuracy (%)	Sensitivity (%)	Specificity (%)
CTC (cells/2mL)	0.25	0.90	84.6	90.9	81.3
CA19-9 (U/mL)	43.7	0.84	80.7	81.8	81.3
CEA (ng/mL)	2.3	0.69	73.0	64.3	78.9

FIGURE 3 Diagnostic performance of biomarkers with regard to distinguishing between controls and patients with non-metastatic PC. A, Comparison of three biomarkers between controls (n = 16) and patients with PC (n = 11). B, C, Diagnostic performance of CEA, CA19-9 and CTC with regard to distinguishing between controls and patients with PC. AUC, area under ROC curve; CA19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen; CTC, circulating tumor cell; PC, pancreatic cancer; ROC, receiver operating characteristic

biomarkers, CTCs again had the highest diagnostic performance (CTCs: AUC 0.89, cut-off 0.25 cells/2 mL, accuracy 92.5%, sensitivity 90.0%, specificity 81.3%), while other markers showed mild to modest diagnostic performance (CA19-9: AUC 0.84, cut-off 28.3 U/mL, accuracy 81.4%, sensitivity 80.0%, specificity 81.3%. CEA: AUC 0.68, cut-off 3.55 ng/mL, accuracy 85.1%, sensitivity 40.0%, specificity 94.7%; Figure 4b,c).

a



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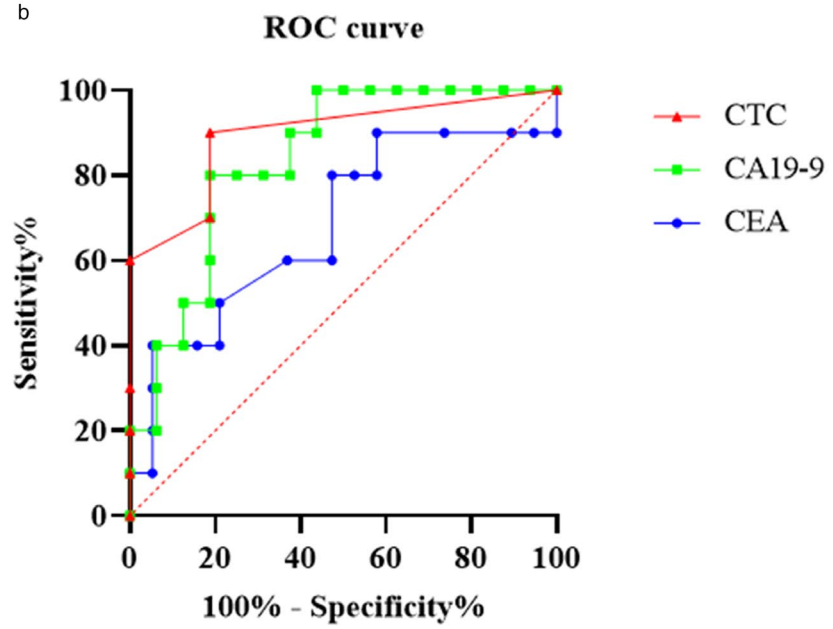

c

Markers	Cut-off value	AUC	Accuracy (%)	Sensitivity (%)	Specificity (%)
CTC (cells/2mL)	0.25	0.89	92.5	90.0	81.3
CA19-9 (U/mL)	28.3	0.84	81.4	80.0	81.3
CEA (ng/mL)	3.55	0.68	85.1	40.0	94.7

FIGURE 4 Diagnostic performance of biomarkers with regard to distinguishing between controls and patients with non-metastatic BC. A, Comparison of three biomarkers between controls (n = 16) and patients with BC (n = 10). B, C, Diagnostic performance of CEA, CA19-9 and CTC with regard to distinguishing between controls and patients with BC. AUC, area under ROC curve; BC, biliary cancer; CA19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen; CTC, circulating tumor cell; ROC, receiver operating characteristic

TABLE 3 Comparison of clinical backgrounds between patients with high CTC and low CTC counts

	≥2.0 CTCs/2 mL (n = 9)	<2.0 CTCs/2 mL (n = 7)	*P*-value
Age (years old), median (range)	70.0 (57.0-73.0)	66.0 (54.0-75.0)	.24
Sex, M, n (%)	5 (55.4)	6 (85.7)	.30
Tumor size (mm), median (range)	38.5 (10.0-88.0)	30.0 (25.0-84.0)	.39
CEA (ng/mL), median (range)	5.2 (2.0-25.0)	7.8 (3.0-539.1)	.12
CA19-9 (U/mL), median (range)	5,833.0 (117.0-135603.0)	1677.0 (9.0-160310)	.46
First-line regimen, combination, n (%)	6 (66.7)	5 (71.4)	.99

Abbreviations: CA19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen; cStage, clinical stage; CTCs, circulating tumor cells; M, male.

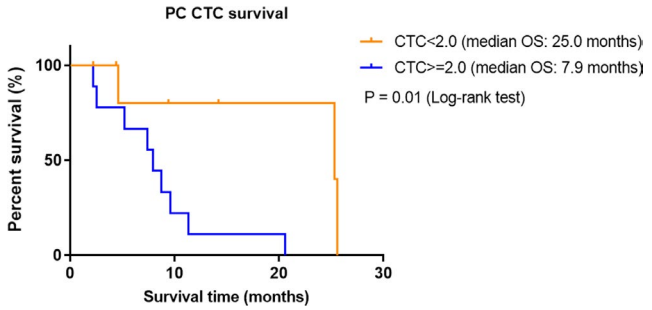

FIGURE 5 Differences in the prognosis of PC patients who underwent systemic chemotherapy based on the CTC count. CTC, circulating tumor cell; OS, overall survival; PC, pancreatic cancer

3.3 | Prognostic yield of biomarkers in PC patients

We included 16 cStage IV PC patients who underwent systemic chemotherapy and divided them into two groups based on the number of CTCs (more than two or fewer than 2). The clinical background of the patients is shown in Table 3. Briefly, there were no significant differences between the two groups in age, sex, tumor size, serum levels of CEA, serum levels of CA19-9 and first-line treatment regimens. We found that the median OS was longer in patients with fewer than 2 CTCs/2 mL than in patients with more than 2 CTCs/2 mL (median OS: 25.0 months vs 8.0 months, $P = .01$; Figure 5).

4 | DISCUSSION

In the current study, we aimed to clarify whether CTCs could be a diagnostic and prognostic marker for PB cancer using a newly developed microfluidic device. CTCs performed well for discriminating between patients with PB cancer in any stage and controls. Moreover, CTCs were already present even in non-metastatic stages (cStage I-III in PC and cStage I-IVA in BC). These results highlighted that CTCs might be useful for cancer screening in the general population, enabling us to detect PB cancer in the relatively early stages. Furthermore, the counts of CTCs appeared to be related to the prognosis of advanced PC patients who underwent systemic chemotherapy. To the best of our knowledge, this is the first study to prove the diagnostic and prognostic role of CTCs in PB cancer using one device.

CTCs are released into the blood from primary tumors at a concentration of approximately 10^6 CTCs/g of tumor per day, even when imaging studies cannot detect apparent metastases.[22] To date, several CTC detection systems have been developed and have been mainly used for research purposes.[23,24] There are several competing modalities of CTC capture technology, but they can be grossly categorized into two types; label-based (or affinity-based) and label-free.[25–27]

Label-based capture is the most widely used strategy. This technology is based on the hypothesis that tumor cells display different surface markers than blood cells and can therefore be separated from the rest of the circulatory cells on this basis. Label-free technologies use differences between tumor cells and blood cells in size, deformability, density and electric change. The CellSearch System is a representative label-based technology and the only tool approved by the United States Food and Drug Administration. This system captures CTCs using epithelial cell-specific EpCAM antibodies and several immunofluorescent markers for epithelial cells (eg, cytokeratins) while omitting leukocytes using CD45.[28] In PC, CTCs detected by the CellSearch System (Veridex) were found to be a prognostic marker; however, CTCs may not be a reliable diagnostic marker because CTCs were only found in 20.0%-75.0% of PC patients.[17,29] A few studies have evaluated the presence of CTCs in BC using the CellSearch System. Yang

et al. investigated the prognostic role of CTCs in patients with various types of BC (41 with intrahepatic cholangiocarcinomas, 42 with peri-hilar cholangiocarcinomas and seven with distant cholangiocarcinomas) and found that one or more CTCs were detected by the CellSearch System in only 28% of these patients with BC.[18] This was probably due to the low positivity rate of EpCAM in BC.[30] Other CTC detection systems, such as the ScreenCell System (ScreenCell, Westford, MA, USA), rely on the relatively larger size of CTCs rather than on the presence of antigens and show higher although still modest CTC detection rates (51.0%-75.8%).[31–33] The newly developed microfluidic chip device used in the current study captures blood cells based on their size first and then selects CTCs based on cell surface antigens. This system had excellent diagnostic performance with a sensitivity of 94.0% and specificity of 100% in 128 cancer patients (95 breast cancer, 27 prostate cancer and five colorectal cancer) and 200 healthy volunteers. Additionally, Horimoto et al. reported that 21 out of 22 patients (95.4%) with breast cancer had one or more CTCs, even though 64 patients had already been treated with chemotherapy.[14] Our results also showed a similar high diagnostic performance in patients with PB cancer.

Several limitations were found in this study. First, this study was conducted in a single referral center, and the results may not be generalizable to all patients with PB cancer. The relatively small sample size also limited the reliability of our statistical analysis. Second, we could not conduct a survival analysis in patients with BC because the majority of patients did not undergo chemotherapy. Third, it is unknown whether CTCs can be detected in high-risk individuals (ie, those with intraductal papillary mucinous neoplasm and chronic pancreatitis for PC screening and those with primary sclerosing cholangitis for BC screening). Therefore, we must conduct a further study including a large number of patients with various clinical backgrounds.

In conclusion, the newly developed microfluidic device described here could diagnose pancreatico-biliary cancers by detecting CTCs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest for this article.

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