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Clinical Significance of Circulating Tumor Microemboli as a Prognostic Marker in Patients with Pancreatic Ductal Adenocarcinoma

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BACKGROUND: Characterization of circulating tumor cells (CTCs) has been used to provide prognostic, predictive, and pharmacodynamic information in many different cancers. However, the clinical significance of CTCs and circulating tumor microemboli (CTM) in patients with pancreatic ductal adenocarcinoma (PDAC) has yet to be determined.

METHODS: In this prospective study, CTCs and CTM were enumerated in the peripheral blood of 63 patients with PDAC before treatment using anti-EpCAM (epithelial cell adhesion molecule)–conjugated supported lipid bilayer–coated microfluidic chips. Associations of CTCs and CTM with patients' clinical factors and prognosis were determined.

RESULTS: CTCs were abundant [mean (SD), 70.2 (107.6)] and present in 81% (51 of 63) of patients with PDAC. CTM were present in 81% (51 of 63) of patients with mean (SD) 29.7 (1101.4). CTM was an independent prognostic factor of overall survival (OS) and progression free survival (PFS). Patients were stratified into unfavorable and favorable CTM groups on the basis of CTM more or less than 30 per 2 mL blood, respectively. Patients with baseline unfavorable CTM, compared with patients with favorable CTM, had shorter PFS (2.7 vs 12.1 months; $P < 0.0001$) and OS (6.4 vs 19.8 months; $P < 0.0001$). Differences persisted if we stratified patients into early and advanced diseases. The number of CTM before treatment was an independent predictor of PFS and OS after adjustment for clinically significant factors.

CONCLUSIONS: The number of CTM, instead of CTCs, before treatment is an independent predictor of PFS and OS in patients with PDAC.

Pancreatic ductal adenocarcinoma (PDAC)[8] is the fourth leading cause of cancer death in the US and European Union *(1, 2)*. PDAC is one of the most aggressive human malignancies, with clinical characteristics of local invasion, early metastasis, and resistance to standard chemotherapy *(3)*. Depending on the extent of disease at diagnosis, the current standard of care includes surgical resection in early disease and chemotherapy in advanced disease. Currently, the surgical outcome in PDAC remains unsatisfactory because many postoperative patients experience distant metastasis shortly after surgery. Most patients (80%) with PDAC are diagnosed with unresectable advanced disease at the time of diagnosis *(3)*. The most commonly used tumor marker for PDAC has been carbohydrate antigen 19-9 (CA19-9), a sialylated Lewis antigen with an overall diagnostic sensitivity of 80% and specificity of 82% *(4)*. However, patients with blood type of Lewis a^- b^- genotype are incapable of synthesizing the CA19-9 epitope *(5)*. In addition, CA19-9 may also be increased in patients with nonmalignant diseases such as cirrhosis, chronic pancreatitis, cholangitis, and cholestasis *(6)*. Better biomarkers are needed for PDAC diagnosis and prediction of clinical outcome.

Circulating tumor cells (CTCs), which are most likely shed from the primary tumor, are rare cells in transit in the bloodstream of patients with solid tumors. CTC burden has been shown to be predictive of survival in

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[8] Nonstandard abbreviations: PDAC, pancreatic ductal adenocarcinoma; CA19-9, carbohydrate antigen 19-9; CTC, circulating tumor cell; PFS, progression-free survival; OS, overall survival; CTM, circulating tumor microemboli; SLB, supported lipid bilayer; EpCAM, epithelial cell adhesion molecule; DAPI, 4′,6-diamidino-2-phenylindole; RECIST, response evaluation criteria in solid tumors; CEA, carcinoembryonic antigen; DM, diabetes mellitus; CK-20, cytokeratin 20; RT, reverse transcription; qPCR, quantitative PCR.

metastatic breast, colorectal, prostate, and lung cancers *(7–10)*. The number of CTCs in patients with breast cancer is an independent predictor of progression-free survival (PFS) and overall survival (OS) *(7)*. The clinical implications of CTCs in PDAC have not been proven to be indicative as in other cancers *(11)*. Circulating tumor microemboli (CTM) have been reported to be associated with prognosis of small cell lung cancer *(12, 13)*. Recently, CTM in PDAC have been examined and described in limited studies with few cases; therefore, its clinical significance is not yet fully known *(14, 15)*. On the basis of these observations, we initiated the prospective study described here to evaluate CTCs and CTM as prognostic and/or predictive markers in patients with PDAC.

Materials and Methods

STUDY DESIGN

This was a prospective, single-center study conducted at the National Taiwan University Hospital. Eligible patients had histologically or cytopathologically confirmed PDAC. All patients gave written informed consent to the ethically approved protocols. Blood samples were collected as described below for analysis before treatment. Data on patient characteristics, including clinical/biochemical factors, were collected. A total of 63 patients with PDAC were enrolled consecutively in our hospital between September 2012 and February 2014. During the same period, 23 noncancer volunteers were examined for numbers of CTC and CTM. Thirty patients with PDAC underwent surgery. For patients who could not undergo resection, chemotherapy was administered unless patients refused it or were in poor condition. Twenty-three patients with advanced PDAC were treated with gemcitabine or TS-1 based therapy and 10 advanced patients received supportive care. Eligibility criteria for treatment in the study included Eastern Cooperative Oncology Group status of 0–1, adequate bone marrow, hepatic, and renal function. This study was approved by the institutional review board of National Taiwan University Hospital.

CAPTURE, ENUMERATION, AND CLASSIFICATION OF CTCS AND CTM

Isolation and identification of CTCs were performed under a biomimetic supported lipid bilayer (SLB) surface–coated microfluidic chip (CMx platform) conjugated with anti–epithelial cell adhesion molecule (EpCAM) as the CTC-capturing antibody *(16, 17)*. In brief, 2 mL of fresh blood sample collected from the patient into an EDTA Vacutainer Tube was transferred and traversed through the anti-EpCAM–coated SLB microfluidic chip under a 1.5 mL/h flow rate without additional processing. After a 9 mL/h flow rate of phosphate-buffered solution wash to eliminate nonspecific bounded cells, the captured cells were released by simply introducing a hydrophobic component such as air bubbles, which disintegrated the SLB assembly.

The cells were released onto the 2-μm–pore size membrane and followed with 4% paraformaldehyde fixation for 15 min, 0.1% triton X-100 penetration for 15 min, and 5% normal–goat serum blocking for 1 h. The polyclonal antibody against wide-spectrum cytokeratin (Abcam, ab9377, 1:200, 4 °C staining overnight) and Alexa Fluor 647 (Life technology, 1:500, room temperature staining for 1 h) were used for epithelial origin cancer cell identification. The antibody of CD45-FITC (Dako, f0861, 1:10, room temperature staining for 1 h) was used for the staining of the leukocytes. Phosphate buffered solution was used to wash out nonbonded antibodies after each staining step. Following with sealing by using 4′,6-diamidino-2-phenylindole (DAPI) containing ProLong Gold Antifade Mountant (Life Technology), the concomitant nuclear staining was allowed in one procedure. The sealed membrane was photographed under a Nikon ECLIPSE Ti-E fluorescence microscope with 10-fold magnification and a Leica TCS SP5 confocal microscope with 40-fold magnification for the following analysis. The Nikon NIS-Elements AR was used for image analysis and CTC selection. Cells with panCK+(red)/CD45−(green)/DAPI+(blue) staining were enumerated as CTCs. Microemboli with multiple cells containing at least 2 distinct nuclei larger than 15 μm in diameter with at least 1 panCK+/CD45−/DAPI+ CTC with/without leukocytes were classified as a CTM. To prevent false assignment of a mitotic CTC as a microembolus, CTM were defined as groups of cells containing 2 or more distinct nuclei. The images and detailed classification of CTCs are shown in Fig. 1.

CELL-SPIKING EXPERIMENTS

The human pancreatic cancer AsPC1 cell line was used as the control cell model in each of the CTC-capturing experiments. The cells were first prestained with cell tracker green CMFDA (ThermoFisher Scientific) and spiked into glass-bottom wells (length × width × height, 6 mm × 6 mm × 5 mm) with waiting for 10 min to allow cells to settle down. The cells in the glass-bottom well were counted and transferred directly into the medium or blood from noncancer volunteers after counting for capture efficiency control of the CMx platform. The capture efficiency showed a positive linear correlation with mean 80% and 60% capturing efficiency and linear regression coefficient R^2 values of 0.95 and 0.93, respectively. For QC of the CMx chip, the capturing efficiency of each batch (20–30 chips) was tested by use of a prestained HCT116 colorectal cancer cell line spiked in medium on a randomly selected chip. The mean binding

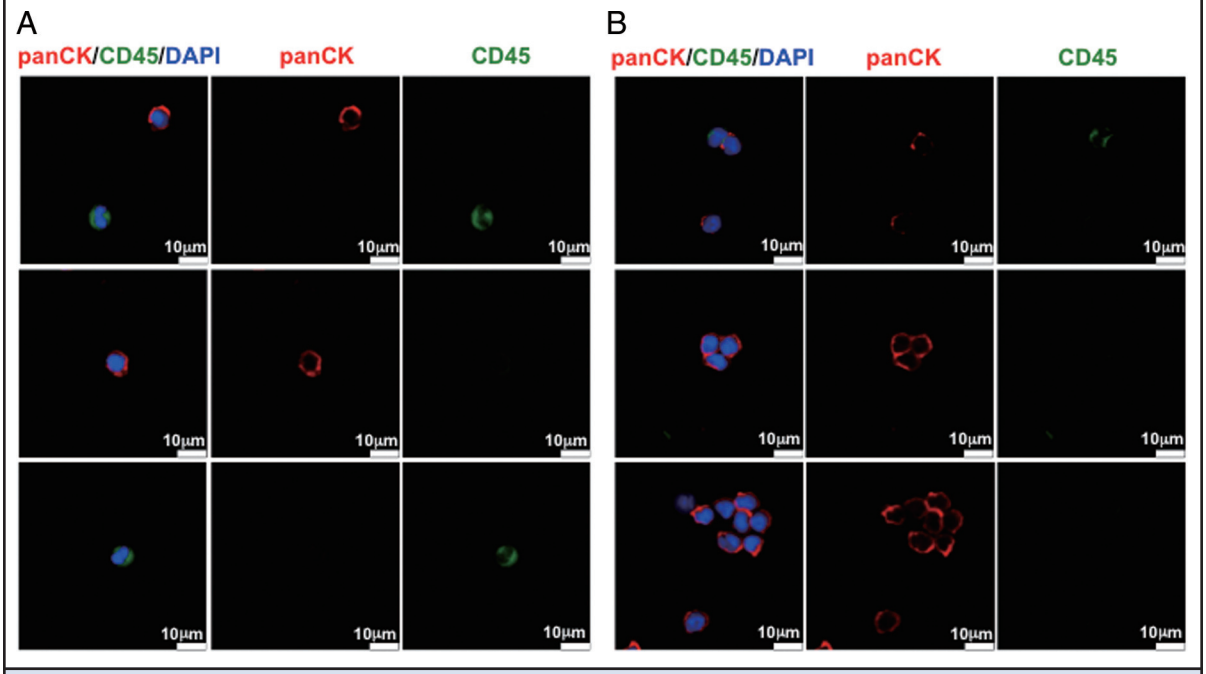

Fig. 1. (A), Confocal images of representative CTCs and leukocytes obtained from CMx platform.
CTCs were defined as panCK+(red)/ CD45−(green)/DAPI+(blue) cells (middle lane), and leukocytes represented as panCK−/CD45+/DAPI+ cells (lower lane). (B), Confocal images of representative CTM captured by a CMx platform. The clusters representing at least 2 distinct nuclei contain at least 1 CTC with or without leukocytes.

efficiency of HCT116 on the total 852 CMx chip was 92% (SD, 1%). When the binding efficiency was lower than 80%, the entire batch was discarded.

EFFICACY ASSESSMENT

Standard response evaluation criteria in solid tumors (RECIST) criteria were used to determine the objective tumor response by computed tomography or MRI. At the time of analysis of CTCs and CTM, the tumor markers CA19-9 and carcinoembryonic antigen (CEA) in sera of all patients were also examined by a chemiluminescent microparticle immunoassay (Architect i4000, Abbott Inc.). The intraassay and total imprecision values of CA19-9 were 5.8% and 6.5% at 45.03 U/mL, 3.8% and 5.4% at 157.66 U/mL, and 5.7% and 6.4% at 781.68 U/mL. Imprecision values for CEA were 3.6% and 4.0% at 5.05 ng/mL, 2.5% and 3.2% at 20.17 ng/mL, and 3.1% and 3.2% at 99.45 ng/mL.

STATISTICAL ANALYSIS

Associations of baseline CTC number and CTM number with individual clinical and biochemical factors were compared using the χ^2 test or Fisher exact test. Correlations between baseline CTC number and CTM number were compared using Spearman's ρ analysis. Values are expressed as mean (SD). The strength of association was estimated by calculating the hazard ratio. The Kaplan–Meier test was used for survival and time to progression. The log–rank test was applied to compare survival and time to progression between subgroups. CTC number and CTM number and standard clinical/biochemical factors were subjected to univariate Cox proportional hazards regression analysis for PFS and OS. Univariately significant parameters were included in a multivariate Cox regression analysis. PFS and OS were measured from date of diagnosis to date of confirmed clinical progression, death, or censoring at last follow-up. A value of $P<0.05$ was considered to indicate significance. All analyses were performed with the SPSS software package version 17 (SPSS).

Results

PATIENT DEMOGRAPHICS

A total of 63 patients were enrolled in this study. At the time of analysis, 24 (38.1%) of the 63 patients had experienced disease progression and 17 (26.9%) of the 63 patients had died, resulting in a mean PFS of 7.4 months (95% CI, 5.9–8.9 months) and OS of 11.2 months (95% CI, 7.2–14.2 months). The mean length of follow-up time for the 46 patients still alive was 8.2 (5.6) months (range 3.0–26.0 months). The clinical charac-

Table 1. Patient Characteristics and the circulating tumor cells and CTM.[a]

	Early (n = 33)	Advanced (n = 30)	Overall (n = 63)	*P* value
Age	64.0 (10.8)	65.6 (12.1)	64.8 (11.4)	0.587
Sex, male/female[b]	26/7	14/16	40/23	0.008
Location of tumor, head/body/tail[b]	29/3/1	15/11/4	44/14/5	0.005
DM	19 (57.6%)	15 (50.0%)	34 (54.0%)	0.547
Smoking	7 (21.2%)	6 (20.0%)	13 (20.6%)	0.577
TNM[c] stage[b]				<0.0001
1	1 (3%)	0 (0.0%)	1 (1.6%)	
2	32 (97.0%)	0 (0.0%)	32 (50.8%)	
3	0 (0.0%)	10 (33.3%)	10 (15.9%)	
4	0 (0.0%)	20 (66.7%)	20 (31.7%)	
Performance status ECOG 0–1	33 (100.0%)	23 (76.7%)	53 (84.1%)	0.223
CA199	673 (1239.1)	1267 (2155.9)	931 (1706.2)	0.212
CEA	5.0 (9.8)	290.3 (1139.0)	145.1 (803.9)	0.183
Albumin	4.1 (0.6)	4.6 (2.6)	4.4 (1.8)	0.282
Leukocyte count	7145 (1926.0)	7504 (4611.4)	7316 (3448.7)	0.684
Neutrophil count	5000 (2023.1)	5361 (4253.2)	5175 (3273.1)	0.668
Lymphocyte count	1512 (569.3)	1504 (521.3)	1508 (542.2)	0.952
Neutrophil/lymphocyte ratio	5.1 (7.8%)	3.7 (2.5%)	4.4 (5.9%)	0.342
C-reactive protein	0.76 (1.21)	2.40 (6.12)	1.76 (4.90)	0.267
Chemotherapy	19 (57.6%)	19 (63.3%)	38 (60.3%)	0.641
CTC number	49.9 (109.8	92.4 (102.4)	70.2 (107.6)	0.119
CTM number	8.9 (16.7)	52.5 (143.6)	29.7 (101.4)	0.88
Unfavorable CTM	2 (6.1%)	7 (23.3%)	9 (14.3%)	0.07

[a] Values presented as mean (SD), n, or n (%).
[b] $P < 0.05$.
[c] TNM, tumor, node, metastasis; ECOG, Eastern Cooperative Oncology Group.

teristics of the 63 patients with PDAC are shown in Table 1. The mean age was 64.8 (11.4) years; 40 patients were men. The PDAC were located in the head of the pancreas in 44 patients (69.8%), body in 14 patients (22.2%), and tail in 5 patients (7.9%). The stage of PDAC was stage I in 1 patient, II in 32 patients, stage III in 10 patients, and stage IV in 20 patients. There were 12 patients with liver metastasis at diagnosis. Regarding treatment, the 30 patients who underwent surgery had attempted curative resections, pancreatoduodenectomy in 26 patients, pancreaticosplenectomy in 3 patients, and 1 total pancreatectomy. Twenty-three of the 33 nonsurgical patients received chemotherapy with gemcitabine or TS-1–based regimen. These 23 patients received a median of 5.2 (4.1) cycles (range, 1–17 cycles) of chemotherapy. Of the remaining 10 patients, 3 refused chemotherapy because of active hepatitis B and 7 had poor general conditions. Partial response was observed in 1 patient, and stable disease was observed in 21 patients, whereas 24 patients developed progressive disease. The OS time for all 63 patients was 11.4 (1.7) months. The OS in early diseases (stage I, II) was 20.1 (1.9) months, advanced diseases (stage III, and IV) 15.4 (2.9) months. There was 1 patient who had grade 3 neutropenia who received combined gemcitabine and TS-1 therapy. There were no grade 3–4 infections or grade 3–4 thrombocytopenia.

CTCs AND CTM IN PATIENTS WITH PDAC

CTCs were detected in 81% of patients (51 of 63 patients) before treatment. The mean CTCs per 2 mL of blood in PDAC was 70.2, in contrast to 3.7 CTCs in the noncancer volunteers ($P = 0.004$). On the basis of a cutoff of ≥70 CTC per 2 mL of blood, 17 (27.0%) patients had an unfavorable CTC number before treatment. An unfavorable CTC number was not significantly associated with stage, leukocyte count, lymphocyte count, neutrophil count, or neutrophil/lymphocyte ratio, CEA, and CA19-9.

CTM were observed in 81% (51 of 63) patients. The mean CTM per 2 mL of blood in PDAC were 29.5, in contrast to 0 CTM in the noncancer volunteers (P = 0.014). There was no significant correlation between CTC number and CTM number (P = 0.476) (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue3). The mean CTM in stage I, II, III, and IV of PDAC were 0, 9.2, 15.2, and 71.2 (P = 0.176). The mean CTM in early (stage I and II) and advanced (stage III and IV) PDAC were 8.9 and 52.5 (P = 0.088). On the basis of these results, we defined a CTM count over 30 CTM per 2 mL of blood as "unfavorable CTM." On the basis of a cutoff of ≥30 CTM per 2 mL of blood, 9 patients had unfavorable CTM numbers before treatment, including 2 patients with early stage (both stage II) and 7 with advanced stage disease (2 stage III and 5 stage IV). An unfavorable CTM number was not significantly associated with stage, tumor size, leukocyte count, lymphocyte count, neutrophil count, or neutrophil/lymphocyte ratio.

PROGNOSTIC SIGNIFICANCE OF CTCS

For patients with an unfavorable CTC number (≥70 CTCs), the mean PFS (3.6 months; 95% CI, 1.8–6.9 months) and OS (15.7 months; 95% CI, 8.6–22.8 months) were not statistically different from those for patients with favorable CTC numbers (mean PFS, 3.9 months; 95% CI, 2.5–18.0 months; mean OS, 19.1 months; 95% CI, 15.6–22.7 months, P = 0.118 and 0.112).

PROGNOSTIC SIGNIFICANCE OF CTM

In univariate analysis for CTM, patients were categorized into favorable and unfavorable groups (<30 CTM vs ≥30 CTM per 2 mL blood). For patients with an unfavorable CTM, there was a significantly shorter PFS (mean 2.7 months; 95% CI, 1.4–3.9 months) and OS (mean 6.4 months; 95% CI, 3.5–9.3 months) compared with patients with favorable CTM (mean PFS, 12.1 months; 95% CI, 8.9–15.3 months; mean OS, 19.8 months; 95% CI, 16.5–23.2 months; Figs. 2A and 3A). In patients with early disease, there were 2 patients with unfavorable CTM. These 2 patients died about 3 months after surgery because of distant metastasis. The mean PFS of patients with unfavorable CTM in early stage PDAC was statistically significantly shorter than that of patients with favorable CTM in early stage PDAC (3.3 months; 95% CI, 2.8–3.8 months vs 12.2 months; 95% CI, 8.8–15.6 months; P < 0.001) (Fig. 2B). In advanced stage, the mean PFS of patients with unfavorable CTM was also statistically significantly shorter than that of patients with favorable CTM (1.4 months, 95% CI, 1.4–1.4 months vs 7.9 months, 95% CI, 6.7–9.2 months, P < 0.0001) (Fig. 2C).

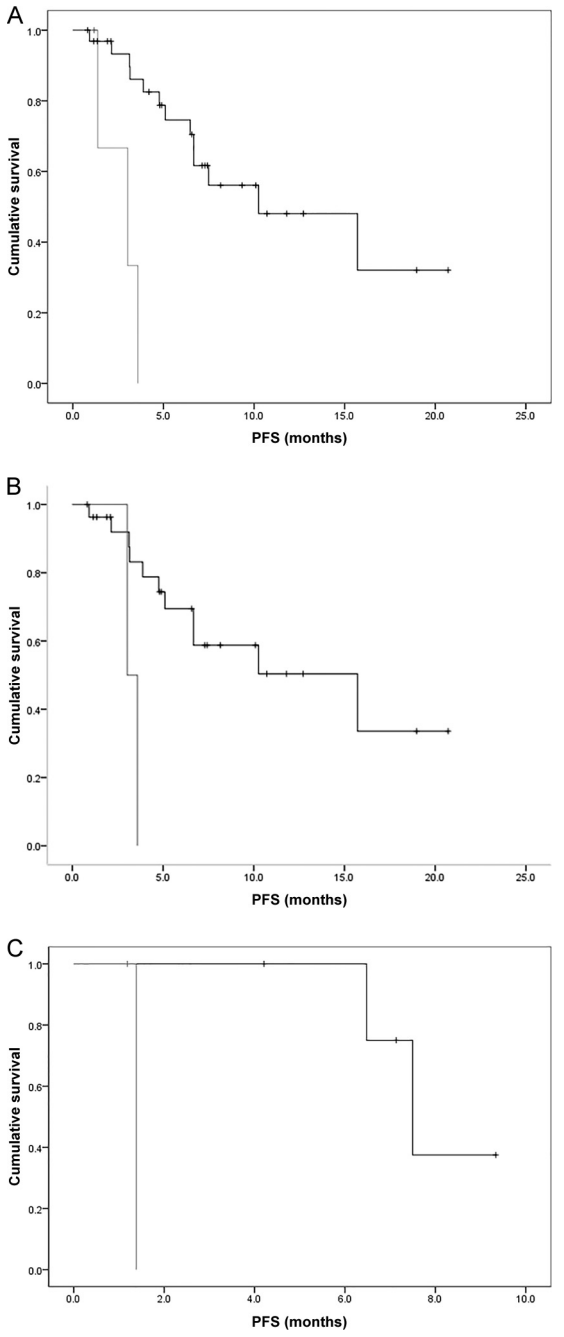

Fig. 2. (A), Kaplan–Meier life-table analysis of the PFS time in all patients.

(B), Kaplan–Meier life-table analysis of PFS time in patients with early PDAC. (C), Kaplan–Meier life-table analysis of PFS time in patients with advanced PDAC. For each Fig. section the gray line represents unfavorable CTM and the black line represents favorable CTM.

The mean OS of patients with unfavorable CTM in early stage PDAC was statistically significantly shorter than that of patients with favorable CTM in early stage PDAC (7.3 months, 95% CI, 1.0–13.5 months, vs 21.7 months, 95% CI, 18.3–25.0 months, $P < 0.0001$) (Fig. 3B). In advanced stage, the mean OS of patients with unfavorable CTM was also statistically significantly shorter than that of patients with favorable CTM (5.5 months, 95% CI, 2.9–8.1 months vs 17.0 months, 95% CI, 10.8–23.4 months, $P < 0.0001$) (Fig. 3C).

CTM AND CEA, CA19-9

The mean CA19-9 concentration was 931.1 (1706.2) U/mL (range, 1–8030 U/mL) in all patients, 905.16 (916.2) U/mL (range, 26–2646 U/mL) in the unfavorable CTM patients, and 934.4 (1788.6) U/mL (range, 1–8030 U/mL) in the favorable CTM patients, showing no significant difference between the unfavorable CTM and favorable CTM patients ($P = 0.969$). The mean CEA concentration was 145.1 (803.9) U/mL (range, 1–5784 U/mL) in all patients, 288.7 (746.5) U/mL (range, 2–1981 U/mL) in the unfavorable CTM patients, and 125 (816.7) U/mL (range, 1–5784 U/mL) in the favorable CTM patients, showing no significant difference between the unfavorable CTM and favorable CTM patients ($P = 0.618$).

PREDICTORS OF PFS

Univariate analysis results, including age, sex, diabetes mellitus (DM), smoking status, advanced stage, poor differentiation, chemotherapy, and unfavorable CTM, were analyzed for predictors of PFS. Only unfavorable CTM was identified as a predictor of poor PFS. Multivariate analysis indicated that unfavorable CTM, in addition to age, sex, and DM, was a predictor of poor PFS, as determined by multiple logistic regression analysis after adjusting for age, sex, DM, smoking status, advanced stage, poorly differentiation, chemotherapy ($P = 0.003$, Table 2). Chemotherapy was a predictor of better PFS ($P = 0.008$, Table 2).

PREDICTORS OF OS

Univariate analysis results, including age, sex, DM, smoking status, advanced stage, poor differentiation, chemotherapy, and unfavorable CTM were analyzed for predictors of OS. Unfavorable CTM was identified as a predictor of poor survival. Multivariate analysis indicated that unfavorable-CTM, in addition to advanced stage, was a predictor of poor OS, as determined by multiple logistic regression analysis after adjusting for age, sex, DM, smoking status, advanced stage, poor differentiation, and chemotherapy ($P < 0.0001$, Table 3).

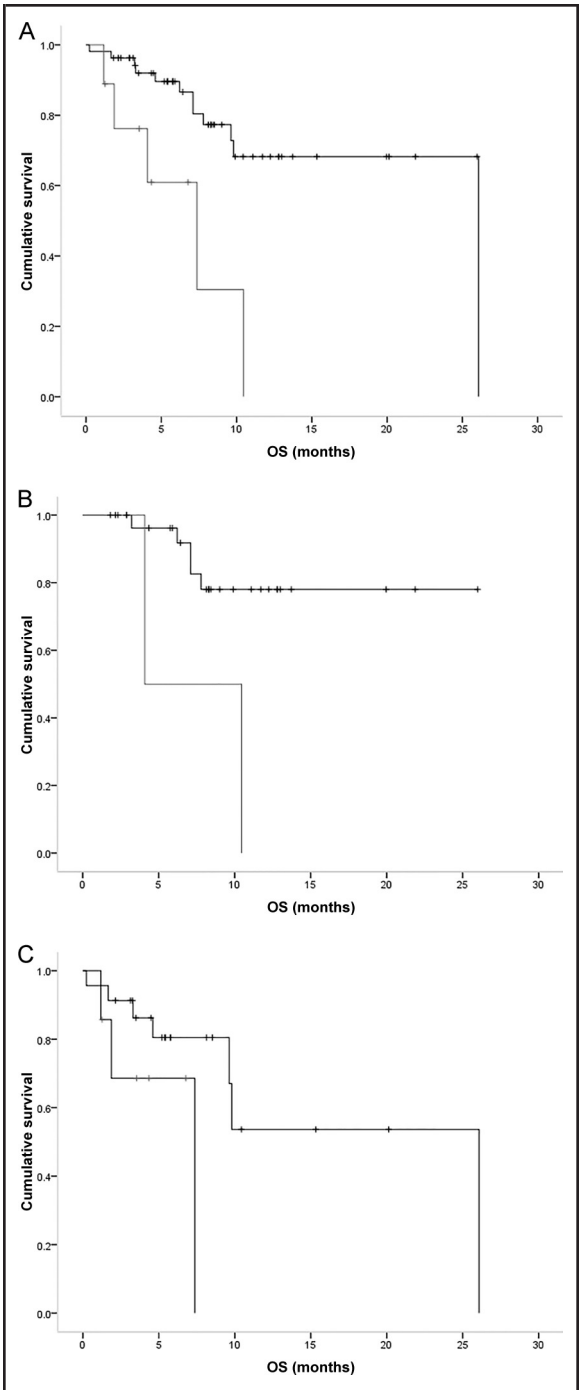

Fig. 3. (A), Kaplan–Meier life-table analysis of OS time in all patients.

(B), Kaplan–Meier life-table analysis of OS time in patients with early PDAC. (C), Kaplan–Meier life-table analysis of OS time in patients with advanced PDAC. For each Fig. section the gray line represents unfavorable CTM and the black line represents favorable CTM.

Table 2. Predictors of PFS by Cox regression model.[a]

	P value			
	Univariate	Multivariate	Hazard ratio	95% CI
Age[b]	0.900	0.012	1.09	1.02–1.16
Sex[b]	0.103	0.016	6.35	1.41–28.58
DM[b]	0.992	0.039	5.35	1.09–26.29
Smoking	0.896	0.205	0.35	0.07–1.79
Advanced stage	0.106	0.275	0.39	0.08–2.09
Poorly differentiation	0.824	0.745	1.29	0.28–5.93
Chemotherapy[b]	0.348	0.008	0.03	0.003–0.416
Unfavorable CTM[b,c]	0.002	<0.0001	486.66	12.38–12884.94

[a] Unfavorable CTM defined by the cluster number over the mean of all cases (mean: CTM = 30).
[b] $P < 0.05$ in multivariate analysis.
[c] $P < 0.05$ in univariate analysis.

Discussion

The prognostic value of CTM has been demonstrated in breast cancer *(18)*, colorectal cancer *(8)*, and small cell lung cancer *(12, 13)*. Our current study demonstrated that CTM has a prognostic value for predicting OS and PFS in patients with PDAC. The presence of unfavorable CTM (≥30 CTM per 2 mL of blood) detected before treatment was significant for inferior PFS and OS and was an independent clinical prognostic factor. Our report is the first one on the presence of CTM in PDAC and with their association with worse survival. In our study, the unfavorable number of CTM was associated with shorter survival in all patients and in stratified groups (early or advanced) with PDAC. The PFS and OS were both shorter in patients with unfavorable CTM. The prognostic role of CTC for predicting OS in PDAC has had differing results in the literature. Soeth et al. reported that the detection of circulating cytokeratin 20 (CK-20)-positive cells by reverse transcription (RT)-PCR in 33.8% of patients with PDAC was associated with shorter OS *(19)*. Kurihara et al. reported that patients with CTC-positive cells detected by CellSearch platform in 42% (11 of the 26) patients with PDAC had shorter OS *(20)*. However, Khoja et al. reported that there was a nonsignificant trend toward decreased survival for patients with detectable CTC by CellSearch or ISET (isolation by size of epithelial tumor cells) platforms in 54 patients with PDAC *(15)*. In our study, the number of CTC was not associated with OS. The discrepancies of the impacts of CTC enumeration on OS of pancreatic cancer in different studies might result from several factors. First, different platforms might capture different subsets of CTCs with distinct biological properties with

Table 3. Predictors of overall survival by Cox regression model.

	P value			
	Univariate	Multivariate	Hazard ratio	95% CI
Age	0.996	0.834	0.99	0.94–1.05
Sex	0.267	0.208	2.72	0.57–12.95
DM	0.277	0.141	1.68	0.84–3.35
Smoking	0.641	0.618	1.42	0.36–5.53
Advanced stage[a]	0.115	0.025	4.37	1.21–15.81
Poorly differentiated	0.446	0.675	1.29	0.39–4.25
Chemotherapy	0.061	0.071	0.29	0.77–1.11
Unfavorable CTM[a,b]	0.005	0.003	8.18	2.05–32.67

[a] $P < 0.05$ in multivariate analysis.
[b] $P < 0.05$ in univariate analysis.

varied impacts on the OS in PDAC patients. Most of the platforms for detecting CTC, including CellSearch and our platforms, are dependent on CTC expression of epithelial markers such as EpCAM and cytokeratin. The expression of EpCAM on pancreatic CTCs is heterogeneous and the relevance of EpCAM expression to CTC behavior and metastatic potential is undetermined *(21, 22)*. Besides, the study that demonstrated that CTC numbers were associated with shorter survival in pancreatic cancer had limited cases *(20)*. Larger studies with adequate patient numbers as well as characterization of the CTC population need be conducted to assess the clinical significance of CTC number in pancreatic cancer.

The common approaches for detection of CTCs in patients with PDAC include (*a*) immunological assays using antibodies directed against cell surface antigen, (*b*) technologies based on physical or biological properties of cancer cells, and (*c*) PCR-based molecular assays for tumor-derived DNA or RNA extraction from CTCs *(11)*. Most of the current reported platforms usually detect small numbers of CTCs in late-stage PDAC. The CellSearch system is the most commonly used platform, which usually detects small numbers of CTC even in advanced-stage PDAC. In our study, we detected much larger numbers of CTCs in both patients with early and those with advanced stage. The detection rates of CTCs (51of 63 patients, 81%) and CTM (51 of 63 patients, 81%) were higher than in previous reports. In most cancers studied, CTCs were rarely detectable in early stages of disease, including PDAC. In this study, CTCs were detected in 19 of 30 (63.6%) of patients with an early stage of PDAC [median, 3; range, 0–424, mean (SD), 50.0 (109.8)]. We did not find any correlation or association of CTCs/CTM with white blood cells (leukocyte, neutrophil, or lymphocyte). Additionally, there was no significant correlation between CTC number and CTM number in our study as in a previous study of non–small cell lung cancer *(23)*. The disparity in the range of CTC numbers of PDAC and other cancers might have originated from tumor characteristics and the platforms used.

The presence of CTM supports the speculation that cells within CTM have a survival advantage via protection from anoikis *(24–26)*. The heterogeneity of CTM regarding epithelial vs mesenchymal cell phenotypes has been demonstrated in small cell lung cancer *(13)*. The lack of proliferation of CTM, compared to proliferating single CTCs, would theoretically make cancer cells relatively resistant to chemotherapy. Furthermore, it implies that CTMs are not groups of cells actively dividing during transit in the blood; rather, they are cell clusters breaking off from the primary tumor, intravasating via "leaky" and chaotic tumor vessels and appearing in the blood as a result of collective migration. Interestingly, an alternative model for metastasis involving tumor cell cooperativity has been postulated. In that model, noninvasive epithelial cells could transform into mesenchymal cells with invasive capability to access to the blood for easier metastases *(27)*. CTMs have been demonstrated to possess increased metastatic potential compared to single CTCs *(18)*. In mouse models, knockdown of plakoglobin abrogates CTC cluster formation and suppresses lung metastases *(18)*. CTMs derived from multicellular groupings of tumor cells that hold together through intercellular adhesion greatly contribute to the metastatic spread of cancer *(18)*. Furthermore, whether there are additional contributions from nonmalignant cells within larger microemboli *(28–30)*, including potential stromal-derived tropism signals *(31)*, awaits further study. Increased metastatic potential in CTMs might explain why CTMs play a more important role than CTCs as an independent prognostic marker in addition to tumor stage in this study.

In 2 patients with stage II disease, unfavorable CTMs were detected preoperatively. These 2 patients had died of liver metastasis and peritoneal metastasis 3 months after surgery. Preoperative positron emission tomography did not show any distant metastasis in these 2 patients. The observation implied that preoperative unfavorable numbers of CTM in peripheral blood might reflect the aggressiveness of tumor or the understaging of PDCA by the current staging modality. In resectable PDAC, the preoperative circulating CK-19 mRNA [by PCR or nested RT–quantitative PCR (qPCR)] *(32)* and EpCAM mRNA (by RT-qPCR) *(33)* show a possible association with poor survival. Preoperative CTM might add more information to stratify patients to the better treatment option in addition to the current staging system.

There are some limitations to the current study. First, we did not fully characterize the CTCs or CTM in our detection. The character of CTC/CTM with correlation of PDAC survival was more valuable than the numbers themselves. The evaluation of CTCs and CTM will be fully realized if molecular characteristics can be measured and monitored in a real-time manner. Furthermore, more information regarding potential targeted drugs and drug resistance in response to chemotherapy might also be obtained if we could characterize the cells more clearly. Second, we collected samples at only 1 time point (pretreatment) in these cases. Decreased amounts or changes in the character of CTC/CTM after treatment could be anticipated, but these need to be confirmed via further study. Third, the case numbers were small in this pilot study. Further study with large case numbers with a longer follow-up period is needed.

In summary, we report CTM number to be an independent prognostic factor for PDAC both in PFS and OS. Our current platform can detect many more CTCs and CTM than other reported platforms can do. Our

findings suggest that the presence of CTM may provide new insights into PDAC biology and new biomarkers in addition to the current staging system for PDAC.

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