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Original Research

Clinical significance of circulating tumor cells after chemotherapy in unresectable pancreatic ductal adenocarcinoma

Hyemin Kim [a,b,1], Chan Mi Heo [a,1], Jinmyeong Oh [a,1], Hwe Hoon Chung [a], Eun Mi Lee [a], Juhee Park [d,e], Se-Hoon Lee [a,c], Kwang Hyuck Lee [a], Kyu Taek Lee [a], Jong Kyun Lee [a], Yoon-Kyoung Cho [d,e,**], Joo Kyung Park [a,c,*]

[a] Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 81 Irwon-ro, Gangnam-gu, Seoul 06351, Republic of Korea
[b] Medical Research Institute, Sungkyunkwan University School of Medicine, Seoul 06351 Republic of Korea
[c] Department of Health Sciences and Technology, SAIHST, Sungkyunkwan University, Seoul 06351, Republic of Korea
[d] Center for Soft and Living Matter, Institute for Basic Science (IBS), Ulsan 44919, Republic of Korea
[e] Department of Biomedical Engineering, Ulsan National Institute of Science and Technology (UNIST), Ulsan 44919, Republic of Korea

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ABSTRACT

Circulating tumor cells (CTCs) have emerged as liquid biopsy biomarker providing non-invasive assessment of cancer progression and biology. We investigated whether longitudinal analysis of CTCs could monitor disease progression, response to chemotherapy, and survival in patients with unresectable pancreatic ductal adenocarcinoma (PDAC). A total of 52 patients with PDAC were prospectively enrolled in this study. Peripheral blood samples were serially collected at the time of diagnosis and after chemotherapy with clinical assessments. CTCs were isolated through a centrifugal microfluidic disc, enumerated with immunostaining against Epithelial cell adhesion molecule (EpCAM), Cytokeratin (CK), Plectin-1 and CD45, and identified by an automated imaging system. One or more CTCs were detected in 84.62% patients with unresectable PDAC at the time of diagnosis. CTC numbers were not statistically different across tumor sizes, location and metastatic sites. The absolute number of CTCs after chemotherapy was inversely related to overall survival (OS), and the decreased number of CTCs after chemotherapy was significantly associated with longer OS in patients with PDAC. Identifying CTCs and monitoring CTC changes after chemotherapy could be a useful prognostic marker for survival in patients with unresectable PDACs.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common cancer with lethal effects, and the overwhelming majority of patients with PDAC have a locally advanced or distant metastatic disease (80–85%). Despite many efforts to improve survival, it is still a lethal disease with a 5-year survival rate of less than 5% and a median survival of less than 1 year [1]. The reasons for such poor survival are the lack of symptoms and effective ways to screen for pancreatic cancer, which result in a delayed detection of cancer [2]. Carbohydrate antigen (CA) 19–9 is, so far, the only biomarker with somewhat clinical usefulness [3], and is used for therapeutic monitoring and early detection of recurrent disease after treatment in pancreatic cancer [4]. However, it is not a specific biomarker to pancreatic cancer; CA19–9 level is also elevated in other conditions like cholestasis, lung diseases and other malignancy as well. In addition, approximately 10% of patients with PDACs who are negative for Lewis antigen a or b cannot synthesize CA19–9 [5]. Still, there are clinical unmet needs for biomarker to monitor and to predict its prognosis in unresectable PDACs, especially. Several studies have reported the potential clinical utility of liquid

; PDAC, Pancreatic Ductal Adenocarcinoma; CTC, Circulating Tumor Cell; CTx, Chemotherapy; OS, Overall Survival; EpCAM, Epithelial Cell Adhesion Molecule; CK, Cytokeratin.

* Corresponding author at: Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 81 Irwon-ro, Gangnam-gu, Seoul 06351, Republic of Korea.
** Corresponding author at: Department of Biomedical Engineering, Ulsan National Institute of Science and Technology (UNIST), Ulsan 44919, Republic of Korea.
E-mail addresses: ykcho@unist.ac.kr (Y.-K. Cho), mdsophie@gmail.com (J.K. Park).
[1] These authors contributed equally to this work.

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biopsy including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and circulating tumor exosomes for monitoring disease progression and for detecting molecular genetic changes during the courses of treatment [6,7]. The concentration of CTCs, ctDNA and exosomes in patient-derived liquid samples reflect differences in total tumor burden, and diversity in cell populations [8]. Thus, technologies detecting minute amounts of CTCs and ctDNA with high sensitivity and selectivity are expected to provide real-time monitoring of tumor evolution and therapeutic efficacy, with the potential for improved cancer diagnosis and treatment [9,10].

In liquid biopsy, CTCs are rare, viable, motile and tumor-derived epithelial cells identified in the peripheral blood of patients with cancer [11]. Primary and metastatic tumor lesion release subsets of CTCs into the bloodstream, and invasive CTCs spread dependent on chemokine gradients [8]. The ability to detect and analyze these CTCs in PDAC may give us insights into its aggressive biology [12,13]. Several studies have focused on identifying CTCs in the blood for diagnosis, staging and prognostication for various cancers [14,15]. There have been studies in other tumor models, such as lung, breast, and prostate cancers suggesting that the presence of CTCs in the peripheral circulation of patients with metastatic carcinoma is associated with shorter survival [16–18]. Unfortunately, there are only a few studies about the detection of CTCs in PDAC [19,20].

Multiple strategies for CTC isolation and identification have been reported [10,11]; however, there have been emerging problems due to the extreme rarity, short lifetime and heterogeneity of CTCs. For example, antigen-dependent capture using an epithelial marker, Epithelial cell adhesion molecule (EpCAM), is a common isolation method, which can overlook CTCs undergoing an epithelial-to-mesenchymal transition (EMT) [20]. Therefore, we additionally utilised a PDAC-specific marker, Plectin-1, to detect CTCs from patients with PDAC. Plectin is an intermediate filament and important crosslinking element of the cytoskeleton, and modulates protein kinase C (PKC) signaling and mitogen-activated protein (MAP) kinase involved in cellular stress responses and migration in cancer [21,22]. Plectin-1 is known as a novel biomarker for primary and metastatic PDAC [23,24]. It was identified in 100% of invasive PDAC tumor and 60% of pre-invasive Pancreatic intraepithelial neoplasia (PanIN) III lesions and was retained in metastatic deposits. Moreover, Plectin-1 distinguished PDAC from benign inflammatory diseases, like chronic pancreatitis [23]. Recently, we captured CTCs from portal vein blood and peripheral blood of patients with resectable pancreatic cancer by using Plectin-1 and EpCAM antibodies. A single Plectin-1^+ CTC was picked and confirmed to have KRAS mutation [25]. Here, we examined whether Plectin-1 combined with EpCAM could improve CTC detection and the Plectin-1positive PDAC CTCs can be used as prognostic biomarkers. The aims of this study are (i) to enumerate CTCs using EpCAM and Plectin-1 antibodies in the peripheral circulation of patients with unresectable PDACs and (ii) to investigate the clinical significance of CTC profiles correlated with clinical outcomes, such as treatment response, overall survival (OS) and site of metastasis in PDAC.

Materials and methods

Study patients and blood collection

Fifty-two patients with PDAC diagnosed between January and June 2019 at Samsung Medical Center (SMC) were prospectively enrolled (ClinicalTrials.gov Identifier No. NCT02934984) and followed up until the end of 2020. Clinical Records Form (CRF) was prospectively collected and the following medical information was contained: age, sex, staging (The 8th edition American Joint Committee on Cancer (AJCC)), body mass index, Eastern Cooperative Oncology Group (ECOG) performance status, comorbidity, serum levels of Carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19–9, chemotherapy regimen, clinical response and survival data**.** This study was conducted under the principles of the Declaration of Helsinki. The study protocol was approved by the institutional review board (IRB) of SMC. All patients provided written informed consent, and all specimens were collected according to IRB regulations and approval (IRB No. 2018–11–080). Blood was taken from the peripheral vein (cephalic vein) and collected into a Cell-Free DNA BCT® CE tube (Streck, Omaha, NE, USA). It was processed within 3–4 h for CTC enrichment and enumeration.

Microfluidic approach to isolate and enumerate CTCs

The CD-PRIME^{TM} system of the CD-CTC disposable disc and CD-CTC Enrichment kit (Clinomics, Inc., Ulsan, Korea) was used to isolate CTCs efficiently from millions of other blood cells in whole blood samples. This CTC enrichment system called 'Fluid-Assisted Separation Technology (FAST) disc' is based on centrifugal microfluidic separation [26,27] (https://www.youtube.com/watch?v=OgJ8eztIYQA). It is based on the size-selective, clog-free CTC isolation through a polyethylene membrane (8-μm pore size) filled with a stably-held liquid throughout the filtration process. Briefly, the sample-loading chamber, filter zone and fluid assistant chamber were filled with a 1% bovine serum albumin (BSA) solution and rotated at a spin speed of 600 rpm for washing. Then, 3 ml of whole blood was loaded to the sample loading chamber and rotated at 600 rpm. After washing with the 1% BSA solution 3 times, CTCs captured on the disc membrane were fixed with 4% paraformaldehyde for 15 min at room temperature for the next CTC staining and enumeration processes.

Immunostaining and enumeration of CTCs

CTCs fixed on the membrane were immunostained using the CD-CTC cell enumeration kit (Clinomics Inc.) combined with Plectin-1 antibody to identify the number of isolated CTCs. The cells were permeabilised with 0.1% Triton x-100 in phosphate buffered saline (PBS) for 5 min and blocked with IgG (20 g/ml) or goat serum for 20 min after washing with PBS. Anti-Plectin-1 (1:60, Cell Signaling Technology (CST), Danvers, MA, USA) solution was applied at 4 °C overnight. The next day, cells were additionally stained with Alexa 647-conjugated anti-rabbit IgG (1:500, CST), FITC-conjugated anti-CK (CK3–6H5, 1:100), Alexa 488-conjugated anti-pan CK (AE1/AE3, 1:100), FITC-conjugated anti-EpCAM (1:400) and Alexa 594-conjugated anti-CD45 (1:100). All antibodies, including pan-CK, CK, EpCAM and CD45 antibodies, were included in the cell enumeration kit. Considering the possibility that pan-CK antibodies may not detect all CKs, we mixed another CK antibody to detect different subsets of CTCs [28]. CTCs were identified by using an imaging system, consisting of staining with 4.6-diamidino-2--pheylindole (DAPI) for DNA content, fluorochrome-conjugated anti-CD45 for haematologic cells and anti-Plectin-1 or anti-EpCAM/CK for PDAC CTC cells. Then number of CTCs/ml was determined via comprehensive image analysis, scanning the entire membrane (Bioview CCBS system, BioView, Ltd., Nes Ziona, Israel) and identifying CTCs based on cell size, morphology and immunofluorescence staining. Total number of cells was counted by DAPI staining; white blood cells (WBCs) were identified by CD45 staining and capture of PDAC CTCs was confirmed by immunofluorescence staining profiles: CD45 (WBC marker) negative and EpCAM/CK (epithelial marker) or Plectin-1 (PDAC-specific marker) positive cells. PDAC CTCs were defined as EpCAM/$CK^+CD45^-DAPI^+$ cells, Plectin-$1^+CD45^-DAPI^+$ cells and EpCAM/CK^+Plectin-$1^+CD45^-DAPI^+$ cells. Thirty blood samples (3 ml each) of healthy volunteers were analysed on the CTC discs to validate the specificity and sensitivity. A count of one or more CTCs per ml of blood was defined as positive [25–27]. We defined true positive (TP), true negative (TN), false positive (FP) and false negative (FN) as follows; TP= PDAC Patients with one or more CTC detection/total PDAC patients, TN= Healthy volunteer without CTC detection/total healthy volunteer, FN= PDAC Patients without CTC detection/total PDAC

patients, FP= Healthy volunteer with one or more CTC detection/total healthy volunteer. Sensitivity and specificity for CTC detection were defined as follows; Sensitivity=TP/(TP+FN), Specificity=TN/(TN+FP), PPV=TP/(TP+FP), NPV=TN/(TN+FN).

Confirmation of KRAS mutation in captured CTCs

Other ten patients diagnosed with PDAC were randomly selected to screen KRAS mutation in PDAC CTCs. After CTC enrichment from the blood of PDAC patients, DNA was recovered from the captured CTCs on the FAST disc membrane with QIAamp DNA Micro Kit (Qiagen, Germantown, MD, USA). Mutant KRAS was detected by Droplet Digital polymerase chain reaction (ddPCR) to identify three somatic mutations located in codons 12 (p.Gly12Asp (G12D), p.Gly12Arg (G12R), p.Gly12Val (G12V). ddPCR was performed using 2X ddPCR Supermix (Bio-Rad, Hercules, CA, USA) for KRAS probes (KRAS wild-type (WT) 5′-HEX-AGTTGGAGCT**GGT**GGCGTA-BHQ1–3′; KRAS mutant G12D 5′-FAM-AGTTGGAGCT**GAT**GGCGTAG-BHQ1–3′; KRAS mutant G12V 5′-FAM-AGTTGGAGCT**GTT**GGCGTAG-BHQ1–3′; KRAS mutant G12R 5′-FAM-AGTTGGAGCT**CGT**GGCGTAG-BHQ1–3′) by QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA). Data analyses were performed as recommended by the manufacturer using the QuantaSoft Software version 1.7.4. (Bio-Rad).

Statistics

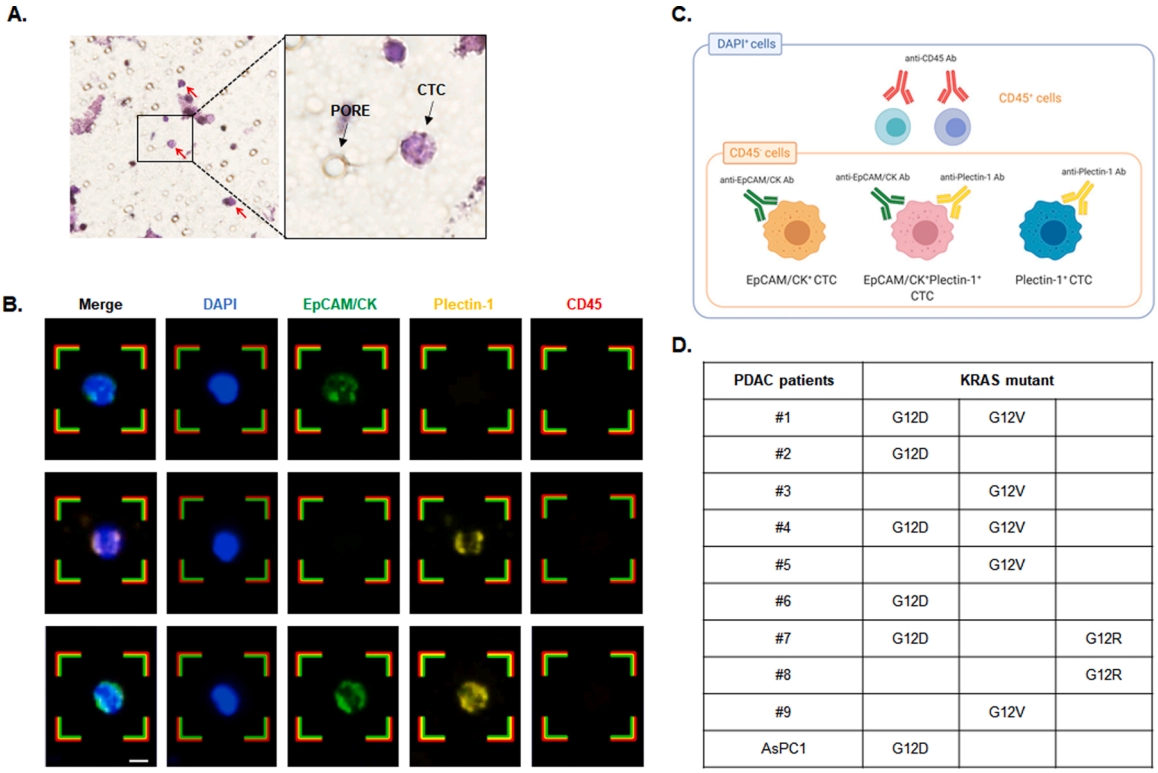
Non-parametric tests were used throughout the study. The difference of CTC numbers was analysed by an unpaired/paired *t*-test or one-way Analysis of variance (ANOVA) test. OS was analysed by the Kaplan–Meier method with the use of one-sided log-rank statistics. *P* values < 0.05 were considered statistically significant. Statistical analysis was carried out using SPSS for Windows (version 17.0, SPSS Inc. Chicago, IL, USA) and GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, San Jose, CA, USA).

Results

Enrichment and identification of CTCs in patients with PDAC

The expression of EpCAM, CK and Plectin-1 was first confirmed in PDAC cell lines to determine if these antigens would be suitable for enumeration of PDAC CTCs. EpCAM, CK and Plectin-1 were expressed in most PDAC cells but not in WBCs (**Supplementary Figs. 1–3**). PDAC CTCs were enriched using 'FAST disc', a centrifugal microfluidic tangential flow filtration device, which allowed rapid, label-free isolation of CTCs from whole blood without sample pre-treatment [26]. First, the membrane of FAST disc was examined by Hematoxylin and Eosin (H&E) staining after running the blood samples of five PDAC patients (Fig. 1**A**). We found CTCs captured on the membrane, which was further confirmed as cancer cells by specialized pathologists. In addition, PDAC CTCs were identified by immunofluorescence staining with anti-EpCAM/CK, anti-CD45 and anti-Plectin-1 antibodies (Fig. 1**B**). Among $DAPI^+$ and $CD45^-$cells, we could find three kinds of CTCs: EpCAM/CK positive (ranged from 0 to 631 cells/3 ml), Plectin-1 positive (ranged from 0 to 123 cells/3 ml), and both EpCAM/CK and Plectin-1 positive (ranged from 0 to 127 cells/3 ml) cells. We decided to consider all three cases (ranged from 0 to 641 cells/3 ml) as PDAC CTCs (Fig. 1**C**).

Next, we performed molecular characterization of the cells captured on the membrane of the FAST disc. KRAS mutation is the most



D.

PDAC patients	KRAS mutant		
#1	G12D	G12V	
#2	G12D		
#3		G12V	
#4	G12D	G12V	
#5		G12V	
#6	G12D		
#7	G12D		G12R
#8			G12R
#9		G12V	
AsPC1	G12D		

Fig. 1. Identification of CTCs in patients with PDAC. From blood samples from pancreatic ductal adenocarcinoma (PDAC) patients, CTCs were enriched with FAST disc and examined. (A) Captured CTCs on the membrane of FAST disc were pathologically identified by H&E staining. Red arrows indicate captured CTCs. Membrane pore size is 8 μm. (B) Three representative images of PDAC CTCs. All nucleated cells were stained by DAPI (Blue), WBCs were identified by CD45 (Red) staining, and CTCs were identified by EpCAM/CK (Green) and Plectin-1 (Gold) staining. Scale bar, 5 μm. (C) PDAC CTCs were defined as the sum of $EpCAM/CK^+$, $Plectin\text{-}1^+$, and $EpCAM/CK/Plectin\text{-}1^+$ cells among $DAPI^+$ and $CD45^-$ cells. Illustration was created with Biorender.com. (D) Digital droplet PCR for KRAS mutants (G12D, G12R, G12V) were performed to confirm PDAC CTCs captured on the membrane of the FAST disc. AsPC-1, a PDAC cell line, was used as a positive control for KRAS G12D mutant (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

frequently detected somatic alteration in PDACs (nearly 100%) [29] and thus the detection of KRAS mutants can be a proof to confirm that the captured cells are CTCs of PDACs. After the enrichment of CTCs from the blood samples of other 10 patients with PDAC, DNAs were extracted from the cells captured on the membrane and followed by ddPCR. Three types of KRAS mutant (G12D, G12V, G12R) were detected in CTCs from patients with PDAC (**Supplementary Fig. 4**). KRAS mutation was detected in 9 out of 10 samples (90%). AsPC-1 was used as a positive control for PDAC, which had KRAS G12D mutant (Fig. 1**D**).

Baseline characteristics of study patients

A total of 52 patients were enrolled in this study, and their baseline characteristics are described in Table 1. There were 26 male and 26 female patients aged 46–85 years (median 67 years). The clinical stage (8th AJCC) was as follows: 21 were stage III (40.4%) and 31 were stage IV (59.6%). Twenty patients (38.5%) had metastasis to the liver, and 11 patients (21.1%) showed metastasis to bone, peritoneum and supraclavicular lymph node (SCN) without liver. The 46 (88.5%) patients received chemotherapy and 6 (11.5%) did not receive chemotherapy due to patient preference, old age or poor performance status. Median (range) of initial CA19–9 and CEA were 203.5 IU/ml (3.75–140,000) and 2.9 ng/mL (0.5–51.39), respectively.

Evaluation of CTCs from the study patients

We previously reported that healthy subjects had negligible CTC counts (mean, 0 CTCs/7.5 mL; median, 0 CTCs/7.5 ml; range, 0 − 5 CTCs/7.5 ml of blood) when EpCAM and CK antibodies were used to detect CTCs [26,27]. Recently, we have also shown the specificity of Plectin-1 to capture CTCs in PDAC [25]. CTC counts in 30 healthy volunteers were less than one (Mean±SD, 0.19±0.26 CTCs/ml; Median 0.00 CTCs/ml). We defined counts of one or more CTCs per ml of blood as positive, like in the previous reports [25–27]. Fifty-two blood samples from patients with PDAC were analysed at baseline. The median number of CTCs was 8.5 in one ml of blood (range 0–641 cells/3 ml), and one or more CTCs per one ml of blood were detected in 44 patients with PDAC (44/52, 84.62%) at baseline. For CTC counting, sensitivity was 84.62%, and specificity was 96.67%. The positive predictive value (PPV) was 96.21% and the negative predictive value (NPV) was 86.27% (Fig. 2**A**). We evaluated the tumor size, stage and metastatic sites based on the number of CTCs to demonstrate the unique clinical potential of CTCs. We calculated the sum of the unidimensional size in centimetres of all significant and measurable tumor sites through the computed tomography (CT) scan of each patient. The absolute number of captured CTCs did not necessarily correspond with tumor size (tumor median size, 36 mm; $p = 0.5139$, Fig. 2**B**) and tumor location ($p = 0.6886$, Fig. 2**C**). In addition, CTC counts were higher in stage III than stage IV ($^{*}p = 0.0237$, Fig. 2**D**). The most common site of metastasis is the liver in PDAC due to the fact that the first venous drainage of pancreatic cancer is the portal circulation. There was no significant difference in the number of CTCs across the metastatic sites ($p = 0.077$) by ANOVA test. Patients with liver metastasis combined with other metastatic sites such as the peritoneum, lung, bone, and SCN showed increased CTC counts compared to patients with liver metastasis only ($^{*}p = 0.0290$, Fig. 2**E**).

Table 1
Patient characteristics ($n = 52$).

Characteristics	$n = 52$
Age, median (range)	67 (46–85)
Sex, n (%)	
Male	26 (50.0)
Female	26 (50.0)
BMI (kg/m^2), median (range)	23.6 (19.0–29.6)
Performance status (ECOG), n (%)	
0: fully active	28 (53.9)
1: light house work	22 (42.3)
2: ambulatory	1 (1.9)
3: limited self-care	1 (1.9)
Stage (AJCC 8th), n (%)	
III	21 (40.4)
IV	31 (59.6)
Metastasis, n (%)	
No metastasis	21 (40.4)
Liver metastasis	20 (38.5)
Other site metastasis, not including liver	11 (21.1)
Location (proximal), n (%)	
Uncinate/head/Neck	23 (44.2)
Body	17 (32.7)
Tail	12 (23.1)
Pancreas mass (mm), median (range)	35 (14.0–130.0)
Treatment	
Best supportive care only	6 (11.5)
Gemcitabine based	24 (46.2)
FOLFIRINOX	21 (40.4)
TS-1	1 (1.9)
CA19–9 (IU/mL), median (range)	203.5 (3.75–140,000)
CEA (ng/mL), median (range)	2.9 (0.5–51.39)

BMI, Body mass index; ECOG, Eastern Cooperative Oncology Group; AJCC, American Joint Committee on Cancer; CA19–9, carbohydrate antigen 19–9; CEA, Carcinoembryonic antigen._

Assessment of CTC numbers before and after chemotherapy

CTCs were evaluated in thirty-nine patients who underwent chemotherapy (CTx). Blood samples were collected at baseline (pre-CTx) and during subsequent clinic visits for treatment (post-CTx). The study patients underwent either of the following regimens; Gemcitabine + Abraxane or FOLRIFINOX. Among 52 patients, 39 patients underwent at least more than #3 Gemcitabine +Abraxane (#1: D1, D8, D15 every 4 weeks) and #4 FOLFIRINOX (#1: D1, D2 every 2 weeks) of chemotherapy. Unfortunately, 7 patients could not draw the blood sampling after the chemotherapy (post-CTx) because they transfered to other hospital or could not continue the chemotherapy with deteriorating general conditions. The exact follow-up schedule varied between patients. CT scan and serum CA19–9 measurement were performed at baseline and at regular intervals according to standard clinical practice (Fig. 3**A**). Upon comparing CTC counts in pre- and post-CTx blood samples of patients with PDAC, paired *t*-test showed no difference in the number of CTCs ($p = 0.5474$, Fig. 3**B**). In addition, we separated good responders with partial response (PR) and stable response (SD) from poor responders with progressive disease (PD) according to RECIST (Response Evaluation Criteria in Solid Tumors, version 1.1) [30]. The number of CTCs at baseline was not correlated with treatment response ($p = 0.3953$, Fig. 3**C**). A higher CTC count at post-CTx was more evident in patients who poorly responded to the treatment than in patients who favourably responded to the treatment, although there was no statistical significance ($p = 0.0687$, Fig. 3**D**). Also, the relative number of CTCs changed after treatment compared to before treatment was not related to the treatment response as well ($p = 0.5117$, Fig. 3**E**).

Clinical significance of CTC changes after chemotherapy in overall survival

Kaplan-Meier analysis was performed to evaluate the absolute and relative number of CTCs with the survival of patients with PDAC. Unlike before treatment ($p = 0.3088$, Fig. 4**A**), higher CTC counts after treatment ($^{*}p = 0.0471$, Fig. 4**B**) was significantly associated with shorter OS. Considering the relative change of CTCs after chemotherapy compared to before chemotherapy (ΔCTC=(Post-Pre)/Pre), OS was longer when ΔCTC were negative. Patients with an increase of CTCs after chemotherapy showed shorter survival (median OS, 16.97 vs. 10.02 months; $^{**}p = 0.0095$, Fig. 4**C**). The association of CA19–9 change (ΔCA19–9) with patient survival was also examined as CA19–9 is the most commonly used biomarker for diagnosis and management of patients with pancreatic cancer [31]. The increase or decrease of CA19–9 level with chemotherapy could not prognose the survival of

A.

	CTC counts
Sensitivity	84.62%
Specificity	96.67%
Positive predictive value (PPV)	96.21%
Negative predictive value (NPV)	86.27%

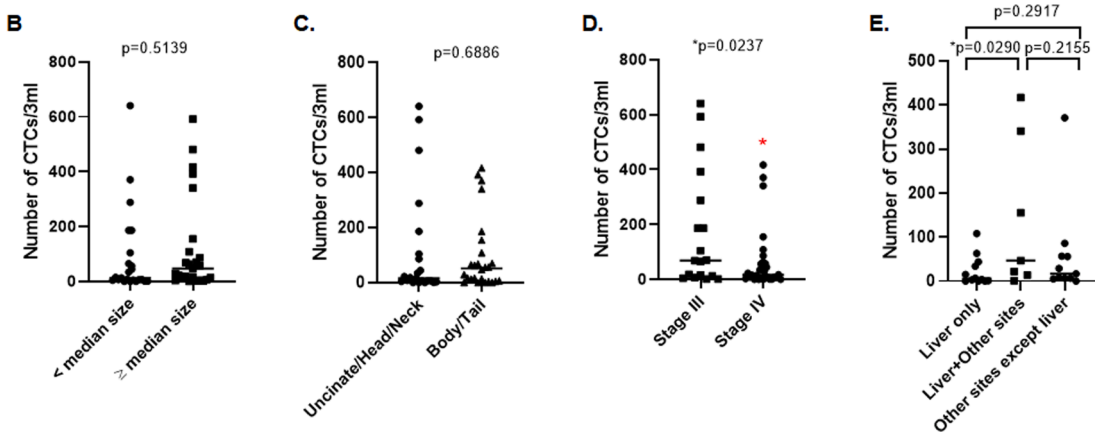


Fig. 2. CTC enumeration in patients with PDAC. CTCs were enumerated in blood samples from 52 patients with PDAC. (A) The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) was calculated with CTC counts. Also, the number of CTCs was analysed depending on (B) Tumor size (median 36 mm, $p = 0.5139$), (C) Tumor location ($p = 0.6886$), (D) Clinical stages ($^*p = 0.0237$) and (E) Metastatic sites ($p = 0.077$). Other sites include lung, bone, supraclavicular lymph node (SCN) and peritoneum. Each bar in the graph represents the median.

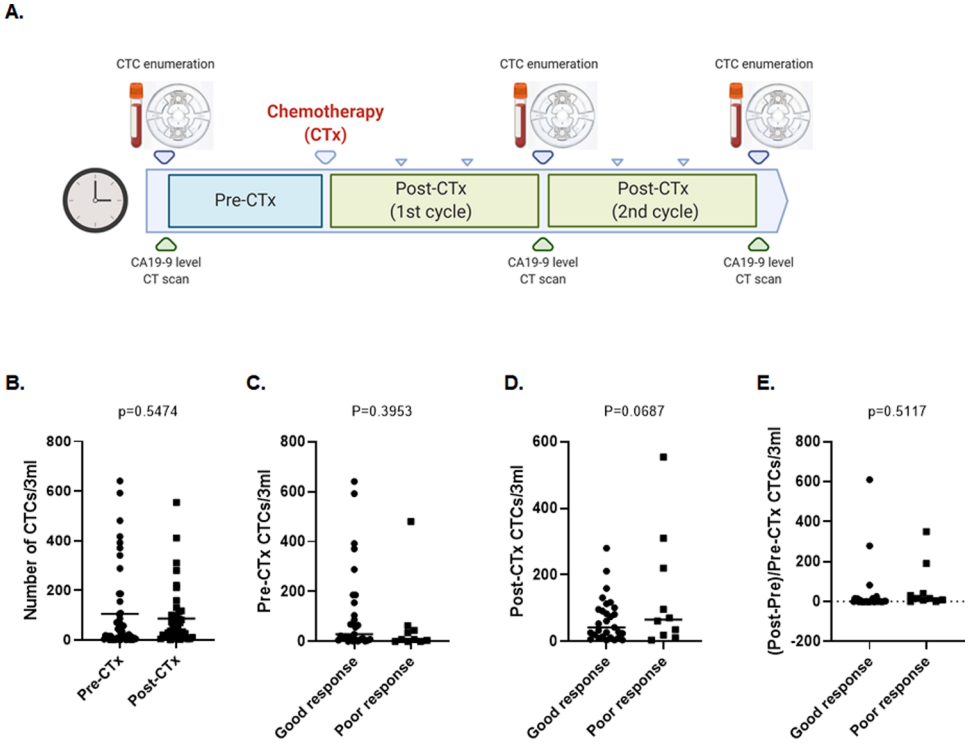


Fig. 3. Evaluation of CTC counts in PDAC patients with chemotherapy. (A) CTCs were enumerated from paired blood samples of 39 patients with PDAC before (Pre-CTx) and after chemotherapy (post-CTx), which was accompanied with measurement of serum CA19–9 level and CT scanning. Illustration was created with BioRender.com. (B) The absolute number of CTCs at the pre-CTx and post-CTx was compared ($p = 0.5474$). Depending on RECIST criteria (version1.1), PR and SD was included in good response, and PD was involved in poor response. The number of CTCs (C) before treatment ($p = 0.3953$) and (D) after treatment ($p = 0.0687$) was evaluated. (E) The relative change of CTC counts was also assessed upon treatment response ($p = 0.5117$). Each bar represents the median. CT, computed tomography; CA19–9, carbohydrate antigen 19–9. RECIST, Response Evaluation Criteria in Solid Tumors; PR, partial response; SD, stable disease; PD, progressive disease.

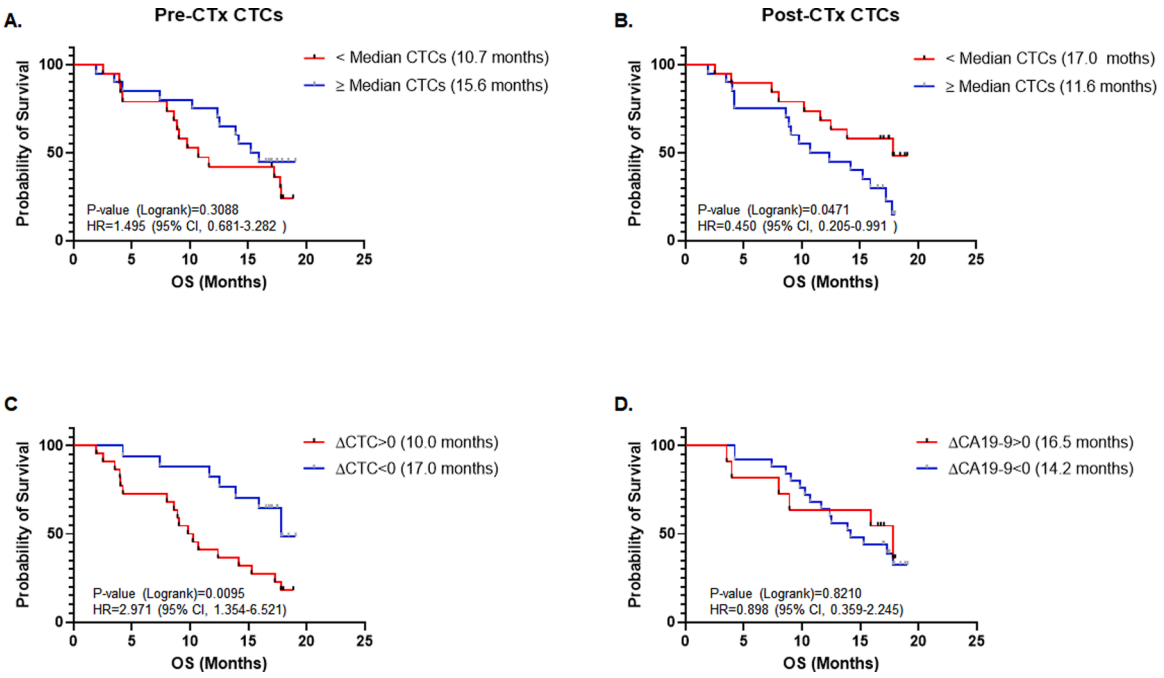


Fig. 4. Kaplan-Meier analysis of CTCs with overall survival in patients with PDAC. Kaplan–Meier curves of overall survival (OS) and log-rank tests for patients with PDAC depending on the number of CTCs (A) before chemotherapy (pre-CTx, $p = 0.3088$) and (B) after chemotherapy (post-CTx, $^{*}p = 0.0471$). The relative change of (C) CTC numbers (ΔCTC) and (D) blood CA19–9 level (ΔCA19–9) after chemotherapy (post-CTx) relative to before chemotherapy (pre-CTx) was evaluated with OS of patients with PDAC. Kaplan–Meier survival curve was stratified by the comparative changes of CTC counts ($^{**}p = 0.0095$), and serum CA19–9 levels ($P = 0.8210$) after chemotherapy.

patients with PDAC ($p = 0.8210$, Fig. 4**D**).

Discussion

Investigation into CTCs may give us insights into the biology of tumor cell dissemination in patients with cancer. Little is known about the biology and pathology of CTCs in PDAC due to difficulty in collecting them in the peripheral circulation. Here, we efficiently collected CTCs from blood samples of patients with PDAC by using size-based centrifugal microfluidic disc and by using EpCAM, CK, and Plectin-1 identification antibodies to estimate their prognostic potential for responses to treatment and survival.

Tumor cells in the peripheral blood are substantially heterogeneous, which offers a unique opportunity to understand how CTCs participate in the tumor dissemination process and tumor heterogeneity [13]. However, these heterogeneous properties of rare CTCs make it harder to detect their variable phenotypes in circulation. The only system currently approved by the Food and Drug Administration (FDA) as an aid in monitoring patients with metastatic breast, colorectal or prostate cancer is CELLSEARCH® (Janssen Diagnostics, Raritan, NJ, UA), which uses antibodies specific to EpCAM and CK of epithelial CTCs [10]. However, CTCs exhibit dynamic changes in epithelial and mesenchymal compositions, and show both epithelial and mesenchymal features [32]. They can show no or low expression of EpCAM during EMT, resulting in missed detection [33]. Furthermore, EpCAM is down-regulated in pancreatic cancer [34,35]. To make up for this, we decided to use a PDAC-specific antibody in addition to EpCAM and CK. The ideal biomarker for PDAC should not only differentiate benign conditions from malignancy but also be able to detect small cancers, ideally at the pre-invasive PanIN III phase. Plectin-1 expression is immunohistochemically positive in all PDAC tissues but negative in all benign tissues, including the normal pancreas and chronic pancreatitis [23]. Moreover, it could detect pre-invasive PanIN III lesions [23]. Therefore, we additionally utilized a PDAC biomarker, Plectin-1, to identify CTCs in the peripheral blood samples of patients with PDAC. As a result, we were able to successfully detect cells positive to only Plectin-1 but not EpCAM/CK in the bloodstream (Fig. 1**B**). Moreover, KRAS mutant was detected in CTCs captured on the membrane, indicating the identity of the captured cells as PDAC tumor cells (Fig. 1**D**). CTC enumeration with an additional Plectin-1 antibody showed lower p-value than CTC enumeration with only EpCAM/CK antibodies to predict OS of patients with PDAC ($^{**}p = 0.0095$ vs. $^{*}p = 0.0152$, HR=2.971 vs. HR=2.689, Fig. 4**C** and **Supplementary Fig. 5**).

The enumeration of CTCs was not statistically different in relation to tumor size, location and metastatic sites (Fig. 2**B, C and E**). Patients with stage III PDAC showed more CTCs than patients with stage IV PDAC (Fig. 2**D**). In pancreatic cancer, more CTCs were reported in stage IV patients than in stage III patients [36]. However, higher number of CTCs were detected in patients with stage III in compared to patients with stage IV in non-small cell lung cancer [37, 38]. CTCs can be expected to include the subpopulations responsible for disease progression [13]. During the aggressive cancer progression, several CTCs could be also detected in blood of patients with stage III. The absolute number of initial CTCs did not relate to the survival of patients with PDAC. Our study was designed such that CTCs were identified both before and after chemotherapy with the clinical assessment of the CT scan and serum CA19–9 measurement (Fig. 3**A**). The number of CTCs was not significantly decreased by chemotherapy across all patients with PDAC (Fig. 3**B**). The detection and existence of CTCs can be a key model of haematogenous spread in the development of metastatic disease. The existence of CTCs expressing the cell surface EpCAM and intracellular CKs is related to poor outcome in patients with both non-metastatic and metastatic disease [39,40]. PDAC patients with a high number of CTCs after chemotherapy may have the potential for worse prognosis (Fig. 3**D**) and poor survival (Fig. 4**B**), which was not evident with the number of CTC before chemotherapy (Figs. 3**C** and 4**A**). Furthermore, the relative change between pre-CTx and post-CTx indicated more critical prognostic significance to predict the probability of survival. Patients with decreased CTCs after chemotherapy indicated a significantly better survival, whereas patients with increased CTCs showed

worse survival (**Fig. 4C**). There is a need for further study to reveal the association of CTC counts with poor or good responses to chemotherapy.

In our study, the sensitivity and specificity of PDAC CTC capture and identification was 84.62% and 96.67%, respectively (**Fig. 2A**), which is comparable to other reports. Sefrioui et al. reported 67% sensitivity and 80% specificity of CTC in solid pancreatic tumor [41], Ankeny et al. reported 75% sensitivity and 96% specificity of peripheral CTC in pancreatic cancer [36]. The sensitivity of CA19–9, the only marker approved by the United States FDA, for use in the routine management of pancreatic cancer is 63.6%, and changes in serum levels are unrelated to disease progression [34]. It coincided with our result where the ΔCA19–9 with chemotherapy was not related to survival (**Fig. 4D**). In contrast to CA19–9, ΔCTC after chemotherapy was significantly correlated with survival of PDAC patients (**Fig. 4C**).

The direction of systemic cancer treatment based on the primary tumor characteristics has limitations due to the tumor heterogeneity and frequent discrepancy between primary and metastatic sites. However, because of both inaccessibility of metastatic sites and procedure morbidity, metastatic biopsies are rarely undertaken [42]. In this sense, the prognostic role of CTC enumeration is the true promise to provide a real-time view of cancer progression just using peripheral blood samples, avoiding the need for repeat invasive biopsies. Understanding the biology of CTCs or cancer cells in transit may give us unique insights into the mechanisms behind metastasis. In addition, further genomic analysis of CTCs needs to be performed.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101321.

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