의학박사 학위논문

수술을 시행한 췌장선암 환자에서 수술 전 순환 종양 세포의 검출에 따른 임상 병리학적 결과의 비교

Comparison of the clinicopathologic outcomes according to preoperative detection of the Circulating Tumor Cells (CTCs) in patients who underwent curative resection for pancreatic ductal adenocarcinoma

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이 논문을 의학박사 학위 논문으로 제출함

2018년 12월

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Comparison of clinicopathologic outcomes according to preoperative detection of Circulating Tumor Cells (CTCs) in patients who underwent curative resection for pancreatic ductal adenocarcinoma

Abstract

Background: This study aims to evaluate circulating tumor cells (CTCs) as a biomarker for diagnosing pancreatic ductal adenocarcinoma (PDAC) at the time of disease presentation and predicting early recurrence of PDAC during outpatient follow-up after surgery.

Method: Among 36 pancreatic cancer patients who were consulted at Asan medical center from December 2017 to August 2018, Whipple's operation or distal pancreatectomy was performed on 32 patients. The Institutional Review Board approved the study design, and all participants enrolled in the study submitted their informed consent. Before the surgery, we took 7.5 ml of a blood sample from each patient. We used a sized-based isolation method for isolating and counting of CTCs, and we divided patients according to CTC detection into two groups: CTCs-positive (n=11) and CTCs-negative (n=21). We separately analyzed 32 patients for the early recurrence analysis.

Results: The total detection rate of the CTCs obtained from preoperative peripheral blood sample was 34.4%, and median CTCs count was 2 cells/7.5ml. 6 patients (18.8%) had a double positive cell, and 15 patients (46.8%) had CTCs-negative. There were 13 patients (40.6%) with recurrence within 6 months, 6 patients (54.5%) with CTCs-positive and 7 patients (66.5%) with CTCs-negative (*P= 0.491*). However, distant metastasis and peritoneal carcinomatosis were more frequent in CTCs-positive, and the differences were statistically significant (*P = 0.043*). When CTCs were detected, p53 mutation in primary tumor was confirmed in 8 (88.9%, *P = 0.077*). However, when analyzed by dividing CTCs by ≥2/7.5mL and <2/7.5mL, the p53 mutation was more frequent in ≥2/7.5mL (100%, *P=0.045*).

Conclusions: Further studies are needed to confirm CTCs as a valuable diagnostic tool marker in patients undergoing curative resection for pancreatic ductal adenocarcinoma. We confirmed that the CTC detection is associated with early recurrence of distant metastasis and peritoneal dissemination. As a preliminary study, all registered patients in this study are constantly being monitored. We hope that CTCs would be analyzed as prognostic biomarkers for long-term survival and disease progression.

Key words: pancreatic ductal adenocarcinoma, circulating tumor cells, tumor marker, recurrence, metastatic recurrence

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most lethal cancer among all the cancer disease worldwide. Up to date, the 5-year survival rate of patients diagnosed with pancreatic cancer is still below 10%.[1, 2)] According to the National Cancer Center report, in South Korea, the incidence of pancreatic cancer is increasing annually, and the pancreas cancer is the only one that its survival rate has not improved in recent decades.[3)] Only 20%-30% of the patient can undergo surgical resection at the time of diagnosis despite recent technical progress in imaging modalities.[2, 4, 5)] But even in these patients, tumor recurrence is frequent because of early lymphatic and hematogenous spread, local recurrences, distant metastases, and peritoneal seeding.[6)] Lack of effective screening for early detection and the symptoms not found until a late stage of cancer leads to the recurrence of cancer. Since metastasis occurs after initial tumor progression, early detection is of utmost importance for successful treatment.[7)]

There are several predictors and prognostic factors of recurrence such as tumor aneuploidy, positive lymph nodes, tumor size, poor histological tumor differentiation, and positive resection margins, but there is a need for additional markers that are accurate and reliable to effectively monitor disease progression.[1, 2, 8)] The most commonly used tumor biomarker in PDAC is carbohydrate antigen 19-9 (CA 19-9). However, CA19-9 levels increase in other non-malignant pancreatic disorders as well such as acute pancreatitis and other gastrointestinal malignancies.[8, 9)] Hence there is still a need for new diagnostic and predictive biomarkers that complement imaging techniques used in patient follow-up to achieve more effective management of patients and improve their survival.

Circulating tumor cells (CTCs), one of the diagnostic and prognostic markers, can be detected in the peripheral blood of cancer patients whom all major organs are affected.[10, 11)] The CTCs originate from the tumor, are shed from the tissue into the bloodstream and may be representative of the systemic disease.[12)] On the other hand, CTCs are rare cells occurring

in very low concentration in the peripheral blood which makes their detection challenging. Besides, some studies are stating that the detection of CTCs is a poor predictor of prognosis.[5, 6, 8, 11, 13, 14)]

CellSearch system(Janssen Diagnostics, Raritan, NJ) is the only platform to have received Food and Drug Administration approval for the isolation of CTCs in cancer patients.[15)] CellSearch involves epithelial cell adhesion molecule (EpCAM)-based immunomagnetic capture of CTCs. However, one significant drawback of EPCAM-based approaches is the heterogeneity in the level of EpCAM or other surface proteins on CTCs in the same patient sample.[15, 16)] Also, a major obstacle for EpCAM-based CTCs isolation is the epithelial-mesenchymal-transition (EMT) often observed with CTCs.[17, 18)] For pancreatic cancer, this has already been described *in vivo* showing the loss of epithelial markers at an early stage of development.[19)] Therefore, an antigen-dependent approach for CTCs isolation is especially challenging in pancreatic cancer. In addition to EMT, other mechanisms of EpCAM downregulation such as internalization, proteolysis and promotor methylation have been known to reduce the success rate of CTC isolation.[18)] Thus, in the present study, we used a size-based isolation method (CD-PRIME^{TM}, Clinomics, Ulsan, Korea) capable of automation and commercialization.[20)] This approach takes advantage of the well-known characteristic that CTCs are larger than normal hematologic cells. Such advantages of the lab-on-a-disc system allow for reduced manual handling steps between the filtration, staining, and detection processes.[20)] The CTC detection rate in pancreatic cancer using a sized-based isolation method studied so far ranges from 50% to 90%.[10, 11, 21, 22)]

Our goal of the study was to evaluate CTCs as a biomarker for diagnosing and predicting early recurrence of PDAC at the time of disease presentation. Thus, our central hypothesis is that detection of CTCs in PDAC patients who underwent resection will form a useful tool for diagnosis, and the tumor biology of patients whom CTCs were detected would be poorer.

Methods

Patients (Figure 1)

Thirty-Six patients with pancreatic cancer consulted our hospital from December 2017 to August 2018. Of these, 34 patients submitted their written informed consent for the analysis of CTCs in peripheral blood. We performed Whipple's operation or distal pancreatectomy in these patients. One case with the intra-papillary mucinous neoplasm (IPMN) in the final histologic examination results was excluded, and one case that could not be operated due to the unexpected peritoneal dissemination was excluded as well, and ultimately 32 patients were included in the study.

Clinical, pathological, and surgical data were collected from our own institution's electronic medical records (EMRs). Follow-up data were also obtained from these records; and the follow-up period was measured from the time of surgery to death or from the time of surgery to the time of the last follow up examination. Postoperative pancreatic fistula (POPF) and overall complications were assessed and graded based on the International Study Group of Pancreatic Fistula criteria [23] and Clavien-Dindo complication classification [24] respectively. Tumor, node, and metastasis (TNM) staging was applied according to the eighth edition of the American Joint Committee on Cancer (AJCC) manual.[25]

Preoperatively, all patients were assessed using computed tomography (CT) with pancreas protocol and magnetic resonance cholangiopancreatography (MRCP). Under the diagnostic strategy of our institution, to identify hidden metastasis, most patients with PDAC underwent 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) at initial cancer staging. After pancreatectomy, on the third day of post-operation, all patients underwent CT to assess postoperative complications, including POPF. All patients who underwent pancreatectomy and were diagnosed with PDAC on surgical biopsy were advised to receive 5-fluorouracil-based or gemcitabine-based adjuvant chemotherapy. For postoperative surveillance, CT was

taken, and CA19-9 levels were checked every 3 months during the first two postoperative years and every 6 months thereafter. FDG-PET, chest CT, or biopsy was taken as necessary to confirm recurrences.

For comparative analyses, we divided patients into two groups according to the CTCs detection: CTCs-positive (N=11) and CTCs-negative (N=21). Firstly, we analyzed the CTCs detection rate. We also compared the demographic, operative, and pathological outcomes according to CTCs detection in all patients. Secondly, we examined the recurrence rates and patterns in CTCs-positive and negative group. We collected data and analyzed according to the institutional guidelines that conformed to the ethical standards of the Declaration of Helsinki. All study participants submitted the informed consent, and the study design was approved by the Institutional Review Board of Asan Medical Center .

Immunohistochemistry for P53, erbB-2(HER-2), DPC4 (SMAD4) in Primary Tumors

Using immunohistochemistry, we examined stained slides to analyze the correlation of mutation of primary tumor according to the CTCs detection. We obtained immunohistochemistry results of the primary tumors from 26 patients among 32 patients and reported for p53, erbB-2, and DPC4. One pathologist examined all 26 slides. The p53 was considered positive when there was a homogeneous staining pattern with more than 10% of cells demonstrating nuclear p53 protein accumulation. The primary antibodies used for p53 protein antigen staining were mouse monoclonal antibodies. Scores of 0 and 1+ were considered to be negative for erbB-2 expression, while 2+ and 3+ were considered to be positive (overexpression). The frequency of DPC4-positive cells in a tumor population were scored as 0 to 3 as follows: 0, less than 10%; 1, 10% to 33% positive; 2, 34% to 67% positive; and 3, more than 67% positive. After scoring, cases were dichotomized as intact/decreased DPC4 expression (score 1-3) and total loss of DPC4 expression (score 0). Representative photographs of immunohistochemistry staining are shown in ***Figure 2***.

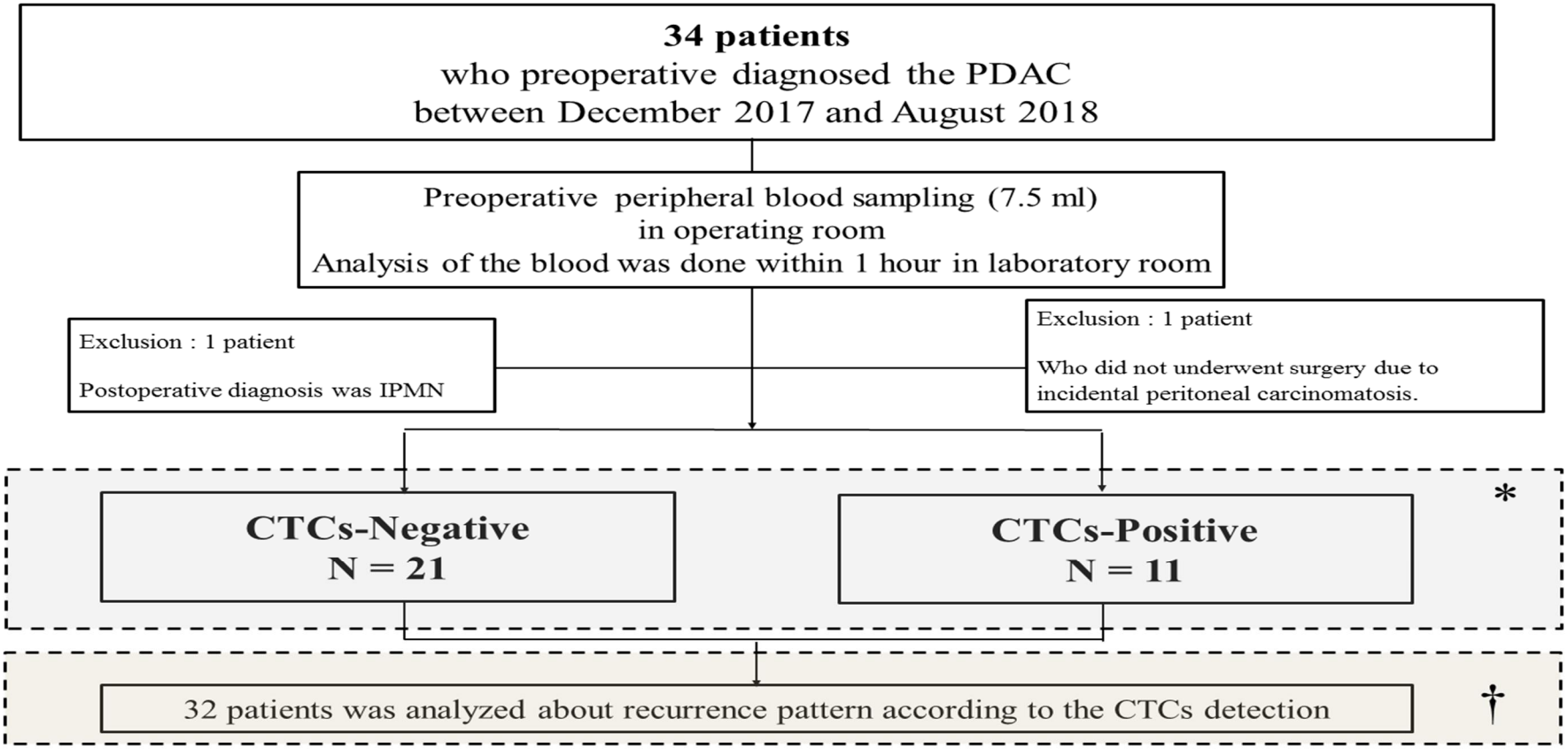


Figure 1. Diagram of this study design. 34 patients submitted their written informed consent for the analysis of CTCs in peripheral blood. Two cases were excluded. Finally, 32 patients were included in this study. *, We analyzed the CTCs detection rate. It is the primary aim in this study. And Clinico-postoperative outcomes and pathological outcomes included the differences of the stage, tumor differentiation were compared between the CTCs (+) and the CTCs (-). †, Secondary aim; 32 patients was analyzed about early recurrence pattern according to the CTC detection. The median follow-up day was 8 months. PDAC : Pancreatic ductal adenocarcinoma, IPMN : Intra-ductal papillary mucinous neoplasm, CTCs : Circulating Tumor Cells

Laboratory methods

1. **Blood samples.** 7.5 ml of blood was drawn from patients who underwent Whipple's operation or distal pancreatectomy for PDAC before surgery. Blood samples were collected in ethylenediamine tetraacetic acid (EDTA)-containing vacutainers. Samples were maintained at 4°C (Celsius temperature scale) and processed within 1-hour of collection.
2. **Isolation of CTCs from Whole Blood.** ***Figure 3*** is a schematic illustration representing the working principle of the CTC isolation disc. Ficoll was injected into the tube first and then the blood was slowly injected through the side of tube wall so that it does not get mixed with the Ficoll. The blood samples were centrifuged at 23°C for 23 minutes (800g). The interface containing the peripheral blood mononuclear cells (PBMC) was removed using a disposable pipette . The CD-PRIME system (Clinomics, Ulsan, Korea) was used for isolating and counting CTCs (***Figure 3A***).[20] A lab-on-a-disc for CTC isolation is shown in Figure 3B, the device contains a sample loading chamber, filtration chamber, waste chamber, ventilation chambers, and channels connected to the chambers. When the disc rotates, the centrifugal force drives a blood sample through the isolation chamber, where target CTCs are trapped on a membrane by size selectivity. Blood cells that are smaller than the size of pores are passed through the membrane and move to the waste chamber (***Figure 3C***). As such, the CTC isolation disc was operated via centrifugal force in a programmable manner using an operating system. The range of G-force used in all the experiments in this study was restricted to 200-3,600rpm (rotations per minute).
3. **CTCs Analysis by Fluorescence Microscopy.** Isolated cells were fluorescently labeled with the nucleic acid dye 4′, 6-diamidino-2-phenylindole (DAPI) and monoclonal antibodies specific for leukocytes (CD45-allophycocyanin) and epithelial cells (cytokeratin 8, 18,19-phycoerythrin). To be considered a CTC, cell must be round or oval, have a nucleus (as determined by positive DAPI staining) contained within the cytoplasm (as determined by positive cytokeratin 8, 18, 19-phycoerythrin staining), and lack the expression of CD 45(as determined by negative CD45-allophycocyanin staining). EpCAM (+)/ DAPI (+)/ CD45 (+) is still controversial with the

concept of "double positive",[12, 26-28] but we defined it as CTCs-negative in this study and included it in the CTCs-negative group. ***Figure 3D*** shows CTCs images from patients with pancreatic cancer. After the immunostaining process, the whole device was mounted on a fluorescence inverted microscope. Isolated cells were analyzed and enumerated using image analysis software. Isolation was done within 30 minutes and the time required for immunostaining was not more than 2 hours. We defined the threshold of CTCs detection using cutoff $\geq$ 1 CTC/7.5ml of peripheral blood. CTC calling was performed by trained personnel and verified by an independent expert.

Follow-up Procedures and Assessment of Recurrence

A follow-up of the study population was achieved using an existing EMR system. We reviewed outpatient outcomes by extraction of information from the EMRs and defined early recurrence as a recurrence within six months after surgery. Recurrence was confirmed by reviewing surgery and oncology EMRs. The final readings of CT or/and PET were checked to determine recurrences. Loco-regional recurrence was defined as cancer recurrence at the pancreatic resection site.[29] Cancer recurrence in the liver, lungs or any other distant site was classified as distant metastasis. The peritoneal dissemination was classified as peritoneal carcinomatosis.

Statistics

Variables are presented as an absolute number, percentage, mean with standard deviation (SD), or median with interquartile range (IQR), depending on the type of variables. Statistical analysis was performed using the Student's *t*-test for continuous outcomes with normal distribution and the Mann-Whitney *U* test as nonparametric test for continuous variables. For the binary outcomes, the χ^2 test, or Fisher exact test, were used as parametric and a non-parametric test, respectively. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, IBM Corp., Armonk, NY) version 21.0.

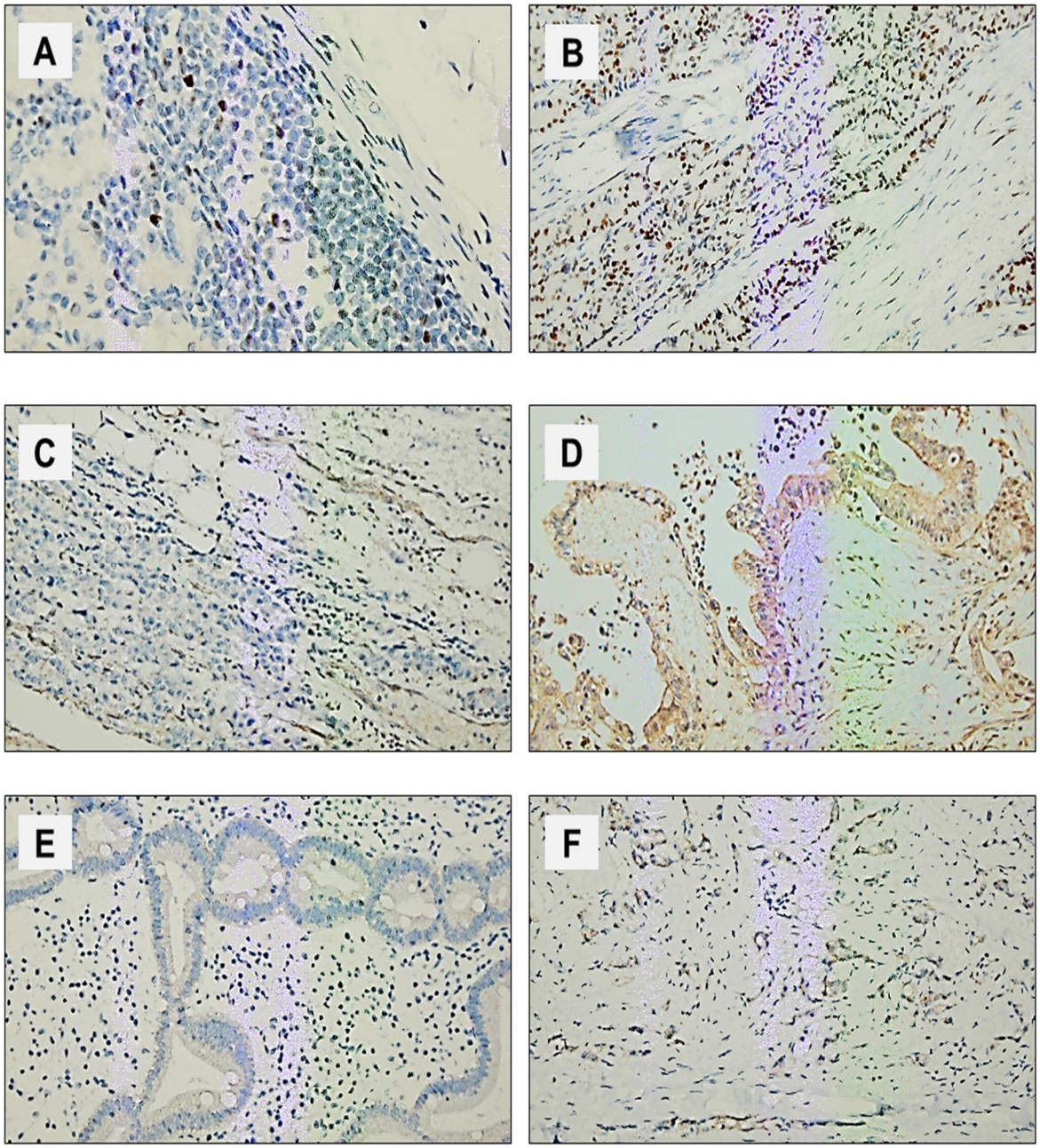


Figure 2. Representative photographs of the immunohistochemistry analysis of p53, DPC4, erbB-2. (A) Normal pattern of p53 immunohistochemical staining. (B) Diffusely positive nuclear staining for p53. (C) Negative staining indicates an inactivated DPC4 gene. (D) Positive staining indicates an expressed DPC4 gene. (E) Negative staining for erbB-2 gene. (F) Positive staining indicates an overexpressed erbB-2 gene

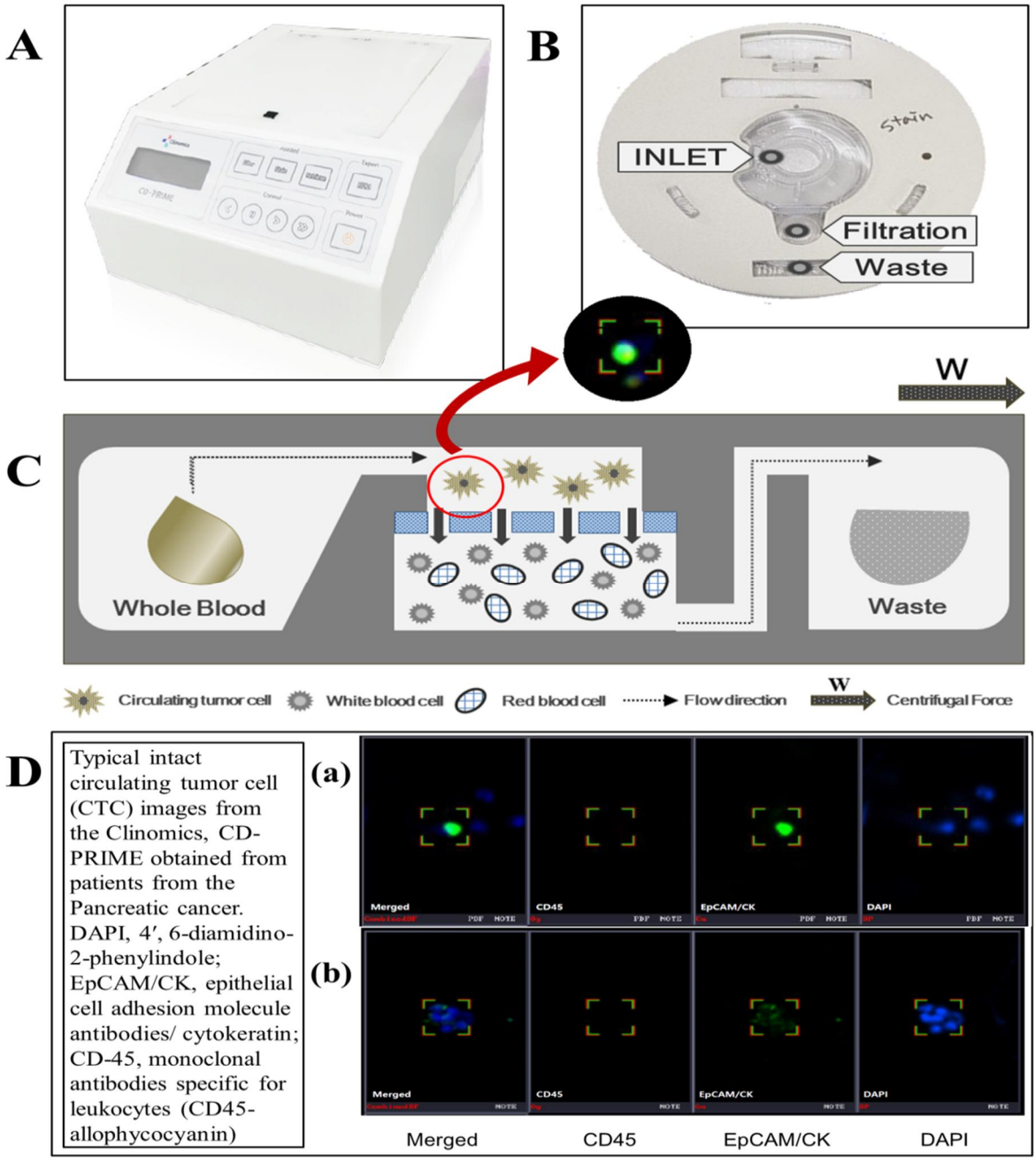


Figure 3. Schematic illustration showing the design and working principle of the CD-PRIME™. (A) This is an operating device; CD-OPR-1000. (B) The CTC isolation disc is composed of three individual filtration units; each unit contains a sample loading chamber, filltration chamber, waste chamber. (C) The schematic illustration showing the working principle of the CTC isolation disc. Operation images of the CTCs isolation disc were taken. (D-a) Fluorescent images of the typical intact circulating tumor cell, No. 21 patients. (D-b) Fluorescent images of the CTCs cluster, No. 34 patients.

Table 1. Clinico-postoperative outcomes according to the CTCs detection

Variable		Total number or median	CTCs (-) (N=21, 65.6%)		CTCs (+) (N=11, 34.4%)	P-value[a]
			*EPCAM-/CD45+ N=15	†EPCAM+/CD45+ N=6	‡EPCAM+/CD45- N=11	
Demographic features						
Age	Median	64	64	72	61	0.953
	IQR	52-72	52-70	52.7-74.5	52-75	
Sex	Male	19 (59.4%)	10 (66.7%)	3 (50.0%)	6 (54.5%)	0.721
	Female	13 (40.6%)	5 (33.3%)	3 (50.0%)	5 (45.5%)	
BMI (kg/m²)	Median	22.02	22.48	21.15	24.18	0.551
	IQR	20.88-24.27	21.39-25.61	20.35-23.01	21.04-24.41	
ASA	1	1 (3.1%)	0 (0.0%)	0 (0.0%)	1 (9.1%)	0.294
	2	30 (93.8%)	14 (93.3%)	6 (100.0%)	10 (90.9%)	
	3	1 (3.1%)	1 (6.7%)	0 (0.0%)	0 (0.0%)	
CA19-9 (U/mL)	Median	60.7	69.2	76.3	27.1	0.350
	IQR	12.1-243.4	1.9-188.1	1.4-7.1	11.9-259.3	
	< 37	15 (46.9%)	6 (40.0%)	3 (50.0%)	5 (45.5%)	
	≥ 37	17 (53.1%)	9 (60.0%)	3 (50.0%)	6 (54.5%)	
CEA (ng/mL)	Median	3.2	3.3	5.7	2.2	0.341
	IQR	1.5-4.5	2.3-4.1	1.4-7.1	1.5-6.4	
	< 6	26 (81.3%)	14 (93.3%)	3 (50.0%)	9 (81.8%)	
	≥ 6	6 (18.7%)	1 (6.7%)	3 (50.0%)	2 (33.3%)	
Preoperative Diabetes	No	23 (71.9%)	10 (66.7%)	5 (83.3%)	8 (72.7%)	> 0.999
Postoperative outcomes						
Procedure	Open	19 (59.4%)	10 (66.7%)	4 (66.7%)	5 (45.5%)	0.491
	Lap.	13 (40.6%)	5 (33.3%)	2 (33.3%)	6 (54.5%)	
Operative type	DP	16 (50.0%)	6 (40.0%)	3 (50.0%)	7 (63.6%)	0.283
	PD	16 (50.0%)	9 (60.0%)	3 (50.0%)	4 (36.4%)	
Venous resection	No	28 (87.5%)	13 (86.7%)	5 (83.3%)	10 (90.9%)	> 0.999
	Yes	4 (12.5%)	2 (13.3%)	1 (16.7%)	1 (9.1%)	
OP time (min)	Median	234	235	210	185	0.487
	IQR	172-370	204-377	149-411	163-338	
POPF	No-BL	31 (96.9%)	15 (100.0%)	6 (100.0%)	10 (90.9%)	0.344
Complication	No	20 (62.5%)	10 (66.7%)	2 (33.3%)	8 (72.7%)	0.493
	Gr 1-2	10 (31.3%)	5 (33.3%)	3 (50.0%)	2 (18.2%)	
	≥ Gr 3	2 (6.3%)	0 (0.0%)	1 (16.7%)	1 (9.1%)	
LOHS (days)	Median	10	10	11.5	9	0.893
	IQR	8-14	7-12	8-15	8-15	

Neo CTx	No	25 (78.1%)	12 (80.0%)	3 (50.0%)	10 (90.9%)	0.374
	Yes	7 (21.9%)	3 (20.0%)	3 (50.0%)	1 (14.3%)	
Adj CTx	No	1 (3.1%)	1 (6.7%)	0 (0.0%)	0 (0.0%)	> 0.999
	Yes	31 (96.9%)	14 (93.3%)	6 (100.0%)	11 (100.0%)	
90-day mortality	Yes	0	0	0	0	> 0.999

a, Comparison between CTCs-Negative and CTCs-Positive, Values in parentheses are percentages.

*, No Circulating tumor cells : EPCAM-DAPI-CD45+

†, No circulating tumor cells, but "double positive" : EPCAM+DAPI+CD45+

‡, Circulating tumor cells : EPCAM+DAPI+CD45-

IQR: Interquartile range, BMI: Body mass index, CA19-9: Carbohydrate antigen 19-9, CEA: Carcinoembryonic antigen, Lap: Laparoscopic, DP: Distal pancreatectomy , PD: Pancreaticoduodenectomy ,BL: Biochemical leakge , OP time : Operative time, LOHS: length of the hospital stays, NeoCTx: Neoadjuvant chemotherapy, Adj CTx : Adjuvant chemotherapy

Results

The total detection rate of the CTCs obtained by preoperative peripheral blood sampling was 34.4 %. Median total cell counts were 2734, and there was no statistically significant difference between the CTCs-positive and the CTCs-negative (2695 vs. 2574, P = 0.151). The median CTCs count detected was 2 (IQR: 1.0-3.5), and the median CTCs / total cell ratio was 0.000561 (IQR: 0.000314-0.000993) in the CTCs-positive group.). At stage III or higher stage(n=8), the CTCs detection rate was 50.0% (n=4). Also, CTCs were detected as a cluster form in one patient. Besides, among seven patients who received neoadjuvant chemotherapy, only one (14.3%) had CTCs-positive. On the other hand, CTCs-positive was observed in 10 (40.0%) out of 25 patients who did not receive neoadjuvant chemotherapy (P = 0.374).

Table 2. Pathological outcomes according to the CTCs detection

Variable		Total number or median	CTCs (-) (N=21, 65.6%)		CTCs (+) (N=11, 34.4%)	P-value[a]
			*EPCAM-/CD45+ N=15	†EPCAM+/CD45+ N=6	‡EPCAM+/CD45- N=11	
Tumor size (cm)	Median	2.7	2.4	2.8	3.2	0.210
	IQR	2.1-3.3	2.0-3.2	2.1-3.4	2.7-4.2	
Differentiation	Well	3 (9.4%)	3 (20.0%)	0 (0.0%)	0 (0.0%)	0.420
	Moderate	21 (65.6%)	9 (60.0%)	4 (66.7%)	8 (72.7%)	
	Poor	8 (25.0%)	3 (20.0%)	2 (33.3%)	3 (27.3%)	
T (AJCC 8th)	T1	6 (18.8%)	5 (33.3%)	1 (16.7%)	0 (0.0%)	0.140
	T2	22 (68.8%)	9 (60.0%)	4 (66.6%)	9 (81.8%)	
	T3	3 (9.4%)	1 (6.7%)	1 (16.7%)	1 (33.3%)	
	T4	1 (3.1%)	0 (0.0%)	0 (0.0%)	1 (9.1%)	
N (AJCC 8th)	N0	14 (43.8%)	7 (46.7%)	4 (66.6%)	3 (27.3%)	0.645
	N1	14 (43.8%)	7 (46.7%)	1 (16.7%)	6 (54.5%)	
	N2	4 (12.5%)	1 (6.7%)	1 (16.7%)	2 (18.2%)	
M (AJCC 8th)	M0	29 (90.6%)	14 (93.3%)	5 (83.3%)	10 (90.9%)	> 0.999
	M1	3 (9.4%)	1 (6.7%)	1 (16.7%)	1 (9.1%)	
AJCC stage	I	14 (43.8%)	8 (53.3%)	3 (50.0%)	3 (27.3%)	0.161
	II	10 (31.3%)	5 (33.3%)	1 (16.7%)	4 (36.4%)	
	III	5 (15.6%)	1 (6.7%)	1 (16.7%)	3 (27.3%)	
	IV	3 (9.4%)	1 (6.7%)	1 (16.7%)	1 (9.1%)	
PNi	No	7 (21.9%)	9 (60.0%)	0 (0.0%)	3 (27.3%)	0.667
	Yes	25 (78.1%)	6 (40.0%)	6 (100.0%)	8 (72.7%)	
LVi	No	16 (50.0%)	9 (69.2%)	3 (50.0%)	4 (36.4%)	0.458
	Yes	16 (50.0%)	4 (30.8%)	3 (50.0%)	7 (63.6%)	
R0 resection	R0	27 (84.4%)	15 (100.0%)	3 (50.0%)	9 (81.8%)	0.891
	R1	3 (9.4%)	0 (0.0%)	2 (33.3%)	1 (9.1%)	
	R2	2 (6.2%)	0 (0.0%)	1 (16.7%)	1 (9.1%)	
p53	Mutation	19 (73.1%)	7 (53.8%)	4 (100.0%)	8 (88.9%)	0.077
	Normal	7 (26.9%)	6 (46.2%)	0 (0.0%)	1 (11.1%)	
DPC4	Inactivation	19 (73.1%)	10 (76.9%)	3 (75.0%)	6 (66.7%)	0.635
	Normal	7 (26.9%)	3 (23.1%)	1 (25.0%)	3 (33.3%)	
erbB-2	Mutation	3 (11.5%)	0 (0.0%)	0 (0.0%)	3 (33.3%)	0.215
	Normal	23 (88.5%)	13 (100.0%)	4 (100.0%)	6 (66.7%)	

a, Comparison between CTCs-Negative and CTCs-Positive, Values in parentheses are percentages.

*, No Circulating tumor cells : EPCAM-DAPI-CD45+

†, No circulating tumor cells, but "double positive" : EPCAM+DAPI+CD45+

‡, Circulating tumor cells : EPCAM+DAPI+CD45-

IQR: Interquartile range, PNi: perineural invasion, LVi: Lymphovascular invasion

1. *Demographic features of the entire cohort*

Demographic features and postoperative outcomes of all patients are described in ***Table 1 and 2***.

7 (21.9%) patients received neoadjuvant chemotherapy, 31 (96.6%) patients received adjuvant chemotherapy or chemoradiotherapy. There was no 90-day mortality, and R0 resection was performed in 84.4% of patient.

2. *Demographic and postoperative outcomes according to the CTCs detection (**Table 1**)*

The two groups did not differ in terms of age, sex, body mass index (BMI), American society of the anesthesia classification (ASA), CA19-9, CEA, and preoperative diabetes. There were no differences in operating procedures, type, and venous resection. Also, there were no statistically significant differences in POPF and complication of Clavien-Dindo classification III or higher.

Of the 32 patients with CA19-9 ≥ 37 U/mL, 17 (53.1%) were CTCs-positive. However, 5 out of 15 patients with CA19-9 < 37 U/mL were CTCs-positive. The proportion of patients with CA19-9 ≥ 37 U/mL or CTCs- positive was 69% (***Figure 4***).

3. *Pathological outcomes according to the CTCs detection (**Table 2**)*

Tumor size, differentiation, peri-neural invasion, and lympho-vascular invasion were not significantly different between the CTCs-positive and CTCs-negative. Also, there were no statistically significant differences between the two resection groups, the R1, and R2 (*P=0.891*).

*4. Sub-analysis of the recurrence pattern in patient according to CTC detection (**Table 3**)*

32 patients were analyzed to identify early recurrence patterns. The median duration of the total follow up was 9.2 months, 7.9 and 9.9 months in CTCs-positive and CTCs-negative respectively. Total of 13 patients (40.6%) recurred within 6 months: 6 patients (54.5%) with CTCs-positive, and 7 patients (33.3%) with CTCs-negative (*P = 0.491*). However, when recurrence type was classified as loco-regional, distant, and peritoneal carcinomatosis as shown in ***Figure 6a***, distant metastasis and peritoneal carcinomatosis were frequent in the CTCs-positive group and statistically significant difference was observed (*P = 0.043*). 1-year progression-free survival did not differ between the two groups (*P=0.060,* ***Figure 7a***). Median progression-free survival time was 5.1 months and 10.0 months respectively. However, in CTCs-positive, there was a poor survival in time to metastatic recurrence (*P = 0.028*, ***Figure 7b***). In ***Figure 7c***, 1-year metastatic progression-free survival is identified as CTCs-positive, CTCs-negative with EPCAM-positive, and CTCs-negative without EPCAM-positive. And survival to metastatic recurrence was 88.9%, 62.5%, and 37.9% respectively.

For the analysis according to CTCs count, we divided the groups depend on the number of CTCs; 8 cases had CTCs less than 2, and 9 cases had CTCs more than 2. There was no difference in recurrence rate between the two groups (CTC < 2/7.5mL:41.7%, CTC ≥ 2/7.5mL: 37.5%, *P > 0.999*). However, there were two disease-related mortalities during the observational period in the CTCs ≥ 2/7.5mL. In this analysis, two patients had more than two CTCs and showed recurrence with a peritoneal carcinomatosis (*P = 0.043*).

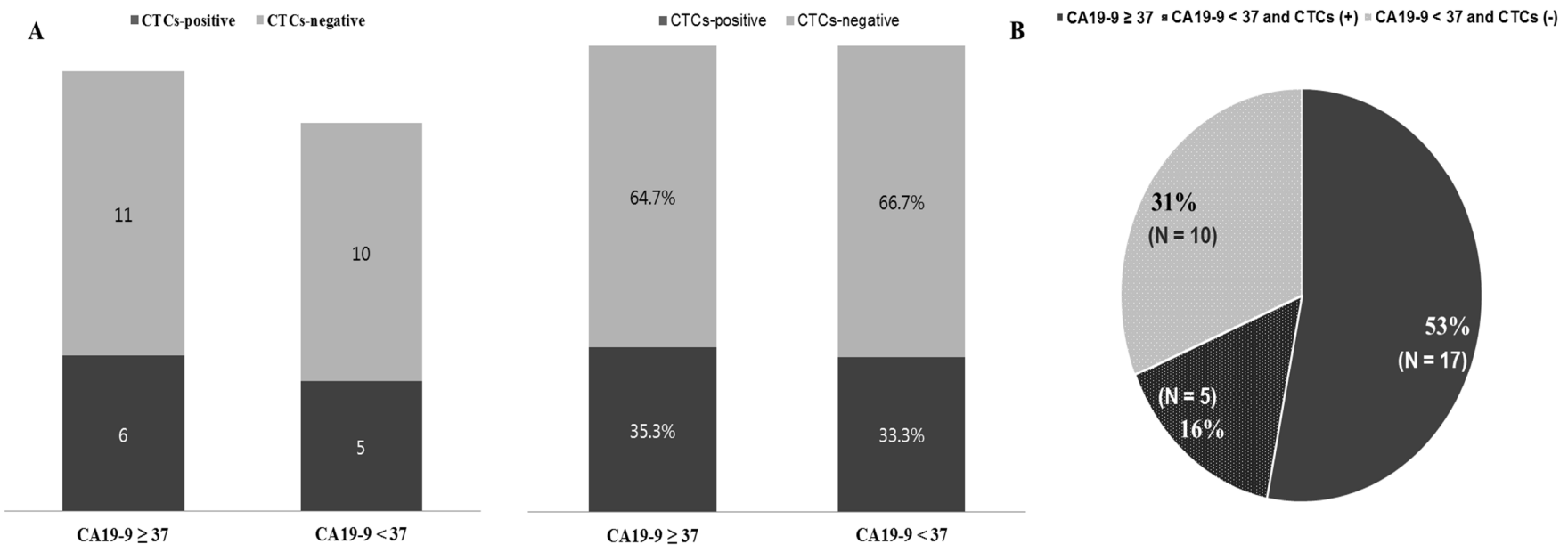


Figure 4. The proportion and number of patients according to the CA19-9 , CTCs detection. Of the 32 patients with CA19-9 ≥ 37 U/mL, 17 (53.1%) were. However, 5 out of 15 patients with CA19-9 < 37 U/mL were CTCs-positive. The proportion of patients with CA19-9 ≥ 37 U/mL or CTCs- positive was 69%.

Table 3. Analysis of the recurrence rate and patterns

Variable		Total number or median	CTCs-negative (N=21, 65.6%)		CTCs-positive (N=11, 34.4%)	P-value[a]
			*EPCAM-/CD45+ N=15	†EPCAM+/CD45+ N=6	‡EPCAM+/CD45- N=11	
Recurrence	No	19 (59.4)	10 (66.7)	4 (66.8)	5 (45.5)	0.491
	Yes	13 (40.6)	5 (33.3)	2 (33.2)	6 (54.5)	
Recurrence type and site	Locoregional	5(38.5)	4 (80.0)	0 (0.0)	1 (16.7)	0.043
	SMA	1	1	0	0	
	Mesentery	2	2	0	0	
	Celiac trunk	1	1	0	0	
	Gastric antrum	1	0	0	1	
	Distant	5 (38.5)	1 (20.0)	1 (50.0)	3 (50.0)	
	Lung	1	0	0	1	
	Liver	4	1	1	2	
	P.carcinomatosis	3 (23.1)	0 (0.0)	1 (50.0)	2 (33.3)	
Rec. duration	Median (months)	4.6	5.1	5.5	4.4	0.056
	IQR	3.6-9.8	3.3-9.9	3.7-10.7	3.1-4.8	
F/U duration	Median (months)	9.2	9.9	10.5	7.9	0.266
	IQR	3.6-10.6	3.8-10.8	6.8-11.1	3.4-9.8	
Death within F/U duration		3 (9.4)	0 (0.0%)	1 (16.7%)	2 (18.2)	0.266

a, Comparison between CTCs-Negative and CTCs-Positive, Values in parentheses are percentages.

*, No Circulating tumor cells : EPCAM-DAPI-CD45+

†, No circulating tumor cells, but "double positive" : EPCAM+DAPI+CD45+

‡, Circulating tumor cells : EPCAM+DAPI+CD45-

IQR: Interquartile range, SMA : Superior mesenteric artery, Rec.duration: Recurrence duration, P.carcinomatosis: Peritoneal carcinomatosis, F/U: follow-up

5. *Correlation of the CTCs detection and mutation of the Primary tumors: p53, erbB-2, DPC4*

Immunohistochemistry results of the primary tumors from 26 patients (81.3%) among 32 patients were reported for p53, erbB-2, and DPC4. There were no statistically significant differences in the distribution of DPC4 inactivation (*P = 0.635,* ***Figure 5a***) and erbB-2 mutation (*P = 0.215,* ***Figure 5b***) according to the CTCs detection . In addition, P53 mutations were found more in CTC-positive, but the levels were not statistically significant (*P=0.077,* ***Figure 5c***). The p53 mutation was confirmed in 88.9% of the CTCs-positive, 100.0% of the CTCs-negative with EpCAM positive, and 53.8% of the CTCs-negative without EpCAM-positive. However, when analyzed based on CTCs counts, meaning, CTCs ≥2/7.5mL and CTCS <2/7.5mL, the p53 mutation was significantly more frequent in the CTCs ≥2/7.5mL. (100.0%, *P=0.045*, ***Figure 5d***)

6. *Recurrence patterns and survival to metastatic recurrence according to the mutation of p53, DPC4 in primary tumor*

Metastatic recurrence occurred in 7 of 11patients with p53 mutation, and there was a statistically significant difference when compared with normal patients (63.6% vs. 0.0%, *P=0.034*,***Figure 6b***). However, metastatic recurrence was observed in 5 (50.0%) of 10 DPC4 inactivated patients and 2 (33.3%) of 6 normal group (***Figure 6c***), and there was no statistically significant difference (*P = 0.633*).

In addition, the time to metastatic recurrence following p53 mutation and DPC4 inactivation was analyzed in this study. Patients with p53 mutations showed poor survival (100% vs. 56.2%, *P = 0.078*, ***Figure 7d***), patients with DPC4 inactivation showed poor survival as well (***Figure 7e***), but there were no statistically significant differences (60.0% vs. 50.0%, *P=0.480*).

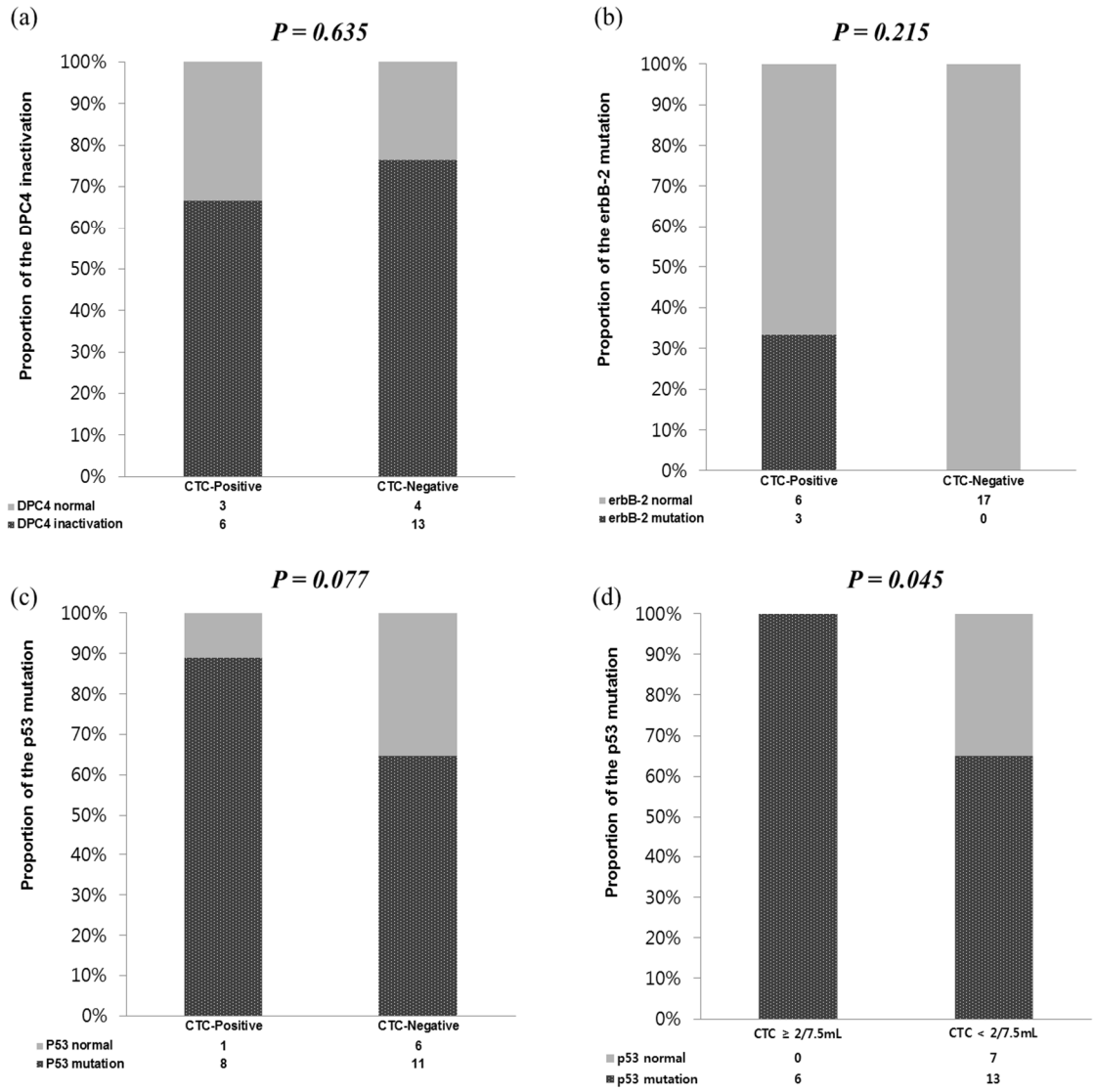


Figure 5. Correlation of the CTCs detection and mutation of the Primary tumors: p53, erbB-2, DPC4. (a) There were no statistically significant differences in the distribution of DPC4 inactivation and (b) erbB-2 mutation according to the CTCs detection. (c) The p53 mutation was confirmed more in CTCs-positive (d) When analyzed by dividing CTCs by ≥2/7.5mL and <2/7.5mL, the p53 mutation was significantly more frequent in the CTCs ≥2/7.5mL.

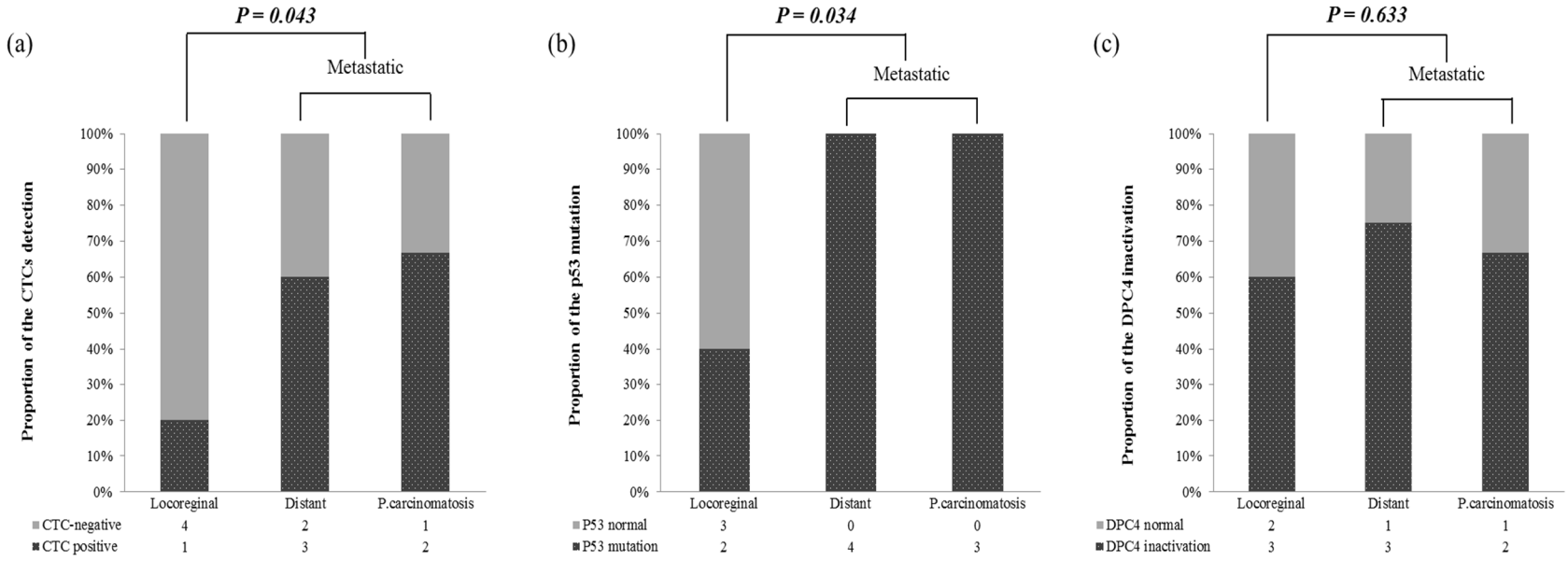
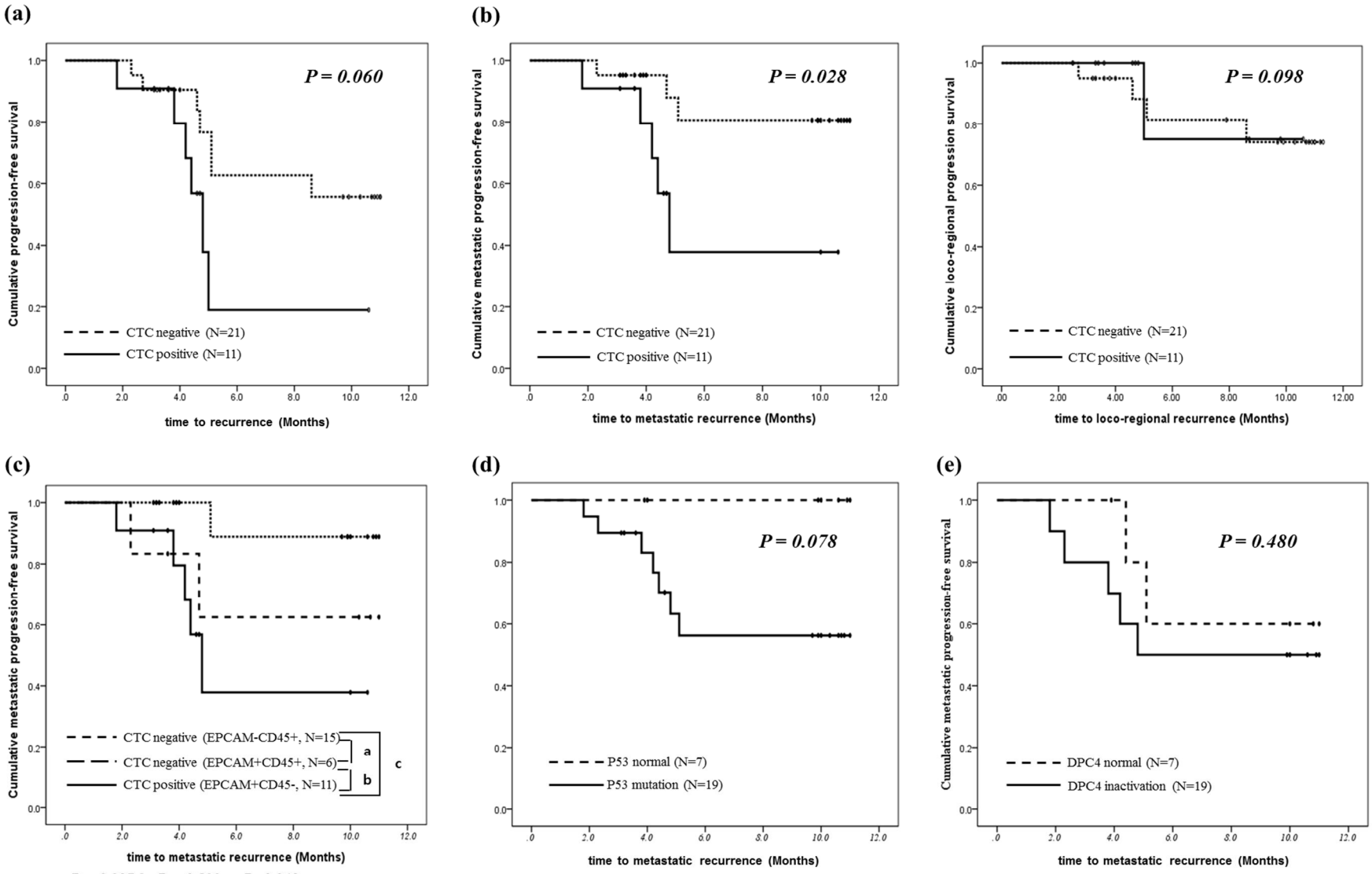


Figure 6. Early recurrence patterns according to the CTCs detection, p53 mutation, and DPC4 inactivation. (a) Distant metastasis and peritoneal carcinomatosis were more frequent in CTCs-positive group and statistically significant difference was found (*P = 0.043*). (b) There was a statistically significant difference when compared with normal patients (63.6% vs. 0.0%, *P=0.034*) (c) metastatic recurrence was observed in 5 (50.0%) of 10 DPC4 inactivation patients and 2 (33.3%) of 6 normal group, and there was no statistically significant difference (*P = 0.633*).



a: *P = 0.137*, **b**: *P = 0.522*, **c**: *P=0.049*

Figure 7. 1-year progression-free survival (a) The 1-year progression-free survival did not differ between the two groups (55.7% vs. 18.9%, *P=0.060*). (b) In CTCs-positive, there was a poor survival in time to metastatic recurrence (80.6% vs. 37.9%, *P=0.028*) (c) 1-year metastatic progression-free survival was identified as CTCs-positive, CTCs-negative with EPCAM-positive and CTCs-negative without EPCAM-positive. And survival to metastatic recurrence were 88.9%, 62.5% and 37.9%, respectively. (d) There was no statistically significant difference in the time to metastatic recurrence according to the p53 mutation (100% vs. 56.2%, *P=0.078*) (e) Patients with DPC4 inactivation showed poor survival (60.0% vs. 50.0%, *P=0.480*).

Discussion

In this study, using a sized based isolation method, the detection rate of CTCs was confirmed to be 34.4%. The purpose of our study was to identify differences in patterns of early recurrence following CTCs detection in the patients who underwent surgery for PDAC. There was no significant difference in the recurrence rate within 6 months of post-operation. However, distant metastasis and peritoneal carcinomatosis were more frequent in the CTCs-positive group (*P = 0.043*). There was no statistically significant association between CTCs detection and p53 mutation (*P=0.077*), but in the case of CTCs ≥ 2/7.5mL, p53 mutation was more frequent (*P=0.045*).

Detection rate of the CTCs in peripheral blood

The CTCs detection rate of our study was calculated as 34.4% while previous studies showed a CTCs detection rate range of 11% -93%, and this difference was depended on the detection method. [5, 6, 8, 11, 13, 14, 30-34)] Iwanicki et al., Kurihara et al., Soeth et al., and Z'graggen et al. found CTCs detection in 56%, 42%, 34%, 26% of patients, respectively. In the study of Iwanicki et al., 18(66.7%) of the 27 were patients with advanced stage cancer.[35)] In a study by Kurihara et al., only one of the 26 were patients with stage I-II, and CTCs were not detected.[5)] And in Soeth et al.'s study of 154 patients, 27 (17.5%) were patients with stage I-II, and CTCs were detected in only 7 of them.[6)] Z'graggen et al. also studied 105 patients, among them, 10 were in stage I-II.[31)] CTCs were detected in 53 patients (75%) of 72 patients with primary PDAC in a study in which CTCs were proposed as diagnostic tool markers (sensitivity=75%, specificity=96.4%, area under the curve =0.0867, *P<0.001*), and of these patients, 31 (43.1%) were patients with stage I-II.[36)] However, there were only 3 patients with stage I cancer. In this study, 24 (75.1%) out of 32 patients were in AJCC 8th stage I-II, and among them, CTCs was found in 7 patients (29.2%). 14 patients (43.8%) were in stage I, and 3 (27.3%) of them had CTCs detection. In the AJCC 7th stage, there were 13 cases of IIA, 16 cases of IIB, and 3 cases of IV. Compared with previous studies, we assumed that the detection rate were relatively low because of the low percentage of advanced stage and high percentage of AJCC 8th stage I-II patients.

Other study has reported that the CTCs detection rate decreases after chemotherapy by 5-Fluoruracil (Before chemotherapy: 80.5% vs. After chemotherapy: 29.3%).[37] In present study, neoadjuvant chemotherapy was performed in 7 of 32 patients, and CTCs were detected in 1 patients (14.3%). Kulemann et al. Reported a detection rate of 72.7% in 11 patients, of whom 62.5% were Stage III and IV.[10] In our study, At stage III or higher (n=8); with more than 4 Lymph node metastases or involvement of the celiac axis, superior mesenteric artery, common hepatic artery were involved, CTCs detection rate was 50.0% (n=4). And in stage III-IV, 3 patients underwent neoadjuvant chemotherapy, of which only one detected CTCs.

Our results suggest that CTCs cannot account for its role as a diagnostic tool marker. However, as shown in Figure 3, among 32 patients with CA19-9 < 37U/mL, 15 patients exhibited less than 37 U/mL, with CTCs-positive in 5 patients(16%). A total number of patients who showed CA19-9≥37U/mL or CTCs-positive were 22 (69%). This suggests that CTCs have potential as a complementary diagnostic tool marker.

True CTCs (EpCAM+CD45-) and False CTCs (EpCAM+CD45+)

The CTC defined by CellSearch is a nucleated cell lacking CD45 and expressing cytokeratin and EpCAM.[38] There is a study stating that a CTCs is not true CTCs unless it is CD45 negative.[39] The currently accepted definition of CTCs (Cytokeratin and/or EpCAM positive nucleated cell that is CD45 negative), multiple groups are beginning to note other atypical cells in the blood of patients with cancer.[12, 27, 28] These include CD45 positive cells that also have cytokeratin or EGFR. These cells have been called ‘‘double positives’’ by some groups. [26] However, the exact origins of these cells are still under debate. The possibilities include the fusion of hematopoietic cells to circulating cancer cells, non-specific binding of CD45 antibodies to isolated cells, or most intriguingly cancer cells originating from the bone marrow with stem cell-like features.[28, 38] 6 patients with double positive were included in the CTCs-negative group in this study, and their clinical information is described in the ***Table 5***. In the case of CTCs-negative with EpCAM-positive, p53 mutation was found in 100.0%. This proportion was similar to the p53 mutation in CTCs-positive (88.9%) and in CTCs ≥ 2/7.5mL

(100.0%). Also, when the survival to metastatic recurrence of CTCs-positive, CTCs-negative with EpCAM-positive and CTCs-negative without EpCAM-negative were compared, the CTCs-negative with the EpCAM-positive group was plotted in the middle (***Figure 7c***). And, 2 patients out of 6 patients who exhibited double positive were recurrent with distant metastases and peritoneal carcinomatosis (***Table 3***). However, there were no other significant differences except for the differences between the CTCs-positive and CTCs-negative without EpCAM-positive.

Recurrence rate and patterns according to CTCs detection

As summarized in ***Table 4***, many studies had reported that there is a difference in overall survival according to CTCs detection.[5, 6, 8, 11, 13, 14] Although there are studies reporting contrast results,[31, 33] it is generally accepted that overall survival is poor when CTCs are detected. However, in our knowledge, there are only a few studies dealing with the recurrence rate or pattern according to the CTCs detection. Mataki et al. reported that, out of 20 patients, CTCs were detected in 6 patients, and among them, 5 patients showed recurrence, and one patient had liver metastasis within 6 months of post operation.[32] In their report, only 2 patients have recurred in the CTCs-negative group (N = 14), and one of them had liver metastases within postoperative 6 months.[32] Bissolati et al. reported that patients with portal vein CTCs-positive had more liver metastases, 2 and 3 years after surgery (57.1% vs. 8.3%, $P = 0.038$).[33] In this study, 32 patients were analyzed to confirm the tendency of recurrence within 6 months of surgery, and their median follow up days were 9.2 months. As described in ***Table 3***, the recurrence rate within 6 months of CTCs-negative and positive were 33.2% and 54.5% respectively ($P = 0.491$), and CTCs-positive group showed a tendency of recurrence with distant metastasis and peritoneal carcinomatosis ($P = 0.043$). Due to the limited number of patients in our cohort and short observational period, the result of correlation or association studies should be considered with caution.

Clinical findings according to CTCs count

Progressive metastatic castration-resistant disease in prostatic cancer has been reported to increase

with increasing CTCs count.[40] And, the CTCs counts in PDAC have been reported account for a large percentage of stage IV. [36] In this study, several clinical significance was confirmed by dividing the CTCs count by two. First, p53 mutation was 100% in patients with CTCs≥2/7.5ml. Also, during the study period, there were 3 disease related deaths, 2 of which were patients with CTCs≥2/7.5ml and all showed peritoneal carcinomatosis recurrence pattern. Finally, in 1-year overall survival, 42.9% (median time : 8.7months) were in CTCs≥2/7.5ml and 93.8 % (median time : 11.1months) in CTCs<2/7.5ml, respectively (*P=0.014*).

Findings with CTC detection and Tumor Stage

Z'graggen et al reported that patients with peritoneal dissemination were found to have more CTCs-positive than CTC-negative patients (67% vs. 22%, *P = 0.001*).[31] Also, in their studies, there was a trend that the CTCs were detected more at UICC Stage IVb with distant metastases than Stage I-IVa (39% vs. 20%, *P = 0.084*).[31] And, in another study, there was a significant difference between CTC detection rates in the test blood samples of stage III and stage IV but not in other stages (*P=0.005*).[6] On the other hand, studies have shown that CTC detection does not vary significantly with tumor stage, which is still controversial.[32] In our study, AJCC 8th stage III and higher were found to be more in the CTC-positive group (***Table 2***), which was not statistically significant (*P=0.161*). The number of patients enrolled in the study was 32, of them, cases of Stage III or higher were 8, which was a very limited number. If we expand the analysis through continuous enrollment in the future, the correlation between cancer stage and CTC detection can be confirmed. The significance of CTC clusters in metastasis development is currently appreciated, but this knowledge does not yet translate into clinical applications.

Table 4. Summarized about previous studies for CTCs detection

Reference (First author)	Patients	Stage	Enrichment methodology	Cell analysis	Detection rate	Summary and Findings
Z'graggen et al., 2001	N = 105	Localized, Locally advanced and Metastatic disease	Density centrifugation	Immunocytochemistry	26%	Not an independent factor of Survival Patients with CTCs detection were more likely to be diagnosed with peritoneal dissemination. (*P = 0.001*) Tumor differentiation is not different.
Mataki et al., 2004	N = 20	Localized, Locally advanced and Metastatic disease	Density centrifugation	Nested PCR	30%	5 of 6 patients with CTCs detected have recurred. 1 patient recurred within 6 months. (liver metastasis)
Soeth et al., 2005	N= 154	Localized, Locally advanced and Metastatic disease	Density centrifugation	Nested PCR	34%	CTCs-positive showed poor survival *(P = 0.05*) and showed a tendency to advanced stage.
Kurihara et al., 2008	N = 26	Localized, Locally advanced and Metastatic disease	Immunomagnetic enrichment	Cellsearch	42%	CTCs-positive correlated with poor survival (*P < 0.001*).
Khoja et al., 2012	N = 53	Localized, Locally advanced and Metastatic disease	ISET (Sized-based) and Immunomagnetic enrichment	Immunocytochemistry and Cellsearch	93% 40%	There were trends toward decreased survival and progression free survival. Tumor differentiation did not differ.
De Albuquerque et al., 2012	N = 34	Localized, Locally advanced and Metastatic disease	Immunomagnetic enrichment	RT-qPCR	47%	Shorter progression-free survival (*P=0.001*)
Bidard et al., 2013	N = 79	Locally advanced disease	Immunomagnetic enrichment	Cellsearch	11%	CTCs-positive correlated with poor tumor differentiation (P=0.04) and OS (*P=0.01*)
Iwanicki-Caron et al., 2013	N = 27	Localized, Locally advanced and Metastatic disease	Sized-based centrifugation	Cytological features	56%	CTCs-positive was not correlated with tumor characteristics, CA19-9, tumor stage.
Bissolati et al., 2015	N = 20	Localized disease	Immunomagnetic enrichment	Cellsearch	45%	CTCs-positive was not correlated with OS. Portal vein-CTCs associated to higher rate of Liver metastasis
Earl et al., 2015	N = 45	Localized, Locally advanced and Metastatic disease	Immunomagnetic enrichment	Cellsearch	20%	CTCs-positive correlated with poor survival (*P=0.023*)
Current study	N= 32	Localized, Locally advanced and Metastatic disease	Sized-based centrifugation	Immunocytochemistry	34%	Stage III or higher, the detection rate of CTCs was 50.0%. There was no difference in early recurrence rate among CTCs-positive.

Association of the CTC detection and p53 mutation, DPC-4 inactivation, erbB-2 mutation in the primary tumors

Patients with p53 mutation or DPC4 inactivation showed a poor survival tendency in time to metastatic recurrence (***Figure 7d, 7e***). All 3 patients with erbB-2 over expression were found to be in the advanced stage (Stage III or more), especially in the node metastasis (*P=0.013*).

It was the p53 mutation that confirmed a statistically significant association with CTCs ≥ 2/7.5mL (*P=0.045*, ***Figure 5d***). However, there was no significant association between p53 mutation and CTCs-detection (*P=0.077*). Zulfiqar et al., reported that CTCs mutation in peripheral blood were invariably identical to those found in corresponding solid tumor samples.[41] They explained that because of the low sensitivity or due to a different size of CTCs, hematogenous cells were mixed. And they also described the possibility of identifying the oncogene mutation of the primary tumor by CTCs mutation identification in a peripheral blood sample. The 8 patients (88.9%) identified with p53 mutations in this study were CTCs-positive. In the case of CTCs-negative, p53 mutations were found in 11 (64.7%) out of 17 patients, of whom 4 (100.0%) were CTCs-negative with EpCAM-positive (Double positive). Herein, metastatic recurrence pattern was significantly higher in the presence of p53 mutation (*P = 0.034*, ***Figure 6b***). There was no statistically significant difference in the time to metastatic recurrence according to the p53 mutation, but there was a tendency for poor survival in the case of p53 mutation (***Figure 7d***). In some studies, p53 mutations have been reported as poor outcome of PDAC, but whether the p53 mutation is a diagnostic factor is still at the center of controversy. [42, 43] However, previous studies on the mechanism of p53 mutation and epithelial-mesenchymal transition (EMT) have been continuing and have been reported to drive EMT, migration and invasiveness.[44-47] When the p53 mutation occurs due to oncogenetic stress, it acts on SLUG, EPCAM, Twist, and weakens cell-cell junction, in turn, leads to weakening of the inhibition of NOTCH, RhoA, PTEN and mirR-143. This is explained as a mechanism to accelerate migration and invasiveness.[48] Considering the similar pattern of metastatic recurrence, time to metastatic recurrence and the association of the p53 mutation in the CTCs≥2/7.5mL, the mechanism of p53 associated with EMT seems to explain the possibility of the association with CTCs detection in this study.

There was no significant association between DPC4 inactivation and CTCs detection (*P = 0.635*, ***Figure 5a***). Inactivation or loss of the TGF-β signaling effector DPC4 is found in approximately 50 percent of pancreatic cancers, resulting in aberrant TGF-β signaling. [42] In previous studies, DPC4 loss appears to be associated with tumor progression, patterns of failure, and the EMT.[49, 50] In our previous study, analysis of 641 patients revealed that genetic status of DPC4 was associated with overall survival and was highly correlated with recurrence patterns, as inactivation of the DPC4 gene was the strongest predictor of metastatic recurrence (odds ratio = 4.28).[51] In this study, metastatic recurrence pattern was more frequent in DPC4 inactivation group and poor survival pattern was observed in time to metastatic recurrence but there was no statistically significant difference (***Figure 6c***, ***Figure 7e***). However, since the two graphs stay parallel and the DPC4 deactivation is consistently depicted as a bad survival pattern, we think that increasing the number of cases will be meaningful. A subsequent large-scale follow-up study is needed to confirm the prognostic value of DPC4 as well as the relationship between CTCs detection and DPC4 inactivation.

The association of CTC detection with erbB-2 mutation could not be confirmed in the present study. Only three patients with the erbB-2 mutation were confined to the analysis. Similar to the results reported in previous studies,[52] all three patients were in the advanced stage (*P = 0.013*) and all patients were node positive.

One patient was presented with unexpected findings; the CTC clusters were identified in enrollment number 34 patient who underwent Whipple's operation for the PDAC (***Figure 3D***). Patient's clinical information is described in the ***Table 5***. Four single CTCs and one CTC clusters were detected in this study. In 1954, Watanabe had shown that these cells have high metastatic potential.[53] CTC clusters are defined as a group of more than two or three tumor cells, with strong cell-cell contacts, detected in the blood of a cancer patient.[54] Several studies have demonstrated reduced apoptosis, enhanced survival and colony-forming potential of CTC clusters.[55] Thus, CTC clusters may have the advantage of survival in the circulation and during dissemination. CTC clusters were found in breast, lung, kidney, prostate cancer, and some tumors, a direct relationship was established with their poor

prognosis.[54, 55] In pancreatic cancer, CTC clusters were reported to be an independent predictor of progression-free survival and overall survival.[56] Long-term follow-up and further studies are needed to confirm the overall survival and clinical characteristics of detection.

Due to the small number of patients included in the study, there is a limitation in confirming recurrence patterns and stage differences in this study. Besides, there is heterogeneity among enrolled patients. Neoadjuvant therapy was performed in 21.9% (n = 7) of patients included in the study, open and laparoscopic methods were mixed in operative type, and 50% of the left-sided PDAC. Moreover, the size-based method we chose is not a sufficiently validated test for internal validity. However, this method is an automated system allowing surface antibodies to capture various CTCs. It is also relatively cheap and an easy method. The role of the diagnostic tool marker could not be confirmed clearly; and because there was no control and the detection rate was relatively low, sensitivity, specificity, AUC could not be identified. Also, the follow-up duration of clinical data was short. Thus, the survival analysis of how the difference in early recurrence patterns will affect overall survival has not been achieved. Above all, gene analysis of CTCs should be accompanied to confirm the relevance of CTCs to PDAC. To our knowledge, this was the first study reporting an association between the early recurrence pattern and CTC detection. We were able to demonstrate that the distant metastases and peritoneal dissemination were more in the CTCs-positive group through subgroup analysis.

Conclusions

Further studies are needed to confirm CTCs as a valuable diagnostic tool marker in patients who underwent curative resection for pancreatic ductal adenocarcinoma. We confirmed that the CTCs detection is associated with early recurrence of distant metastasis and peritoneal dissemination. As a preliminary study, all registered patients in this study are constantly being monitored. We hope that CTCs would be analyzed as prognostic biomarkers for long-term survival and disease progression.

Table 5. Clinical information of the patients who underwent pancreatectomy for PDAC (N=32)

No.	Sex	Age	Neo-adj CTx	OP	CA 19-9	CTCs Detection	CTC count	Total cell	CD 45	EpCAM	Stage	Differ	Rec.	Duration (month)	Type	Rec. Site
1	M	53	Yes	DP	9.1	Double	1	4529	(+)	(+)	IV	Poor	(+)	4.7	D	Liver
2	M	79	No	DP	1362.0	No		7678			IIB	Mod.	(+)	5.1	D	Liver
3	N	52	Yes	DP	12.5	Double	1	3372	(+)	(+)	IA	Mod.	(-)			
4	F	35	Yes	PD	52.3	No		2574			IB	Mod.	(-)			
5	M	40	No	PD	5550.0	No		1084			IB	Mod.	(-)			
6	M	73	No	PD	72.8	No		3360			IIB	Poor	(-)			
7	M	74	Yes	PD	332.6	Double	1	2304	(+)	(+)	IIB	Mod.	(-)			
8	F	75	No	DP	23.1	Yes	6	1851	(-)	(+)	IB	Mod.	(+)	4.4	P	P.carcinomatosis
9	F	64	No	PD	0.6	Yes	2	2054	(-)	(+)	IB	Mod.	(-)			
10	F	52	Yes	PD	0.6	No		1197			IB	Mod.	(+)	5.1	L	Mesentery root
11	F	72	No	DP	140.0	Double	1	2734	(+)	(+)	IB	Mod.	(-)			
12	M	76	No	PD	2.2	Double	3	3593	(+)	(+)	III	Mod.	(-)	3.8	P	P.carcinomatosis
14	F	83	No	PD	1240.0	Yes	7	13434	(-)	(+)	III	Poor	(+)	2.3	P	P.carcinomatosis
15	M	69	No	DP	107.5	No		2397			IIB	Mod.	(+)	4.6	L	Celliac trunk
16	M	61	Yes	DP	27.1	Yes	1	2448	(-)	(+)	III	Mod.	(+)	5.0	L	Stomach antrum
17	M	58	No	PD	21.3	No		2493			IIB	Well	(-)			
18	M	45	Yes	PD	69.2	No		4036			IV	Well	(+)	5.1	L	SMA
19	F	69	No	PD	195.1	Yes	1	6758	(-)	(+)	III	Mod.	(+)	4.2	D	Lung
20	F	64	No	PD	128.0	No		3096			IIB	Well	(-)			
21	M	61	No	DP	259.3	Yes	1	5688	(-)	(+)	IB	Poor	(+)	4.8	D	Liver
22	M	77	No	DP	454.0	Yes	2	3018	(-)	(+)	IIB	Mod.	(-)			
23	F	52	No	DP	11.9	Yes	4	4879	(-)	(+)	IV	Poor	(-)			
24	F	69	No	DP	246.1	No		5046			IB	Mod.	(-)			

25	F	70	No	PD	10.7	No		2029			III	Poor	(+)	2.7	L	Mesentery root
26	M	75	No	DP	35.2	No		3568			IA	Mod.	(-)			
27	F	72	No	PD	1.0	Double	1	968	(+)	(+)	IB	Mod.	(-)			
28	F	52	No	DP	235.6	Yes	5	2695	(-)	(+)	IIB	Mod.	(-)			
29	M	42	No	DP	8.2	Yes	2	1460	(-)	(+)	IIB	Poor	(+)	1.8	D	Liver
30	M	61	No	DP	21.6	No		1854			IB	Poor	(-)			
31	M	67	No	PD	21.1	No		2016			IB	Mod.	(-)			
33	M	56	No	DP	524.1	No		1164			IA	Mod.	(-)			
34*	M	59	No	PD	98.8	Yes	5	2180	(-)	(+)	IIB	Mod.	(-)			

*, One cluster was identified in this patient.

Neoadj. CTx. : Neoadjuvant chemotherapy, Rec. : recurrence, P.carcinomatosis: peritoneal carcinomatosis, NA : Not applicable, M : Mutation, N: No mutation, I: Inactivation, Mod,: moderate, D: distant metastasis, L: local recurrence

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국문 요약

목적

이 연구는 췌장선암 발생시 진단과 수술 후 외래 추적 관찰 중 췌장선암의 조기 재발을 예측하기 위한 위한 생체 표지자로서 순환종양세포의 역할을 평가하는 것을 목적으로 한다.

대상 및 방법

2017년 12월부터 2018년 8월까지 서울 아산 병원에서 36명의 췌장선암 환자가 의뢰되었고 이중 32명의 환자를 대상으로 췌십이지장절제술, 췌원위부절제술을 시행하였다. 연구는 Institutional review board (IRB)의 심의를 받았으며, 연구에 참여한 모든 환자에게 충분한 설명을 시행 후 동의서를 구득했다. 수술 시작 전 동의서를 구득한 모든 환자의 말초 혈액 7.5mL를 채혈 했다. 우리는 순환종양세포를 분리하고 계수하기 위한 방법으로 크기 기반의 분리 방식을 사용하였고, 순환종양세포 검출 여부에 따라 양성 (11명)과 음성 (21명) 그룹으로 나누었다. 또한 전체 32명의 환자를 대상으로 조기 재발 분석을 시행하였다.

결과

수술 전 말초 혈액에서 채혈 후 획득한 순환 종양 세포의 검출률은 34.4% 였고,

중앙값은 2cells/7.5mL였다. 순환종양세포가 음성인 환자는 21명 (65.6%)이었는데 이중 6명의 환자에서 "이중양성세포"가 확인되었다. 6개월 이내 재발한 환자는 13명 (40.6%)이 확인되었는데, 순환종양세포 양성인 환자는 6명 (54.5%), 음성인 환자는 7명 (66.5%)으로 통계적으로 유의한 차이는 없었다 (*P=0.491*). 그러나 원격전이와 복막파종 형태의 재발은 순환종양세포 양성인 환자에서 빈도가 높았으며 통계적으로 유의한 차이를 보였다 (*P=0.043*). 순환종양세포가 검출되었을 때 원발 종양의 p53 돌연변이가 8례 (88.9%, *P=0.077*)에서 확인 되었다. 그러나 순환종양세포를 2개이상, 2개 미만으로 나누어 분석한 결과 2개이상인 경우에서 p53 돌연변이 빈도가 더 높게 확인되었다 (100%, *P=0.045*).

결론

췌장선암으로 수술적 치료를 받은 환자를 대상으로 진단도구로서의 순환종양세포의 역할과 가치를 확인하기 위해서는 추가적인 연구들이 필요할 것이다. 이 연구를 통해 췌장선암 환자의 수술 직전 채혈한 혈액에서 검출된 순환종양세포는 원격 전이와 복막파종의 형태로 조기 재발하는 것과 연관성이 있음을 확인했다. 이 연구는 예비 연구이다. 등록된 모든 환자에 대해 지속적인 추적관찰을 통해 장기 생존 및 췌장선암의 진행의 예후인자로서 순환종양세포의 역할을 확인할 수 있기를 기대한다.