Culture of circulating tumor cells using a microfilter device

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Abstract. Circulating tumor cells (CTCs) are associated with cancer metastasis and prognosis but their scarcity in whole blood prevents their use as a diagnostic tool. The purpose of the present study was to establish a novel approach to capture and cultivate CTCs using a microfilter device. The present study was a prospective study of patients with pancreatic cancer at the University of Tsukuba Hospital (Tsukuba, Japan). From each patient, 5 ml of whole blood was collected into an EDTA collection tube. Whole blood was filtered to isolate CTCs and cells captured on the microfilter were cultured in place. A total of 15 patients were enrolled. CTCs and/or CTC clusters were detected in 2 of 6 cases on day 0. In all cases, CTCs and/or formed clusters and/or colonies were observed during long-term culture periods of up to 103 days. In samples where CTCs were not immediately evident, CTC clusters and colonies emerged after long-term culture. To confirm activity of the cultured CTCs on the filters, staining with Calcein AM was performed and epithelial cellular adhesion molecule-positive cells were observed. The system enables the capture and culture of CTCs. Cultured CTCs may be used for patient-specific drug susceptibility testing and genomic profiling of cancer.

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Abbreviations: IPD, invasive ductal carcinoma; IPMC, intraductal papillary mucinous carcinoma; PDAC, pancreatic ductal adenocarcinoma; DP, distal pancreatectomy; TP, total pancreatectomy; SSpPD, subtotal stomach-preserving pancreatoduodenectomy; Lap-DP, laparoscopic distal pancreatectomy

Key words: circulating tumor cell, pancreatic cancer, microfilter device, cluster, colony

Introduction

Circulating tumor cells (CTCs) are malignant cells, which may metastasize from a primary site to a secondary site (1,2). Their number may serve as a prognostic marker in cancer patients, since increases in CTCs after treatment indicate therapy failure (3-6). Of note, to achieve the goal of personalized therapy, CTC phenotype details are more important than total counts (7-9). For this reason, the analysis of CTCs in liquid biopsy enables us to improve the management of therapies for patients with cancer and elucidate the mechanisms of cancer metastasis.

Conventional technologies mainly rely on antibody-based methods for detecting CTCs with an epithelial phenotype, since epithelial-mesenchymal transition (EMT) has a core role in promoting metastasis by facilitating high mobility and invasiveness (10,11). Since CTCs in the bloodstream gain a mesenchymal phenotype, current antibody-based methods (including the CellSearch system, which detects CTCs based on epithelial characteristics), have decreased detection sensitivity in these cases. Therefore, sized-based CTC isolation methods were developed to overcome antibody-based limitations and enable EMT-independent detection of CTC (12-14).

For such blood-based cell analyses to work, *in vitro* expansion is required, as the analysis of physiological functions, such as drug sensitivity, requires a large number of cells and whole blood may only contain a small number of total cells in circulation. Several studies have attempted to cultivate CTCs isolated from whole blood; however, the periods of such reported *in vitro* CTC cultures are short (15-17). The establishment of long-term CTC cultures therefore remains an urgent issue. The purpose of the present study was thus to establish a novel approach to culture captured CTCs using a microfilter device. The primary aim was to reduce damage to cultured CTCs captured on the filter and investigate the effects of the filter material and a culture medium exchange method on long-term culture viability. It was hypothesized that the less invasive peripheral collection and microfiltering are able to rapidly isolate and culture CTCs (including CTC clusters), which may be subjected to functional and genome analyses to better predict treatment responses and design antitumor strategies.

Materials and methods

Study design. The present study was a prospective study performed in patients with pancreatic cancer at the University of Tsukuba Hospital (Tsukuba, Japan). A total of 15 whole-blood specimens from patients with pancreatic cancer were collected between October 2021 and April 2022. The study protocols were approved by the Institutional Review Board of the University of Tsukuba Hospital (Tsukuba, Japan; approval no. H30-150) and all patients provided written informed consent.

Single and cluster CTC isolation. From each patient, 5 ml whole blood was collected into an EDTA blood collection tube (Venoject II vacuum blood collection tube; Terumo) and aspirated with a 10-ml syringe (SS-10SZ; Terumo), and the syringe was attached to an automatic pump (KDS100; KD Scientific, Inc.). The following operations were all performed at a flow rate of 50 ml/h. A 13-mm diameter 15±0.5-μm pore filter (φ15-RM-P30d-t10; Optnics Precision) and a 8±0.5-μm pore filter (φ8-RM-P17d-t10; Optnics Precision) were respectively mounted on the filter holders (CSS-00352; Optnics Precision), and these two filter devices were connected in tandem to the syringe containing the sample. Blood samples were passed through the 15-μm pore filter and 8-μm pore filter, and cells were collected by each filter. Subsequently, the filter was washed by flushing with 10 ml PBS/2 mM EDTA. In cases 1 to 6, 2 ml of staining solution [PE-conjugated mouse anti-human epithelial cellular adhesion molecule (EpCAM; 1:200 dilution; IgG2b clone 9C4; cat. no. 324206; BioLegend) and Hoechst 33342 (1:2,000 dilution; cat. no. H3570; Invitrogen; Thermo Fisher Scientific, Inc.) in PBS], was added and incubated at room temperature for 30 min. Subsequently, the filter was washed by flushing with 10 ml PBS. Finally, the filter device was removed from the syringe and placed on the 35-mm dish (MS-10350; Sumitomo Bakelite Co., Ltd.) in all cases. The cells on the filter were cultured at 37˚C in a humidified atmosphere containing 5% CO_2 in Iscove's Modified Dulbecco's Media (IMDM; cat. no. 098-06465; Fujifilm) 1:1 with Nutrient Mixture F-12 Ham (cat. no. N6658; Sigma-Aldrich; Merck KGaA), 20% FBS (cat. no. 35-079-CV; Corning, Inc.), Matrigel® (cat. no. 354234; Corning, Inc.), recombinant mouse EGF (cat. no. 053-07751) and recombinant human basic fibroblast growth factor (FGF; cat. no. 064-04541; both from Fujifilm) and B27 (cat. no. 17504-044; Gibco; Thermo Fisher Scientific, Inc.). The culture medium was made to flow between the inside and outside of the filter device. The culture medium was exchanged by aspirating from the outside of the filter device and then adding the medium to the inside of the filter device (Fig. 1). After being cultured, the cells were washed 3 times with 200 μl PBS and 200 μl staining solution (PE-conjugated mouse anti-human EpCAM and Hoechst 33342, as specified above) was added and the mixture was incubated at room temperature for 30 min. For cases 14 and 15, 200 μl of staining solution (Calcein-AM; 1:2,000 dilution; cat. no. 341-07901; Dojindo; and PE-conjugated mouse anti-human EpCAM-PE; 1:200 dilution; cat. no. 324206; clone 9C4; Biolegend; in PBS) was added and cells were incubated at room temperature for 30 min. After the above-mentioned staining, the cells were washed 3 times with 200 μl PBS.

Enumeration of CTCs, CTC clusters and CTC colonies. The collected filter was placed on a 35-mm dish. For observation, an automatic fluorescence microscope (MSZ25; Nikon Corporation) was used and an image of the entire area of the filter was acquired with a x10 objective lens (excitation wavelengths of 395, 472 and 545 nm and emission wavelengths of 460, 520 and 615 nm), before automatically generating a single image file (software: Nikon NIS-Elements BR v.5.21; Nikon Corp.).

Results

Patient and tumor characteristics. The clinicopathological characteristics of the patients are presented in Table I. The age range of the 15 cases included was 44-87 years with a median age of 74 years. The cohort comprised 6 males and 9 females. The histologic type was invasive ductal adenocarcinoma in 13 cases and intraductal papillary mucinous carcinoma in 2 cases. The blood was collected from all patients just before the surgery. Among the 15 patients, 4 patients with borderline resectable or unresectable locally advanced pancreatic cancer received pre-operative treatment, 2 patients received systemic chemotherapy and 14 underwent a course of gemcitabine/nab-paclitaxel and 2 cycles of gemcitabine (1,000 mg/m^2) with S1 oral intake prior to surgery. A total of 2 patients received chemoradiotherapy, including 2 cycles of gemcitabine infusion with concurrent proton beam radiation (67.5 Gy) (Table I).

Enumeration of single CTCs, CTC clusters and CTC colonies. CTCs isolated on the precision microfiltration membrane with 8- and 15-μm pore size were cultured, and enumeration and evaluation of the state of the cancer cells on the filter was performed by fluorescent labeling in the real-time (approximate culture time: 0, 25, 45 and 100 days). CTCs captured on the filter were cultured using IMDM-F12 medium containing 20% FBS, -2% Matrigel, EGF, bFGF and B27. A CTC was defined as a single, intact round oval cell with a visible nucleus (Hoechst 33342-positive) that stained positive with anti-EpCAM (Fig. 2A and B). Clusters were defined as aggregates of two or more CTCs and <50 μm in the long diameter (Fig. 2C-E), and colonies were defined as aggregates of >50 μm in the long diameter (Fig. 2F-H). The numbers of single CTCs, clusters and colonies for cases 1-6, which were observed at three sequential time-points, are provided in Table II. In several cases, there were no CTCs immediately after filtration, but single cells, clusters and colonies were detected after cultivation in each case. The numbers of single CTCs, clusters and colonies for cases 7-13, after culturing for different durations, at one time-point, are presented in Table III.

Activity of cultured CTCs. The number and state of cancer cells on the filter were evaluated by staining with Calcein AM to confirm cultured CTC activity. The cells were observed after exposing them to Calcein AM in cases 14 and 15. The EpCAM-positive cells cultured on the filter stained with Calcein AM were considered viable CTCs (Table IV, Fig. 3).

Table I. Patient characteristics.

Case	Age, years	Sex	Stage	Pathology	Chemotherapy	Radiotherapy	Surgical procedure
1	68	M	ypStageIIB	IPD	+	-	DP
2	78	M	pStageIIA	IPD	-	-	TP
3	71	F	pStageIA	IPD	-	-	DP
4	64	M	pStageIIA	IPMC	-	-	DP
5	75	F	ypStage0	IPD	+	+	SSpPD
6	75	F	ypStageIA	IPD	+	+	SSpPD
7	78	M	ypStageIIB	IPD	+	-	TP
8	77	F	pStageIIB	IPD	-	-	SSpPD
9	68	M	pStageIIA	IPD	-	-	SSpPD
10	71	F	pStageIA	IPD	-	-	SSpPD
11	78	F	pStageIA	IPMC	-	-	SSpPD
12	81	F	pStageIA	IPD	-	-	SSpPD
13	87	M	pStageIA	IPD	-	-	Lap-DP
14	44	F	pStageIIB	IPD	-		SSpPD
15	73	F	pStageIIB	IPD	-		SSpPD

IPD, invasive ductal carcinoma; IPMC, intraductal papillary mucinous carcinoma; DP, distal pancreatectomy; TP, total pancreatectomy; SSpPD, subtotal stomach-preserving pancreatoduodenectomy; Lap-DP, laparoscopic DP; F, female; M, male.

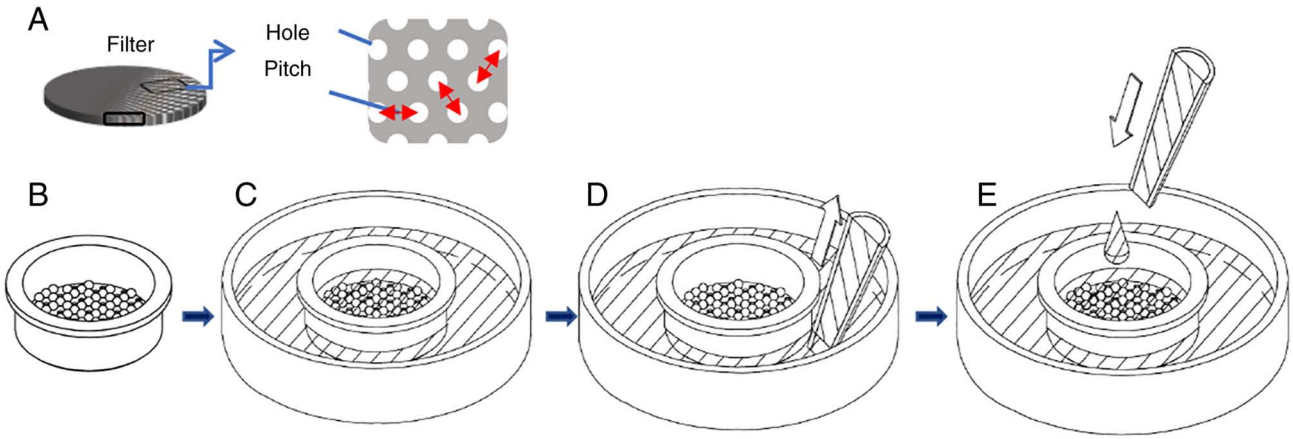

Figure 1. Schematic of the filter material and culture medium exchange method. (A) A 13 mm diameter 15-μm pore filter or 8-μm pore filter; (B) the filter device containing the filter; (C) the filter device placed on a 35-mm dish; (D) medium may be aspirated from outside of the filter device; (E) medium may be injected into the inside of the filter device.

Discussion

In the present study, a culture system was constructed using a microfilter device to isolate and culture both CTC and CTC clusters simultaneously from peripheral blood samples of patients with pancreatic cancer. In addition, staining was performed to confirm cell viability in clinical practice.

Long-term *in vitro* CTC culturing poses multiple challenges, particularly with regard to cellular viability after filtering. Yusa *et al* (18) reported that filters electroformed from pure Pd or a Pd alloy (Pd/Nickel, 4:1) had lower toxicity to cultured cells during three days of culture than a pure nickel filter. However, while an electroformed Ni-Pd alloy does not elute toxic nickel, the cells on the filter may be damaged when the medium is exchanged. Therefore, in the present study, a method for exchange, in which the CTCs would not detach from the filter, was devised. In this system, the medium was aspirated from the outside of the filter device with a pipette, followed by the addition of medium directly into the filter device for replacement of the medium. In this way, the cells remained adsorbed on the filter and were able to be viable over a long culturing period.

CTCs reduce or eliminate the expression of EpCAM through EMT to acquire invasive potential. Our group previously developed a simple and inexpensive system for accurate detection, in which CTCs are concentrated by using a microfilter and 5-aminolevulinic acid (12). Kitz *et al* (13) have additionally developed EMT-independent CTC enumeration

Table II. Enumeration of single CTCs, CTC clusters and CTC colonies for cases 1-6 at three sequential points.

Case no.	First observation				Second observation				Third observation			
	Day	Cells	Clusters	Colonies	Day	Cells	Clusters	Colonies	Day	Cells	Clusters	Colonies
1	0	0	0	0	21	5	0	0	50	0	1	0
2	0	0	0	0	23	3	1	0	44	0	1	0
3	0	0	0	0	27	2	0	3	42	0	0	2
4	0	18	2	0	43	3	1	1	103	8	0	2
5	0	0	1	0	42	1	1	0	102	1	2	2
6	0	0	0	0	38	0	1	2	98	0	1	0

CTC, circulating tumor cell.

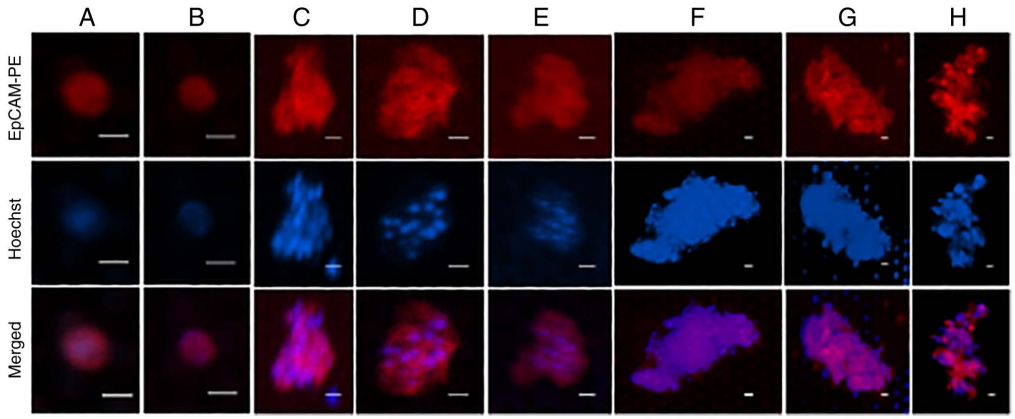

Figure 2. Definition of CTCs (single cells), CTC clusters and CTC colonies. Cells were identified by epidermal staining with anti-EpCAM antibody and nuclear staining with Hoechst. (A and B) Double-positive cells by anti-EpCAM and Hoechst staining were identified as CTCs. (C-E) CTC clusters were defined as ≥2 CTCs and an aggregate length of <50 μm. (F-H) CTC colonies were defined as aggregates of >50 μm in length (scale bars, 10 μm). EpCAM, epithelial cellular adhesion molecule; CTC, circulating tumor cell.

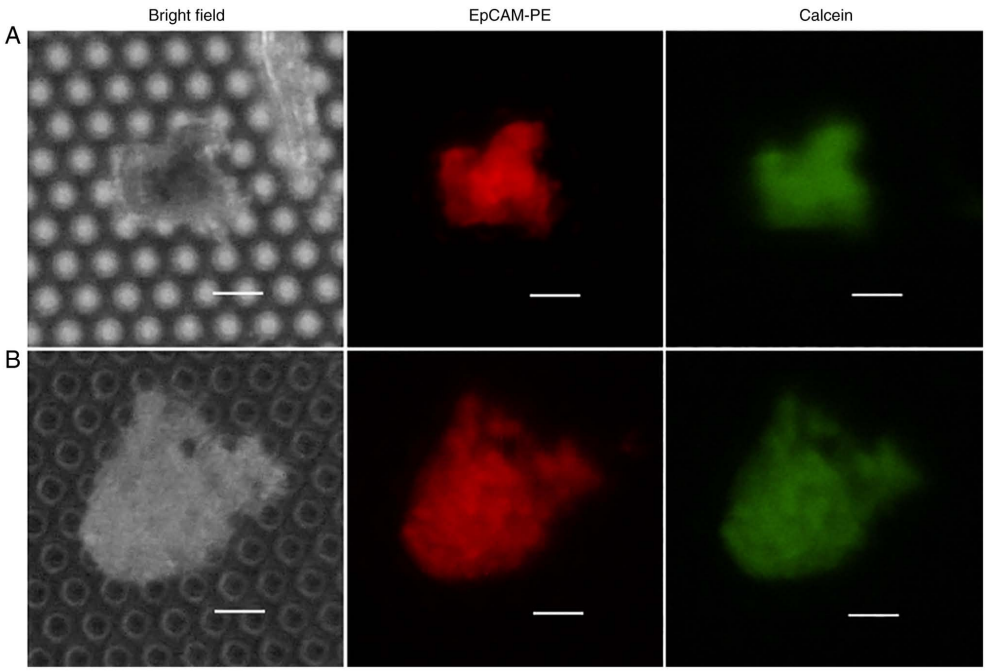

Figure 3. Detection of Calcein AM-positive cells. Cluster-forming cells (A) and colony-forming cells (B), identified by epidermal staining with anti-EpCAM antibody, were additionally stained with Calcein AM (scale bars, 20 μm). EpCAM, epithelial cellular adhesion molecule; BF, bright field; EpCAM, epithelial cellular adhesion molecule.

Table III. Enumeration of single CTCs, CTC clusters and CTC colonies for cases 7-13 at one point of measurement only.

		Observation			
Case no.	Filter	Day of culture	Cells	Clusters	Colony
7	R8	23	7	5	0
8	R8	23	7	0	0
9	R8	40	0	0	0
	R15	40	0	5	3
10	R8	41	0	0	0
	R15	41	0	2	1
11	R8	54	0	0	0
	R15	54	0	2	0
12	R8	54	0	4	44
	R15	54	0	0	0
13	R8	47	0	0	3
	R15	47	0	1	1

CTC, circulating tumor cell.

Table IV. Enumeration of single CTCs, CTC clusters and CTC colonies for cases 14 and 15.

		Observation			
Case no.	Filter	Day of culture	Cells	Clusters	Colonies
14	R8	49	0	1	1
	R15	71	2	0	5
15	R8	61	0	1	2
	R15	62	0	0	0

CTC, circulating tumor cell.

and harvesting protocols using the Parsortix®. In the present study, CTCs were not detected immediately after filtering the blood, presumably due to attenuated EpCAM expression in certain cases, but were able to be eventually detected after recovering the epithelial phenotype in the process of culturing on the filter. In CTCs undergoing EMT, the transition may be reversed by attachment on the filter. Furthermore, this result indicates that size-based CTC isolation methods may capture EMT-independent CTC.

To confirm whether cultured cancer cells on the filter retain cell viability, Calcein AM staining was used, indicating a positive result. This means that cultured cells proliferating from cancer cells may be analyzed for morphological and genetic markers of cancer. Long-term culture of filtered CTCs may result in a more specific analysis of the physiological characteristics of these cells, which, in turn, will translate into individualized pancreatic cancer treatments.

The present data are limited by the small sample size and focus on only pancreatic cancer. It has been reported that the size of CTCs varies depending on the cancer site (19); therefore, it is necessary to examine the filter pore size for each cancer type. Future studies require to be prospectively considered in larger populations.

In conclusion, a non-invasive, size-based filtering modality was developed in the present study, which is able to effectively isolate and culture CTCs and CTC clusters from clinical patients with cancer to characterize these peripheral cells for therapy response predictions. Future validation studies will serve to facilitate functional and genomic analyses, such as in patient-specific anticancer drug susceptibility testing.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AF: Methodology (equal); formal analysis (equal); investigation (lead); data curation (lead); writing-original draft (lead); visualization (equal). TM: Methodology (equal); investigation (equal); resources (equal); writing-original draft (equal). OS: Investigation (equal); writing-review & editing (equal). KA: Investigation (equal); writing-review & editing (equal). TO: Investigation (equal); writing-review & editing (equal). SM: Conceptualization (equal); methodology (equal); resources (equal); writing-review & editing (equal); supervision (lead); project administration (equal); funding acquisition (equal).

Ethics approval and consent to participate

The study protocols were approved by the Institutional Review Board of the University of Tsukuba Hospital (Tsukuba, Japan; approval no. H30-150) and all patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

SM received a research grant and free filters and filter devices provided for from Optnics Precision Co., Ltd. The other authors have no competing interests to disclose.

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