Prostate International 7 (2019) 131–138

Contents lists available at ScienceDirect

Prostate International

journal homepage: https://www.journals.elsevier.com/prostate-international

Original Article

Initial detection of circulating tumor cells from metastatic prostate cancer patients with a novel small device

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ARTICLE INFO

Article history:
Received 1 November 2018
Received in revised form
21 January 2019
Accepted 22 January 2019
Available online 8 February 2019

Keywords:
Circulating tumor cells
CTC-chip
Epithelial cell adhesion molecule
Prostate cancer

ABSTRACT

Background: Various devices for isolating and detecting circulating tumor cells (CTCs) have been developed, whereas the CellSearch® system has been clinically used in numerous prostate CTC studies. CTCs might become more useful surrogate markers of prostate cancer, and they should be measured in all settings, but a smaller, low-cost CTC capture system is required.
Methods: An inexpensive and highly sensitive microfluidic CTC-capture polymeric chip, developed by the Toyama Industrial Technology Center, as described in the following text, was used to assess the number of CTCs from patients with metastatic prostate cancer. After verifying that cultured human prostate cancer cells (PC3 and LNCaP) could be captured with the chip coated with anti–epithelial cell adhesion molecule (CD326) antibody, whole blood samples of 14 patients with prostate cancer were screened.
Results: The average capture efficacy of PC3 cells was 94.60% in phosphate-buffered saline (PBS) and 83.82% in whole blood. The average capture efficacy of LNCaP cells was 82.73% in PBS and 75.78% in whole blood. CTCs were detected by the chip device in all 14 patients with metastatic prostate cancer using 2-mL blood samples. Although fewer CTCs were detected in patients with oligometastases, all patients with multiple distant metastases had CTCs. The average CTC count was 48 cells/mL (range 1–81 cells/mL).
Conclusion: This CTC-chip will be able to capture CTCs and be useful to check CTCs as a surrogate marker in prostate cancer with smaller samples and lower cost in any small institution.

1. Introduction

Malignant cells that detach from primary tumors and stream in blood vessels are called circulating tumor cells (CTCs). CTCs are clinically isolated and detected in various solid cancers. In prostate cancer, CTCs are generally accepted as prognostic biomarkers. Numerous studies have evaluated the baseline CTC level in patients with prostate cancer treated with androgen deprivation therapy and chemotherapy as a prognostic factor.[1–6] A phase III, double-blind, randomized, placebo-controlled trial (COU-AA-301) evaluated whether CTC enumeration using CellSearch® (Veridex, Raritan, NJ, USA) could be used as an efficient biomarker of overall survival (OS) in metastatic castration-resistant prostate cancer (CRPC).[5]

Among a variety of CTC-capture systems using the epithelial cell adhesion molecule (EpCAM) antibody, including magnetic bead–based ones such as CellSearch®, size-based, or density-based separation systems[7], a microfluidic system called a CTC-chip has an advantage due to its capability to capture specific cells with an antibody attached to microposts. Nagrath et al[8] and Maheswaran et al[9] first reported higher sensitivity in the detection of CTCs with a CTC-chip coated with anti-EpCAM antibodies.

A novel polymeric CTC-chip comprising light-curable resins has been designed, and its advantages in comparison with other systems are lower cost and higher sensitivity.[10] The usefulness of this CTC-chip for capturing human prostate cancer cells was confirmed by using cell lines and clinical samples from patients with prostate cancer.

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https://doi.org/10.1016/j.prnil.2019.01.003

2. Materials and methods

2.1. Cell line and cell culture

The human prostate carcinoma cell lines, PC3 (ATCC CRL-1435 Lot No. 61777391) and LNCaP (ATCC CRL-1740 Lot No. 61777383), were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were plated on a 24-well dish and cultured in a Dulbecco's modified eagle medium (DMEM) with 10% deactivated fetal bovine serum and 1% streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO2. The media were changed every 48 hours, and the cells were subcultivated every four days. Subconfluent cell layers were dissociated by 0.25% trypsin solution.

2.2. Immunocytochemistry

The immunocytochemical method was designed based on the existing literature.[11] PC3 and LNCaP cells were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100. The cells were incubated with a primary antibody, a mouse anti-human EpCAM monoclonal antibody (clone HEA125), for 60 min at room temperature. They were then incubated with a secondary antibody, a goat anti-mouse IgG conjugated with Alexa Fluor 594 (Life Technologies, Carlsbad, CA, USA), and 4′,6-diamidino-2-phenylindole (DAPI) for 30 min at room temperature and. Images were acquired with a CKX41 fluorescence microscope equipped with a DP73 digital camera (Olympus, Tokyo, Japan). Cells captured from patients with prostate cancer were additionally stained with anti-human cytokeratin 18 conjugated with Alexa Fluor 488, anti-human EpCAM conjugated with Cy3, and CD45 conjugated with allophycocyanin.

2.3. Principle and method of collection and concentration in the polymer CTC-chip

The microstructure of the chip consisted chiefly of an array of two different types of microposts, modified from the previous design to prevent clogging by whole blood. The gap between microposts was enlarged to 200 μm in the area around the chip inlet (Fig. 1A). Goat anti-mouse IgG antibody (cat. no. 1032-01; Southern Biotech, Birmingham, AL, USA) and mouse anti-human EpCAM antibody were used for the chip coating (Fig. 1B). The polymer CTC-chip immobilized with surface antibody was set in a holder, which enabled a liquid sample to flow through a channel, and then the two ports of the holder were connected to a syringe pump and a sample tube with tubing and fittings (Fig. 1C). Capture efficiency was evaluated using the method described previously with cancer cell suspensions.[10] The capture efficiency was calculated by counting the number of cells remaining on the chip after sample passage compared with the number of cells that passed through the chip inlet.

2.4. Sample preparation and flow test

Cells were labeled using the CellTrace™ CFSE Cell Proliferation kit (Life Technologies) as per the manufacturer's protocol, and then about 1,500 PC3 and LNCaP cells were suspended in 2 mL of PBS containing 5% BSA or in 2 mL of blood sampled from a healthy volunteer. A cell suspension sample of 1 mL (500 cells/mL) was applied to the CTC-chip system.

Each sample was sent into the chip using a syringe pump at a constant flow rate (1.5 mL/h when suspended in PBS or 1.0 mL/h when suspended in blood). Each sample tube was shaken to ensure that the cell suspension was homogeneous. Images and videos of the cells in the chip were monitored and recorded with a fluorescence microscope (CkX41; Olympus) and a digital video camera (Sony Biotechnology, Inc., Tokyo, Japan). Each experiment, sample preparation, and flow test were performed five times.

2.5. Evaluation of cell capture efficiency

The actual number of cells that were sent into the chip (N-total) was determined by counting the number of cells that passed through the inlet of the chip. The number of captured cells (N-captured) was also determined by counting carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled cells remaining on the chip after completion of the flow test. The cell capture efficiency was evaluated as N-captured/N-total. The average capture efficiency was calculated from the results obtained in five experiments.

2.6. Patient characteristics

A total of 14 patients not initially treated in our hospital were reviewed, as shown in Table 1. The average age of the patients was 72 (range 60–86) years, and the median prostate-specific antigen (PSA) level was 619.7 (range 9.85–6822.8) ng/mL. Thirteen patients had multiple bone metastases, and one patient had only paraaortic lymph node metastasis (Table 1). All study participants provided both verbal and written informed consent, and the study design was approved by an ethics review board at our institution.

3. Results

A captured PC-3 cell within human whole blood on the chip in a bright field and immunocytochemical staining fields of DAPI, CK18, EpCAM, and CD45 are shown in Fig. 2. A stained cell was classified as an epithelial cell because its morphologic features were nearly round or oval, with a visible nucleus within the cytoplasm (A, B) and staining patterns consistent with DAPI positive/CK18-Alexa Fluor 488®-positive/EpCAM-PE-positive/CD45-allophycocyanin-negative (B–F). The size of the captured cell was about 20 μm, with large heterogeneity.

From five flow counting tests, CSFE-labeled PC3 and LNCaP cells could be captured in the system, and average cell capture efficiencies are shown in Table 2. When PC3 and LNCaP cells were suspended in PBS, the average cell capture efficiency of the EpCAM-chip was 94.6% ± 2.01% (mean ± standard deviation [SD]) in PC3 and 82.73% ± 2.06% in LNCaP. Using the same method for PC3 and LNCaP cells in whole blood, the average capture efficiency of the EpCAM-chip in PC3 cells was 83.82% ± 3.11% and 75.78% ± 3.10% in LNCaP. Statistical analyses were performed, including the Kolmogorov–Smirnov test (PC3 cells p-value = 0.6799 in PBS and 0.422 in whole blood, LNCaP cells p-value = 0.4544 in PBS and 0.1538 in whole blood), F test (PC3 cells p-value = 0.421, LNCaP cells p-value = 0.449), and t-test (PC3 cells p-value = 0.000188, LNCaP cells p-value = 0.00311). Fig. 3 shows the significant differences in cell capture efficiencies between PBS and whole blood samples. Because the capture efficiency *in vivo* was not low, the same study was performed in clinical patients.

Following the PC3 and LNCaP study, 2-mL blood samples were taken from 14 patients with metastatic prostate cancer and the samples were sent into the chip device. The characteristics of the 14 patients with metastatic prostate cancer are shown in Table 1. All patients were pathologically diagnosed with high-grade (Gleason score 8–10) prostate cancer by transrectal ultrasound-guided prostate biopsy. Captured CTCs from clinical samples are shown in Fig. 4. Several captured CTCs in clinical samples were positive for DAPI (A), CK18 (B), and EpCAM (C). CTCs were detected in all

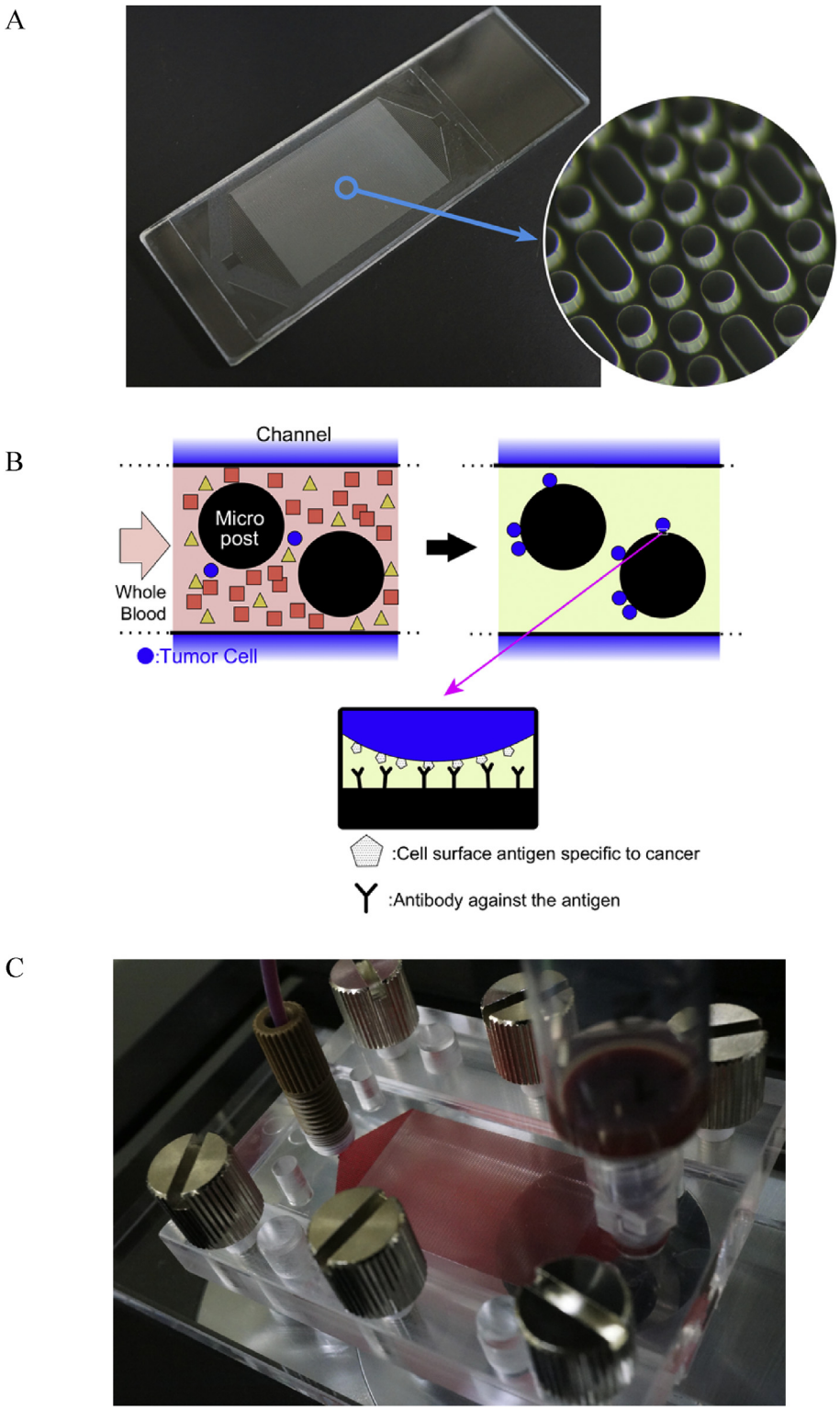

Fig. 1. Gross pictures and illustrations of the CTC-chip device. (A) The size of the polymer CTC-chip is 25 mm × 75 mm. The diameter and height of the microposts in the magnified image are 100 μm. (B) Illustrations of the microposts and captured CTCs with the polymer CTC-chip. The antibody for capture is selectable. (C) The chip set in a holder enables a liquid sample to flow through the channel. The two ports of the holder were connected to a syringe pump and a sample tube with tubing and fittings. CTC, circulating tumor cell.

patients with distant metastases, but fewer CTCs were detected in patients with oligometastases (Table 1).

When 2-mL clinical samples were streamed into the chip, a total of 2 to 168 CTCs were captured from every patient. The maximum number of CTCs was 162 cells/2 mL in high-volume prostate cancer. Although the count of captured CTCs differed greatly among patients, the average count was 48 cells/mL (range 1–81 cells/mL).

Table 1
Patients' characteristics and the number of captured CTCs

Patient no.	Age (y)	T factor	N factor	M factor	GS	PSA (ng/mL)	CTCs (/2 mL)
1	80	T4 (bladder invasion)	N0	M1b (whole body bone)	9	6822.8	121
2	65	T4 (bladder invasion)	N0	M1b (whole body bone)	8	6432.8	162
3	86	T4 (bladder invasion)	N1	M1b+c (lung and whole body bone)	9	2648.8	94
4	67	T4 (bladder invasion)	N1	M1a+b (mediastinal lymph node and whole body bone)	9	2487.1	84
5	68	T4 (bladder invasion)	N1	M1b+c (pleural and whole body bone)	9	1130	57
6	64	T3b (seminal vesicle invasion)	N1	M1a+b (paraaortic lymph node and rib bone)	9	765.8	3
7	72	T4 (bladder invasion)	N1	M1a+b (paraaortic lymph node and whole body bone)	9	680	45
8	64	T4 (bladder invasion)	N1	M1a+b (mediastinal and paraaortic lymph node, and 3rd lumbar bone)	9	559.4	5
9	78	T2c (both lobes of prostate)	N0	M1a (paraaortic lymph node)	9	540.9	2
10	77	T4 (bladder invasion)	N0	M1b+C (lung and whole body bone)	9	305.8	35
11	60	T3b (seminal vesicle invasion)	N0	M1b (whole body bone)	9	187.4	10
12	78	T4 (bladder invasion)	N1	M1a+b (multiple lymph nodes and sacral bone)	9	182.9	14
13	82	T3b (seminal vesicle invasion)	N1	M1b+c (lung and scapula)	8	162.3	17
14	69	T4 (bladder invasion)	N1	M1b (whole bods bone)	9	9.85	23

GS, Gleason score; PSA, prostate-specific antigen; CTCs, circulating tumor cells.

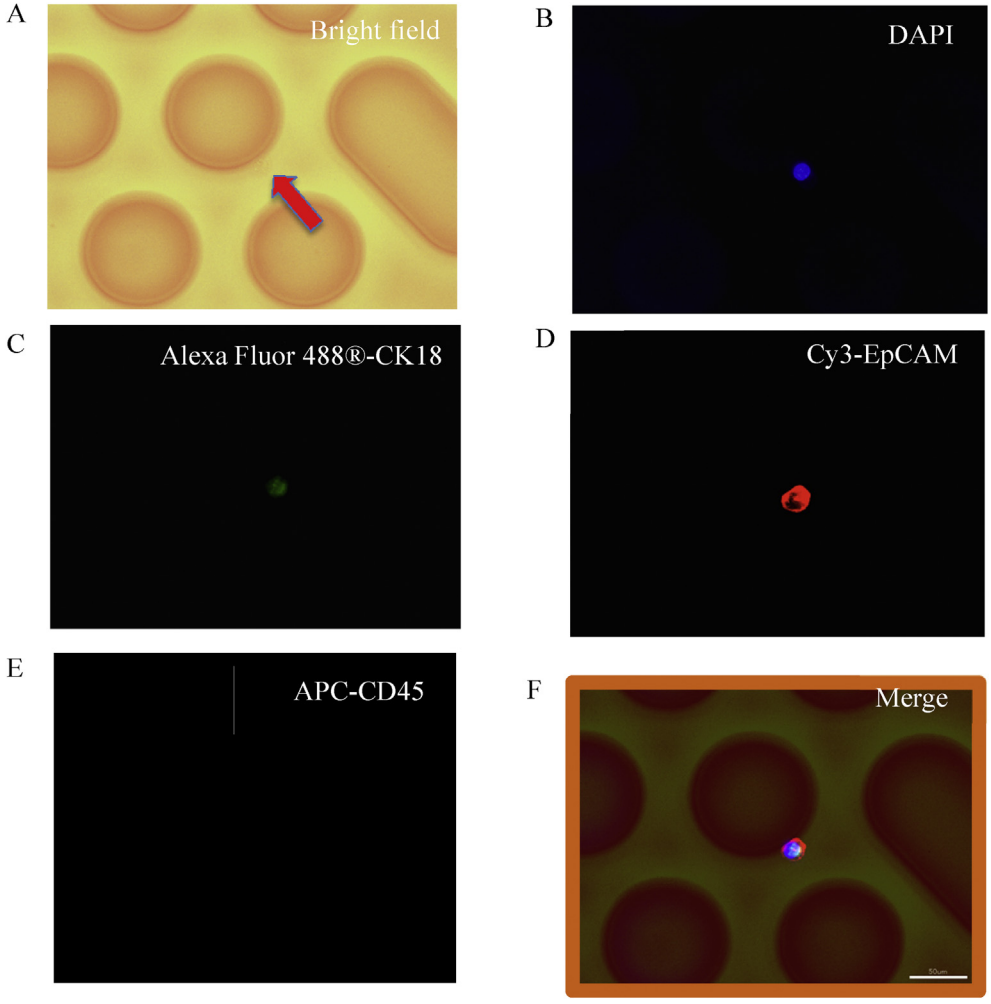

Fig. 2. A single PC3 cell captured on the chip. (A) A red arrow shows a single PC3 cell captured in a bright field. (B)–(E) A captured cell with immunofluorescence staining by DAPI, CK18 with Alexa Fluor 488, EpCAM with Cy3, and CD45 with APC. (F) A merged image of all stained images. EpCAM, epithelial cell adhesion molecule; APC, allophycocyanin; DAPI, 4′,6-diamidino-2-phenylindole.

4. Discussion

The number of CTCs has been assessed for use as a prognostic marker in several reports. It has been reported that when there were 5 or more CTCs in 7.5 mL of blood in 28 patients with CRPC, they had poor OS rates.[13] According to another report, the number of CTCs was strongly related to cancer survival in 67 (57%) of 120 patients with progressive CRPC with more than 5 CTCs.[14] From a pilot study in Japan, the CTC count provides useful information about patients with metastatic CRPC undergoing zoledronic acid treatment.[15]

The number of CTCs has also been used as a marker of effects of treatments in clinical trials. For example, an additional *post hoc*

Table 2
Average cell capture efficiencies from five flow tests counting CSFE-labeled PC3 and LNCaP cells in phosphate-buffered saline and in whole blood

PC3 (PBS)	No. of cells captured	No. of cells streamed	Capture efficiency (%)
1	635	687	92.43
2	518	558	92.83
3	527	550	95.81
4	597	614	97.23
5	517	546	94.69
Total			94.60 ± 2.01
PC3 (whole blood)	No. of cells captured	No. of cells streamed	Capture efficiency (%)
1	427	523	81.64
2	450	545	82.57
3	473	549	86.16
4	558	634	88.01
5	424	525	80.76
Total			83.82 ± 3.11
LNCaP (PBS)	No. of cells captured	No. of cells streamed	Capture efficiency (%)
1	478	590	81.02
2	529	636	83.18
3	463	539	85.9
4	568	686	82.8
5	504	624	80.77
Total			82.73 ± 2.06
LNCaP (whole blood)	No. of cells captured	No. of cells streamed	Capture efficiency (%)
1	472	582	81.1
2	457	622	73.47
3	414	562	73.67
4	489	650	75.23
5	387	513	75.44
Total			75.78 ± 3.10

PBS, phosphate-buffered saline.

analysis of data from patients in the prospective IMMC-38 (chemotherapy) and COU-AA-301 (abiraterone) trials with baseline CTCs $\geq$ 5 cells/7.5 mL was performed in 2016, and the value of a level of 30% of baseline at 4, 8, and 12 weeks of treatment was evaluated. The OS in patients with metastatic CRPC after abiraterone and chemotherapy was associated with a 30% decrease in the CTC level from the original number of $\geq$5 cells/7.5 mL.[6] A comparison between the reduction in CTCs and the reduction in PSA at earlier time points showed the limitation of PSA as a biomarker for survival and response rates to chemotherapy.[16]

PSA is not a useful marker of a change toward a neuroendocrine phenotype (NEPC) in CRPC, although it is useful in early stage adenocarcinoma. According to a report by Himisha et al, in a 27-patient cohort, patients with CRPC, including 12 with NEPC and 5 with atypical clinical features suggestive of NEPC transition, demonstrated a higher frequency of liver metastases and lower PSA than typical patients with CRPC. It was recognized that CTCs from patients with NEPC have unique morphologic characteristics, which were also identified in a subset with aggressive clinical features potentially undergoing NEPC transition.[17]

The CellSearch® system represents the first automated, standardized, and regulatory agency—approved system for detecting and quantifying CTCs in peripheral blood.[12] A system was developed that processes and analyzes 7.5 mL of blood for the presence of epithelial-derived tumor cells.[18] It is widely used and has become a major device in the field of research dealing with detecting and counting CTCs in prostate cancer, but there are few reports in other urological cancers because of its reliance on EpCAM-positive selection for the surface of CTCs.[19–21] The present study is the first report of the capture and identification of CTCs from clinical prostate cancer using a novel EpCAM-coated microchip device with advantages such as high capture rate sensitivity and universality of coating any antibody. Overall, 94.6% ± 2.01% (mean ± SD) of PC3 cells were detected in PBS, a higher capture rate than the 69% ± 3% in the same materials using the CellSearch® system.[22] Some reports showed EpCAM-positive CTCs from prostate cancer are consistently smaller than cultured cancer cells.[23] Morphological differences may affect CTCs enrichment efficiency because this device uses microfluidic technology. The reason why capture efficacy of PC3 is higher than that of LNCaP in this study may be that PC3 is larger than LNCaP. It will be required to evaluate the difference between CTCs from patients with prostate cancer and cultured prostate cancer cells in the next step.

Although conventional immune-based capture of CTCs relies on immunomagnetic enrichment, recent advances in microfluidic technologies have allowed improving CTC isolation methods. Because immune-based capture depends on the molecular interaction between cell surface antigens and antibodies, frequent contact between the target cell and antibody-immobilized surface is needed for highly efficient capture. Many devices could not always detect CTCs, partly because they mostly used antibodies only against EpCAM. EpCAM appears exclusively in epithelia and epithelial-derived cancers; anti-EpCAM antibody is broadly applied to immune-based capture of CTCs. However, EpCAM expression varies among cancer cells and is upregulated or downregulated in response to an external stimulus and environment.[24] Downregulation of EpCAM by epithelial mesenchymal transition leads to the failure in CTCs detection by EpCAM-based techniques.[25,26] Therefore, it is important that CTCs could be detected using EpCAM antibody with not only the CellSearch® system but also our device. The developed type of CTC-chip device, called 'polymer CTC-chip' produced with UV light—curing resins, is transparent to visible and UV light and mechanically tough compared with conventional silicon chips and can be commercially provided at low cost. Moreover, because the resin contains functional groups which react with proteins just by contacting them and has lasting surface

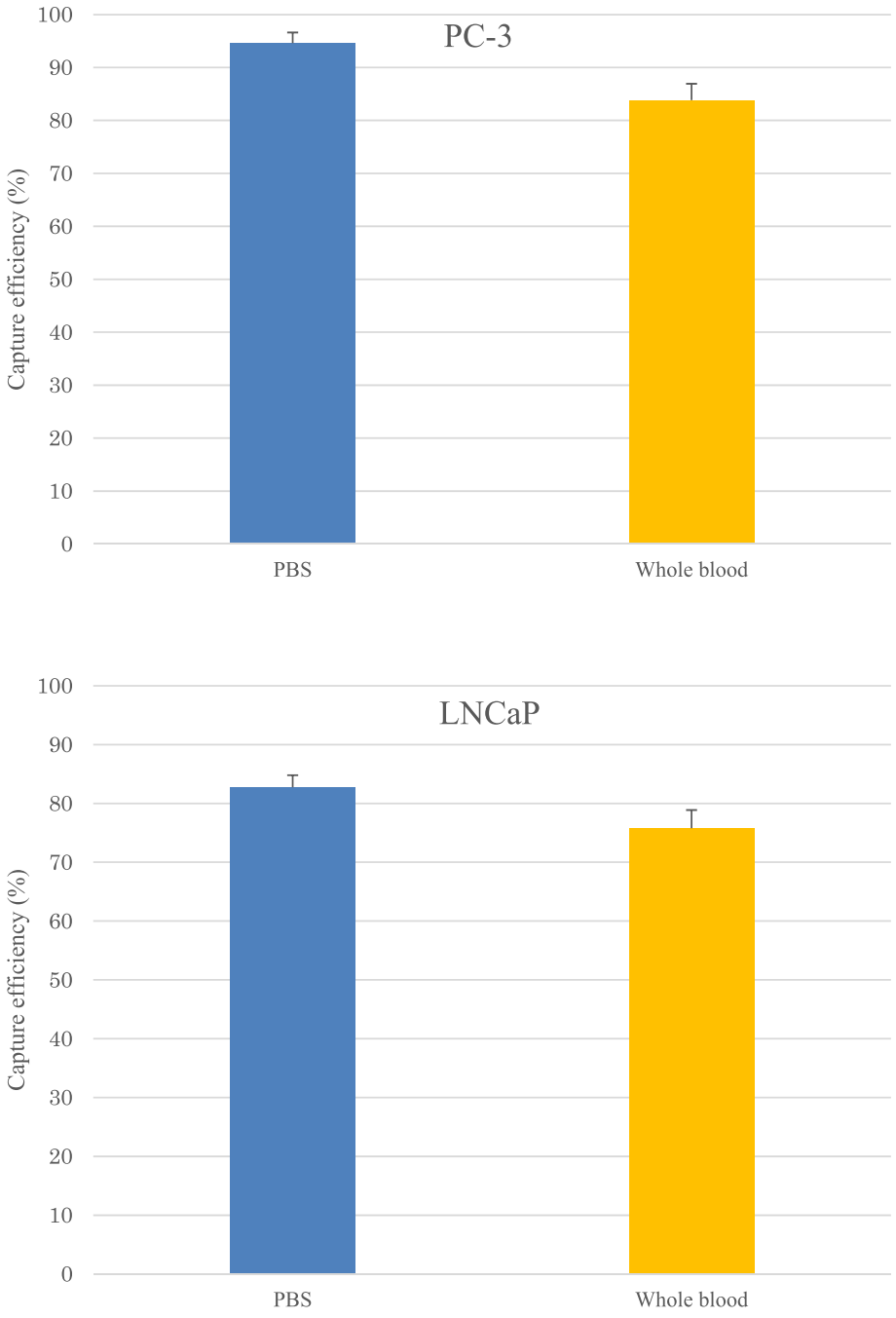

Fig. 3. There is a significant difference in cell capture efficiencies between in phosphate-buffered saline and whole blood (t-test p-value = 0.000188 in PC3 cells and 0.00311 in LNCaP cells). PBS, phosphate-buffered saline.

reactivity, antibodies can be selected by chip-users arbitrarily at any time and immobilized onto chip easily. Therefore, we recognize this device as a modified CTC-chip.[27]

Chikashi et al[11] reported the usefulness of the same CTC-chip using an EpCAM-independent coating against podoplanin for human mesothelioma cells. It was confirmed that human prostate cancer cells (LNCaP) appeared to be stained by anti-CD47 antibody–conjugated Cy3 (data not shown), so the sensitivity of capturing CTCs should be higher and less blood volume should be required when the antibody is added on the surface of the novel chip device in prostate cancer. Moreover, it will be able to capture more CTCs in other urological cancers by coating multiple antibodies on the chip.

The size of this microfluidic chip and the space of the present system are about 25 × 75 × 12 mm^3 and 30 × 40 × 50 cm^3, respectively; therefore, it is only a half-meter square without the computer, which is enough to operate the novel chip device, smaller than the CellSearch® system. The initial cost of the novel device is about 3,000 US dollars, and the running cost is about 100 US dollars/sample, lower than for CellSearch®, at 220,000 US dollars and 1,000 US dollars/run. Immediate decisions on treatment for patients with prostate cancer will reduce wasteful treatments, because it takes about half a day, shorter than CellSearch® (a few weeks), to obtain the results of one test.

It is obvious that analyzing exosome, DNA, RNA, and other molecular materials in CTCs will be more and more important. In

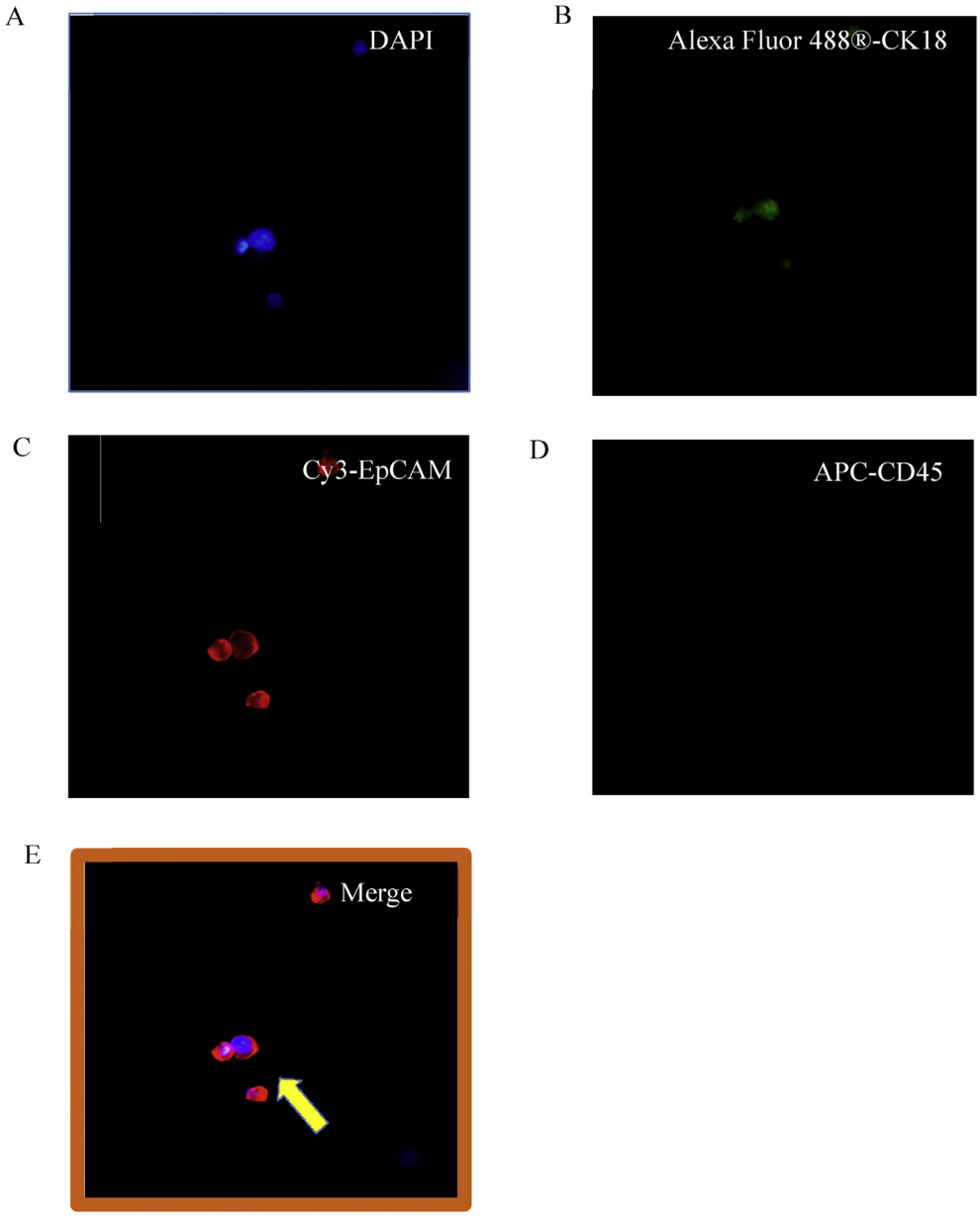

Fig. 4. Several CTCs captured on the chip from clinical samples. (A)–(D) Several CTCs and merged images of cells with immunofluorescence staining by DAPI, CK18 with Alexa Fluor, EpCAM with Cy3, and CD45 with APC. (E) A yellow arrow shows several CTCs in a merged image of all images. CTC, circulating tumor cell; EpCAM, epithelial cell adhesion molecule; APC, allophycocyanin; DAPI, 4′,6-diamidino-2-phenylindole.

the near future, it will be possible to collect, culture, and analyze the CTCs captured by the chip. Additional complementary experiments including analyses of molecular expression and genetic abnormality, such as AR-V7 and AR mutation, in captured CTCs would reinforce the characterization of our device function. We could find the usefulness of the device in this study and consequently should prepare to detect CTCs from patients with CRPC after treatment when the improved device is obtainable.

There is growing interest in the field of research examining technologies to detect, isolate, and characterize CTCs. A device that is faster, more efficient, and easier to capture and detect CTCs is needed. It seems that the present CTC device can be introduced in all settings at every institution because of its small size, low cost, and ease of use.

5. Conclusion

The advantages of this modified CTC-chip are likely to have rapid important implications for our treatment of patients with prostate with a smaller sample and lower cost in any small institution.

Conflict of interest

All authors have no conflict of interest to declare.

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