

RESEARCH PAPER

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Identifying cancer origin using circulating tumor cells

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ABSTRACT

Circulating tumor cells (CTCs) have become an established clinical evaluation biomarker. CTC count provides a good correlation with the prognosis of cancer patients, but has only been used with known cancer patients, and has been unable to predict the origin of the CTCs. This study demonstrates the analysis of CTCs for the identification of their primary cancer source. Twelve mL blood samples were equally dispensed on 6 CMx chips, microfluidic chips coated with an anti-EpCAM-conjugated supported lipid bilayer, for CTC capture and isolation. Captured CTCs were eluted to an immunofluorescence (IF) staining panel consisting of 6 groups of antibodies: anti-panCK, anti-CK18, anti-CK7, anti-TTF-1, anti-CK20/anti-CDX2, and anti-PSA/anti-PSMA. Cancer cell lines of lung (H1975), colorectal (DLD-1, HCT-116), and prostate (PC3, DU145, LNCaP) were selected to establish the sensitivity and specificity for distinguishing CTCs from lung, colorectal, and prostate cancer. Spiking experiments performed in 2 mL of culture medium or whole blood proved the CMx platform can enumerate cancer cells of lung, colorectal, and prostate. The IF panel was tested on blood samples from lung cancer patients ($n = 3$), colorectal cancer patients ($n = 5$), prostate cancer patients ($n = 5$), and healthy individuals ($n = 12$). Peripheral blood samples found panCK⁺ and CK18⁺ CTCs in lung, colorectal, and prostate cancers. CTCs expressing CK7⁺ or TTF-1⁺, (CK20/CDX2)⁺, or (PSA/PSMA)⁺ corresponded to lung, colorectal, or prostate cancer, respectively. In conclusion, we have designed an immunofluorescence staining panel to identify CTCs in peripheral blood to correctly identify cancer cell origin.

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Introduction


Cancer constitutes an enormous burden and is a leading cause of death worldwide due to the growth and aging of the population.¹ The majority of cancer deaths are caused by metastasis, when circulating tumor cells (CTCs) leave the primary tumor site, travel in blood through the circulatory system, and lead to the formation of distant, secondary tumors.^{2,3} Quantity of CTCs has shown to correlate with the severity of the cancer disease, and the emerging evidence showed that the characteristic of CTCs may provide the source of primary tumors as a simple, fast alternative to tumor biopsy.⁴ Thus, the combination of both could be a powerful tool in the ongoing battle against cancer.

The most common method of CTC enumeration is the CellSearch→ system. In the CellSearch→ system, CTCs are isolated by epithelial cell marker, epithelial cell adhesive molecule (EpCAM) and identified as cell with pan-cytokeratin (pan-CK)⁺/CD45⁻/DAPI⁺. CD45⁻ represents the cells that are not blood cells, while panCK⁺ (CK8/CK18/CK19) representing epithelial cells.⁵ CK18 and CK19 are useful markers for carcinomas,⁶ and are also positively expressed by lung adenocarcinoma,⁷ colorectal cancer (CRC),⁸ and prostate cancer, among many others.^{9,10} Because of this, the markers used in the CellSearch→ system can enumerate CTCs but cannot discern the type of cancer that generated the CTCs.

Different kinds of markers have been routinely used to identify specific tumor origins and were applied for CTC characterization: For example, CK7 is a potential marker for CTCs of lung cancer.¹¹ Prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) have been used to identify CTCs for prostate cancer patients.^{12–14} CK20, and gastrointestinal tract specific marker- caudal type homeobox 2 (CDX2), were used to enumerate CTCs of CRC,^{6,15} was reported to detect CTCs in 81% of CRC patients (73 of 90).¹⁶ Another study suggested that thyroid transcription factor 1 (TTF-1), which is selectively expressed in the lung, thyroid and diencephalons.¹⁷ could be a diagnostic marker to confirm non-small-cell lung cancer (NSCLC) CTCs.¹⁸

Current CTC detection methods provide good correlations for prognosis prediction and therapy monitoring on known cancer patients. However, a more advanced CTC platform with high sensitivity has shown that even early-cancer or pre-cancer patients may already have “CTCs.” For example, in a previous study, we reported that by analyzing the blood samples of 158 people, whose health ranged from cancer-free to patients with polyps or CRC patients, with a microfluidic chip coated with a supported lipid bilayer (SLB) that was conjugated with anti-EpCAM (the “CMx platform,” Figs. 1A, 1B),^{19,20} we could detect CTCs in CRC patients in all stages of cancer and in 30%

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of the patients who only had polyps based on colonoscopic finding. The CTCs found in the patients with polyps show that CTCs can be detected before currently available imaging technology or markers can detect a tumor. Therefore, CTC may be useful as a screening tool for the general population. In addition to detecting CTCs, it would be helpful to construct a system to identify the most likely origin of any CTCs that are detected. Such a system would be beneficial for the screening on the patients without known cancer and symptoms-free individuals.

In this work, we present a process for using a sequence of CMx chips to identify the cancer origin of CTCs. To demonstrate the concept, we focused on CTCs of NSCLC, CRC, and prostate cancer, the 3 most common male cancers in the 2012 GLOBOCAN survey (Table 1). We constructed an immunofluorescence (IF) staining panel that consisted of 6 individual CMx chips that captured EpCAM-positive CTCs and stained them with different markers: chip1-panCK, chip2-CK18, chip3-CK7, chip4-TTF-1, chip5-CK20/CDX2, and chip6-PSA/PSMA (Fig. 1C). The CMx chip overall efficiency was tested by standard cancer cell lines, and the IF staining efficiency and the potential cross reactivity were evaluated to verify the stability of

staining panel and the blood processing. We collected cancer patient blood samples for validating the applicability. In this study, we hypothesized that we could use the IF staining panel results to predict the tissue origin of CTCs, with NSCLC CTCs detected in chips1, 2, 3, and 4, CRC CTCs detected in chips1, 2, and 5, and prostate cancer CTCs detected in chips1, 2, and 6. CTCs that were detected only in chip1 and chip2 most likely would be from a cancer other than NSCLC, CRC, or prostate cancer. If no CTCs were present in a patient (e.g., patients with no known cancer), all chips would be CTC negative. Our clinical sample tests verified our hypothesis in that the positive and negative expression of CTCs matched with our staining panel design.

Results

Establishing the immunofluorescence staining panels for CTC origins from NSCLC, CRC, and prostate Cancer

The representative IF staining images of NSCLC, CRC, prostate cancer, and lymphoma cell lines for the selected markers are shown in Fig. 2, and are summarized in Table S1. PanCK and

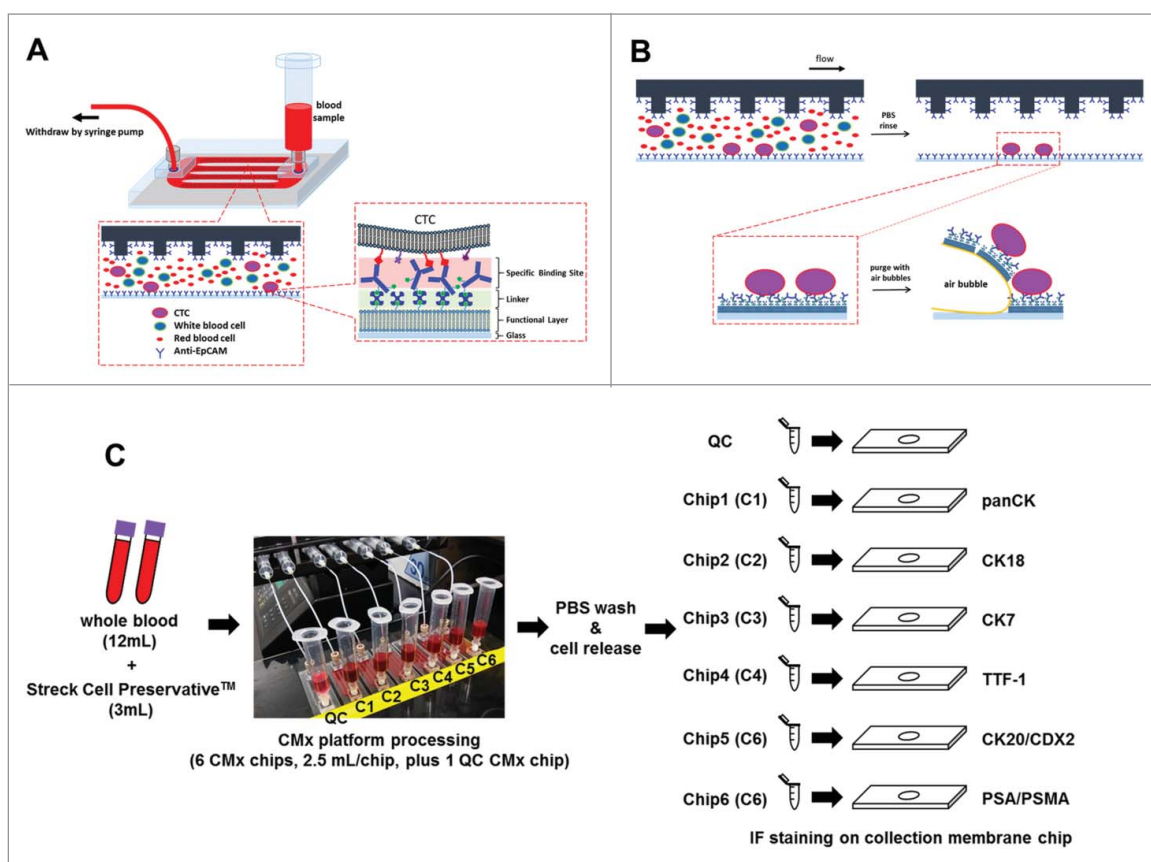


Figure 1. The CMx system for binding, releasing, and analyzing circulating tumor cells (CTCs). (A) Schematic of CTC capture on a CMx chip. A blood sample is drawn from a 5 mL volume syringe connected at the inlet of CMx chip and processed through the microfluidic channel on the CMx chip by syringe pump withdraw. CTCs can be captured by the anti-EpCAM antibody layer that is linked to the functional supported lipid bilayer (SLB) coated on the glass substrate. (B) Cell release mechanism of the CMx platform. Once the blood sample has flowed through the microfluidic channel, the channel is rinsed with PBS to remove excess blood cells. Captured CTCs are then released by injecting air foam into the channel, which disrupts the SLB coating without damaging the cells. (C) CMx platform workflow for the IF staining panel. Blood samples are drawn in EDTA tubes. 12mL of blood is mixed with 3mL of Streck Cell Preservative and processed within 24 hours. Six CMx chips are used for blood processing resulting in a sample volume of 2.5 mL/chip. An additional chip with a baseline sample is used for quality control (QC). After sample processing and PBS rinse, captured cells are released into individual Eppendorf tubes and are transferred to separate collection membrane chips for staining with immunofluorescence (IF) markers. The QC chip processed a control sample 200 cells of CMFDA-labeled cancer cells, in or culture medium. The efficiency of detection in the control sample must be >70% to ensure the CMx chip quality.

Table 1. The 3 most common male cancers worldwide, in terms of new cases, based on the 2012 GLOBOCAN report.

Cancer	New Cases			Deaths		
	Rank	Number (millions)	% of New Cases	Rank	Number (millions)	% of Deaths
Lung	1	1.2	16.7	1	1.1	23.6
Prostate	2	1.1	15	5	0.3	6.6
Colorectal	3	0.7	10	4	0.4	8

CK18 immunostaining were positive in all cell lines from all cancer cell lines except blood originated lymphoma cell line. CK7 and TTF-1 were only positive in NSCLC cell line; CK20 and CDX2 were only positive to CRC cell lines; PSA and PSMA were only positive in prostate cancer cell line LNCaP, but negative in all other cell lines. Our result suggests that this staining panel design is sensitive and can distinguish among 3 carcinoma origins, while negative to the blood cells. However, PSA and PSMA may not be sensitive to detect all prostate cancer cells, as it is negatively expressed in both prostate cancer cell lines PC3 and DU145.¹²⁻¹⁴ The sensitivity and specificity of the antibody mixtures, anti-CK20/anti-CDX2, and anti-PSA/anti-PSMA remain consistent as single antibody staining. Hence, based on these IF staining results, the possible origin(s) of CTCs from lung, colorectal, prostate, or other unknown primary site could be predicted with the decision tree shown in Fig. 3. Chip1 and chip2 can be the first line to detect epithelial cells in blood. CTCs that came from the NSCLC would be detected in chip3 and chip4. Similarly, CTCs that came from CRC or prostate cancer would be identified in chip5 or chip6, respectively.

Sufficient capture efficiency of CTCs using CMx platform processing

To verify the ability of our CMx panel to capture CTCs from different tumor origins, we tested the overall efficiency

of CMx chip with a baseline test with a known sample. The sample consisted of 200 cells that were fluorescence-labeled with CellTracker CMFDA and spiked in 2 mL of culture medium or whole blood from a healthy individual. After CMx chip processing, the captured cells were released on the collection membrane chip as previously reported.¹⁹ The overall efficiency was calculated by dividing the cell number on the staining membrane by the known number of cells in the sample (Fig. 4). The capture efficiency was better in culture medium than in whole blood, but still sufficient in whole blood for the work to continue. The staining efficiency of the cells released from CMx chip was evaluated and was very high (~90–100%, Table S2), except CK20/CDX2 on HCT116 cell line, which is known to be low in CK20/CDX2 expression level.^{21,22} In summary, the staining efficiency did not change between 2 spiking conditions (culture medium or whole blood) or after blood processing, indicating that cells remained intact and there were no interferences caused by blood.

PanCK⁺ CTC count can distinguish healthy individuals from cancer patients

Once the capture and staining efficiency of the CMx platform was analyzed, we evaluated the panel's ability to determine the origin of CTCs with 13 cancer patients (Table 2), and 12 individuals with no known cancer diseases, tested in triplicate, that served as a baseline. For the panCK and CK18 panels, as shown on Table 3, the median CTC counts were 0 for the healthy individuals, while cancer patients have apparent elevated panCK⁺ CTC counts. Using ≥ 3 per 2mL blood as a cutoff, all cancer patients (except one prostate cancer patient (P-2)) were above the cutoff value. On the contrary, in the CK18 panel, only 7 out of 13 cancer patients had CK18⁺ CTC counts ≥ 2 / 2mL, though the average or median values of CTCs of cancer patients are significantly higher than those of healthy individuals whose median value is 0 and none has CK18⁺ CTC counts ≥ 2 / 2mL ($p < 0.002$).

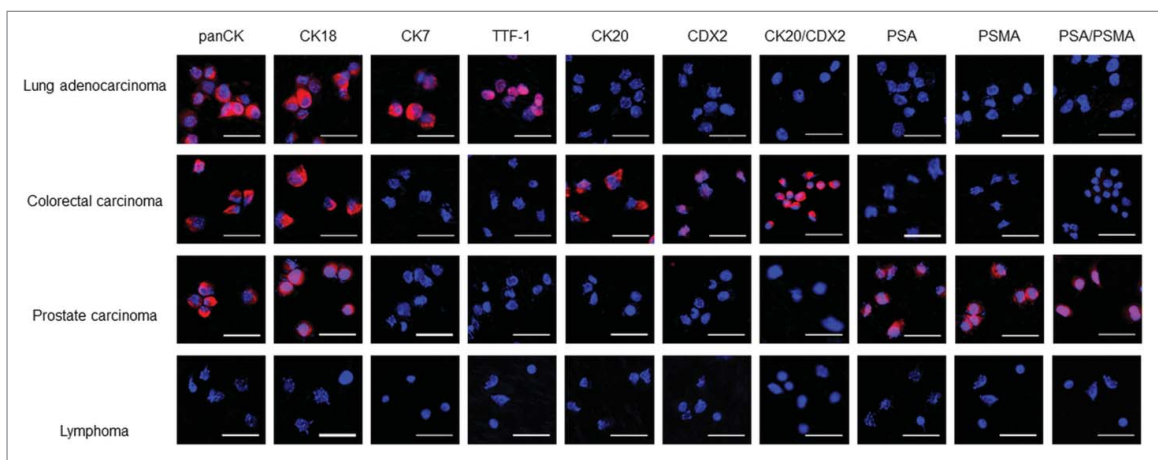


Figure 2. Immunofluorescence (IF) staining of cancer markers on cell lines from lung, colorectal, and prostate cancer origin. The array of images were representative of NSCLC (H1975), CRC (DLD-1), prostate carcinoma (LNCaP), and lymphoma (HL-60) cell lines stained with anti-panCK, anti-CK18, anti-CK7, anti-TTF-1, anti-CK20, anti-CDX2, anti-CK20/anti-CDX2 mixture, anti-PSA, anti-PSMA, and anti-PSA/anti-PSMA mixture. 200 cells were dripped and fixed on the collection membrane chip, underwent IF staining with rabbit hosted primary antibody for each marker, and labeled with Alexa Fluor 647 conjugated goat-anti-rabbit IgG secondary antibody (red). Cell nuclei were labeled with DAPI (blue). The scale bar represents 50 μ m.

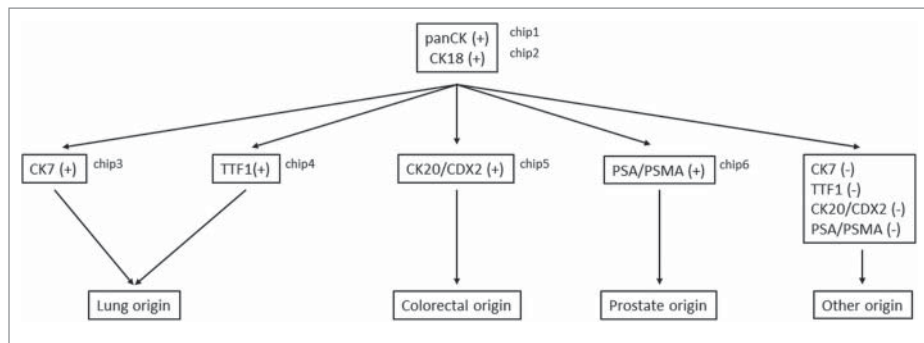


Figure 3. Decision tree for predicting CTC tissue origin. CTCs from lung cancer origin could be identified in chip1/chip2/chip3/chip4. CTCs from colorectal cancer could be identified in chip1/chip2/chip5. CTCs from prostate cancer could be identified in chip1/chip2/chip6. Additionally, CTCs of tissue origins other than lung, colorectal, and prostate could be identified in chip1/chip2.

The panel can distinguish NSCLC, CRC, and prostate cancer patients with statistical significance

CK7⁺ and TTF-1⁺ CTCs were detected in NSCLC patients, (CK20/CDX2)⁺ CTCs were detected in CRC patients, and (PSA/PSMA)⁺ CTCs were identified in prostate cancer patients (Fig. 5). In NSCLC patients, CK7⁺ and TTF-1⁺ CTC count both had significant differences compared with healthy individuals ($p < 0.001$ for both markers). The (CK20/CDX2)⁺ CTC count was significantly higher in the CRC and prostate cancer patients ($p < 0.001$ in CRC and $p = 0.022$ in prostate cancer). The (PSA/PSMA)⁺ CTC counts were significantly high in the prostate cancer patients ($p < 0.001$) (Table 3.). The CTC counts of each patient and healthy individual are presented in Table S3 and Table S4. The CTC positive number cut-off value of each marker was determined by the healthy individual tests. The positive detection corresponded with the origin of the 3 cancer types. It's worth noting that 2 prostate cancer patients, P-1 and P-3, have positive CTC/CTC microemboli (CTM) counts in

addition to panCK⁺, CK18⁺, and (PSA/PSMA)⁺. In the case of P-1 whose cancer panel indicates positive CTCs/CTMs in panCK, CK18, CK7, CK20/CDX2, and PSA/PSMA, but negative in TTF-1. Clinical chart review indicated that this case was also diagnosed as muscle-invasive urothelial carcinoma of urinary bladder. 61% of urothelial carcinoma is CK20⁺/CK7⁺,²³ but only 1% of prostate cancer was CK20⁺/CK7⁺.²⁴ Though dual cancers are rare, this case suggested that our CTC panel is useful in detecting such a scenario. In the case of prostate cancer patient P-3, whose cancer panel detected positive CTCs/CTMs in panCK, CK18, CK20/CDX2, PSA/PSMA, but negative in CK7 and TTF-1. The clinical history indicated T4 disease and all biopsy cords were positive for malignancy with high grade (Gleason score 4+4). High-grade prostate cancer is highly suspected to further develop neuroendocrine differentiation (small cell/large cell), which can be confirmed by CK20⁺ tissue staining.²⁵ Our panel shows 5 (CK20/CDX2)⁺ CTCs/ 13 (CK20/CDX2)⁺ CTMs detected in patient P-3 in addition to the (PSA/PSMA)⁺ CTCs and CTMs. This attests the possibility that this panel offer additional benefit for the detection of urothelial invasion from the primary site.

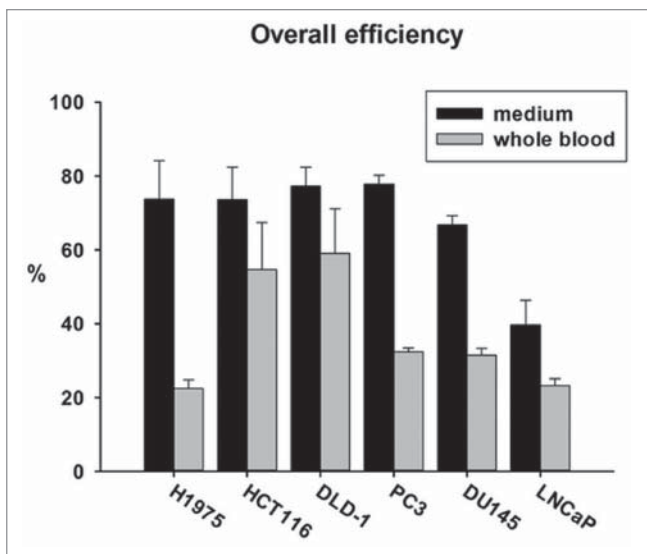


Figure 4. Cell line overall efficiency of CMx chip and staining efficiency test. 200 cells, stained with CellTracker CMFDA, were spiked in 2 ml of culture medium or whole blood. After CMx chip capturing and releasing to an Eppendorf tube, the cells were dripped and fixed on the collection membrane chip. The number of pre-stained cells were counted for the calculation of the system overall efficiency (triplicate individual tests of each cell line). The overall efficiency (%) was defined as: number of CellTracker CMFDA stained cells on the collection membrane chip / number of CellTracker CMFDA stained cells spiked into the CMx chip x 100%

The panel can identify both single CTCs and CTMs in cancer patients

We were able to identify CTMs (images shown in Fig. 6D) in the cancer patients with our CMx panel (Table S3), while no

Table 2. Cancer patient staging and metastasis profile.

Cancer type	Patient No.	Gender	Age	TNM ^a	Metastasis
NSCLC	L-1	F	61	IV (T4N2M1a)	Pleural
	L-2	M	71	IV (T1bN0M1b)	Bone
	L-3	F	46	IV (T2bN3M1a)	Brain
CRC	C-1	M	74	Ila (T3N0M0)	None
	C-2	M	57	Ila (T3N0M0)	None
	C-3	M	58	Ila (T3N0M0)	None
	C-4	M	52	Ila (T3N0M0)	None
	C-5	M	79	I (T1N0M0)	None
Prostate cancer	P-1 ^b	M	69	IV (T4N1M1c)	LN, bladder
	P-2	M	72	IIB (T2cN0M0)	None
	P-3	M	68	IV (T4N0M0)	None
	P-4	M	77	III (T3bN0M0)	None
	P-5	M	74	IIB (T2cN0M0)	None

^aThe 7th edition of the UICC TNM staging system

^bP-1 was diagnosed as dual cancers (prostate cancer and muscle-invasive urothelial carcinoma).

Table 3. Median CTC number and positive detection rate in the cancer patient blood samples.

	NSCLC (n=3)	CRC (n=5)	Prostate cancer (n=5)	Healthy individual (n=12)
panCK (≥ 3) ^a	16 (3/3, 100%) ^b	13 (5/5, 100%)	8 (4/5, 80%)	0 (0/12, 0%) ^c
p value	<0.001*	0.002*	<0.001*	
CK18 (≥ 2)	2 (2/3, 67%)	3 (3/5, 60%)	1 (2/5, 40%)	0 (0/12, 0%)
p value	0.002*	<0.001*	<0.001*	
CK7 (≥ 2)	4 (3/3, 100%)	0 (0/5, 0%)	0 (0/5, 0%)	0 (2/12, 17%)
p value	<0.001*	0.05	0.128	
TTF-1 (≥ 1)	2 (3/3, 100%)	0 (0/5, 0%)	0 (0/5, 0%)	0 (0/12, 0%)
p value	<0.001*	0.212	0.212	
CK20/CDX2 (≥ 2)	0 (0/3, 0%)	2 (3/5, 60%)	0 (2/5, 40%)	0 (2/12, 17%)
p value	0.098	<0.001*	0.022*	
PSA/PSMA (≥ 2)	0 (0/3, 0%)	0 (0/5, 0%)	2 (3/5, 60%)	0 (1/12, 8%)
p value	0.435	0.250	<0.001	

^acut-off number established by healthy individual triplicate test.

^bThe format was shown as "median CTC number (positive detection rate)".

^cThe positive detection rate in healthy individual group was determined by mean CTC count of triplicate tests.

* $p < 0.05$ vs. Healthy individual group.

CTMs were identified in healthy individual samples (Table S4). CTM was defined as a cluster with at least 2 cells and at least one is CTC. The positive detection rate of CTMs in cancer patients are not as high as that of single CTCs, with panCK⁺ CTM median values of 7 for NSCLC, 9 for CRC, and 0 for prostate cancer. Previous studies have reported that in patients with metastatic cancer, the presence of CTMs was strongly associated with a poor prognosis.^{26,27} In our study, we show that CTM is a lower abundant event than single CTCs. Future study with more patient recruitment and long term follow up is necessary to confirm the correlation between CTCs and CTMs.

Discussion

The ability of current CTC panel to detect 3 cancers was primarily dictated by the sensitivity and specificity of the

state-of-the-art biomarkers. In this study, we show that by using 2 types of IF markers, rather than just one, because many other cancers also positively express at least one of the IF markers, we may improve the prediction of cancer origins. For example, CK20 can identify CRC, which was positively expressed in 76% (31/41) CRC tissues,²⁸ but CK20 was also a prevalent marker in ovarian (45%, 10/22).²⁸ and gastric cancer (26%, 227/870).²⁹ On the other hand, CDX2 was positively expressed in over 90% (1087/1249) CRC tissues, but less prevalent in ovarian (10%, 6/62) and gastric cancer (22%, 16/61).³⁰ The combination of CDX2 and CK20 can help identify CRC by eliminating the majority of the other cancers that would be either only CK20⁺ or only CDX2⁺. In the current IF panel, CK20 and CDX2 were mixed in one staining chip as we aim to distinguish CRC originated CTCs from NSCLC and prostate cancer. Since CK20 is expressed in cytoplasm and CDX2

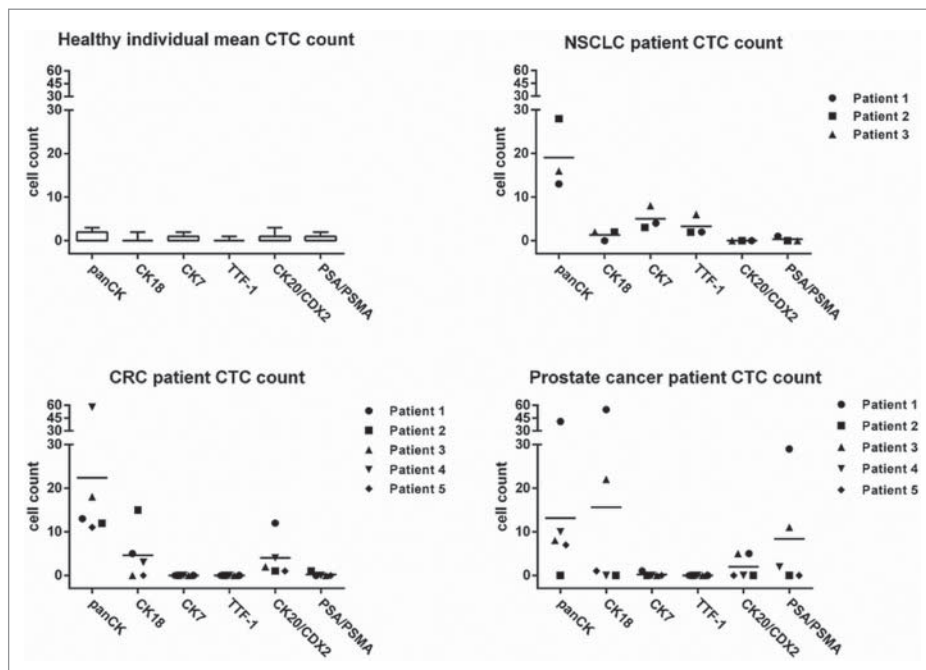


Figure 5. CTC count and analysis of blood samples using the CMx platform and staining panel. Blood from 12 healthy individuals, 3 NSCLC patients, 5 CRC patients, and 5 prostate cancer patients were processed through the CMx platform, and the enumerated CTCs were applied in the IF staining panel. The distribution of CTCs in the panel is shown for each group of patients.

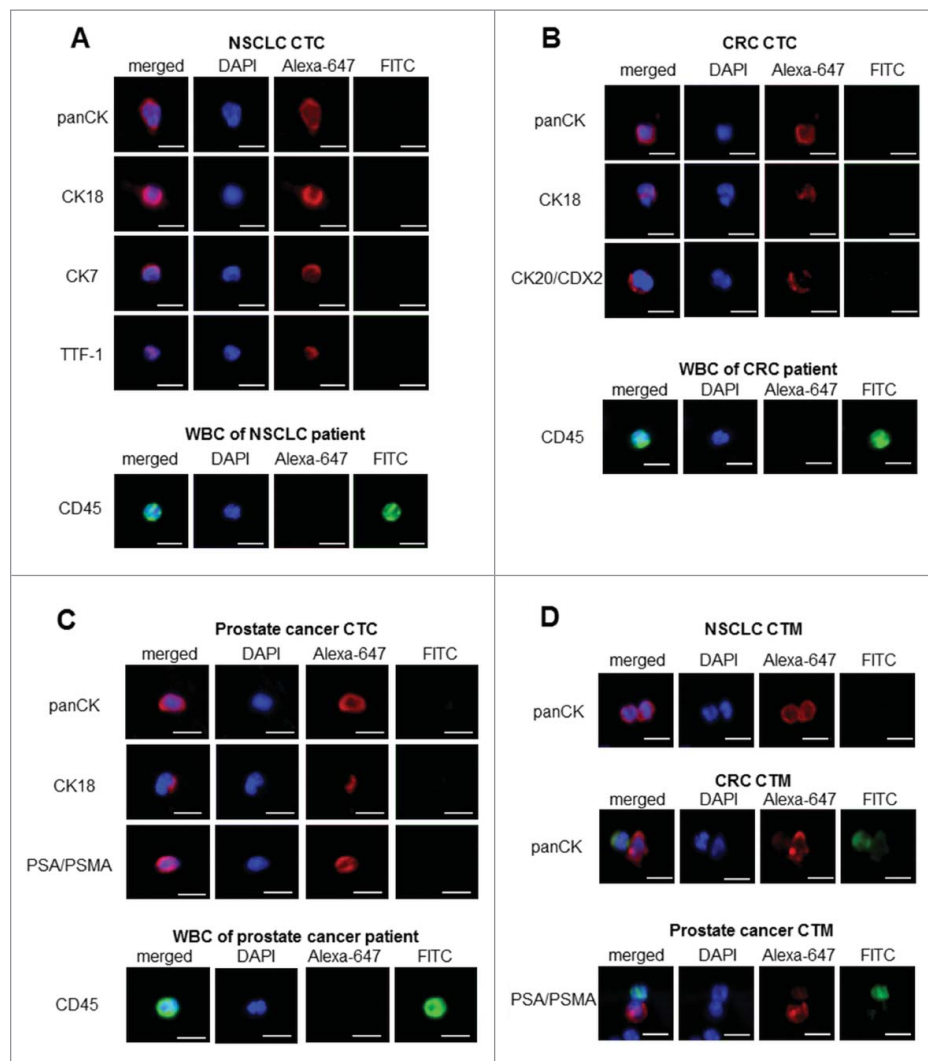


Figure 6. CTCs of cancer patients identified in the IF staining panel. Representative images of CTCs and white blood cells (WBC) from patients of (A) NSCLC, (B) CRC, and (C) prostate cancer. The cancer related markers were labeled with Alexa Fluor 647, and WBC marker CD45 was labeled with FITC. Cell nuclei were labeled with DAPI. (D). Identified circulating tumor microemboli (CTM) morphology. All images were taken with a Nikon Ti-Eclipse microscope at 100X magnification. Scale bar = 10 μ m.

is expressed in nucleus, in the future, using different fluorescent labels could establish $CDX2^+/CK20^+$, $CK20^-/CDX2^+$ $CK20^+/CDX2^+$ CTCs to further improve the accuracy.

Similarly, CK7 was positively expressed in 100% (101/101)³¹ of NSCLC tissue, but was also positively expressed in 98% of metastatic breast cancer,³² 91% (20/22) of ovarian cancer,²⁸ and 96% (48/50) of pancreatic cancer.³³ On the other hand, TTF-1 was positive in 87% (113/130) of NSCLC tissues,³¹ but negative in metastatic breast cancer, ovarian cancer, and pancreatic cancer.³³ A recent report revealed 4% (24/555) of CRC tissue positively expressed TTF-1, all of which were $CK20^+$, and only 2 samples were $CK7^+$.³⁴ The low abundance of $CK7^+/TTF-1^+$ in the cancer tissue other than NSCLC decrease the possibility that CTCs came from another origin which were $TTF-1^+$. With the tissue expression pattern of these markers, we combined CK7 and TTF-1 to increase our specificity in detecting NSCLC CTCs.

Finally, in the case of prostate cancer, we combined PSA and PSMA because in the clinical application, immunohistochemistry for PSA is commonly used for detecting metastatic prostate carcinoma, and PSMA can be strongly expressed in

neoplastic prostatic tissue.³⁵⁻³⁷ The high sensitivity and specificity³⁸ were both the reason for using PSA/PSMA as a detection marker for prostate cancer CTCs. Though PSA was also reportedly expressed in 3.6% (3/84) of breast cancer tissue (tissue samples of both sexes),³⁹ which is considerably lower than the high expression in prostate cancer.³⁸ PSMA was reported to be expressed in various tissues, such as kidney, bladder, testis, and intestine.⁴⁰ However, we could find no quantitative studies on the expression of PSMA by cancers other than prostate cancer. Therefore, it seems safe to assume that at least for initial diagnostic purposes, the probability that a CTC positively expressing PSA/PSMA is from a cancer other than prostate cancer is very low. In our study, the patient P-3 is just an incidental case with dual cancers diagnosed at the same time (within 2 weeks). The CTCs with positive CK20 may come from urothelial cancer itself or prior procedure for urothelial cancer (transurethral resection of bladder tumor). Though it is a rare case, the result demonstrates PSA/PSMA can specifically identified prostate cancer CTCs, and further implicate the importance of using cancer specific markers could successfully identify the CTC origins.

In our clinical study (Fig. 5), panCK⁺ CTC counts were significantly higher in cancer patients than in healthy individuals, which suggested panCK can be a good marker to distinguish cancer patients in CTC enumeration. It is noted that the cancer specific marker expressed CTC amounts were always lower than panCK⁺ CTCs in the clinical samples. This may be explained by the staining efficiency of different antibodies as well as the heterogeneity of CTCs population.⁴¹⁻⁴⁴

In this work, our system focused on 3 specific cancers (NSCLC, CRC, and prostate cancer), but our general method should be applicable to many more cancers, assuming appropriate IF markers are available for those cancers. Although the use of multiple IF markers to identify the origin of a cancer has been reported⁴⁵ and have the accuracy of 88%⁴⁶ and 75%,³³ it is not widely established in pathological diagnosis, probably due to the lack of practical use as the tumor origin is already known. On the contrary, in the case of using CTCs as a surrogate for tumor biopsy, establishing a decision tree as presenting here to sorting out CTC origin likelihood can be very useful in many clinical applications including first line disease screening, and assisting diagnosis.

In conclusion, the panel we designed can reflect the CTC characteristics and marker expression of specific cancer types, and not only provides the ability of identifying CTC origin in clinical use, but can also make CTC detection as an early cancer detection tool. With the number of cancer patients in our study and the IF staining panel that we designed, the CTC count and distribution could reflect a trend to classify lung, colorectal, and prostate cancers. In addition, the panel can be expanded to include more staining markers for other types of cancers. This CTC identification concept can also apply to any types of CTC enumerating systems, which allows the possibility of early cancer detection in a most efficient way.

Materials and methods

Cell culture

Human cancer cell lines were purchased from Bioresource Collection and Research Center (BCRC, Taiwan). We used H1975 for NSCLC, HCT116 and DLD-1 for CRC, and PC3, DU145, and LNCaP for prostate cancer. Lymphoma cell line HL-60 was used as a negative control. HCT116 cells were maintained and grown in Dulbecco's modified Eagle medium (Gibco-RBL Life Technologies). DLD-1, H1975, DU145, PC3, and LNCaP were maintained and grown in RPMI 1640 medium (Gibco-RBL Life Technologies). HL-60 was maintained and grown in Iscove's modified Dulbecco's medium (Gibco-RBL Life Technologies). All cell lines were supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Gibco-RBL Life Technologies) and cultured at 37 °C with 5% CO₂ atmosphere in a humidified incubator.

CMx platform sample processing and overall efficiency

The detailed fabrication and surface modification process of CMx chips were summarized in Supporting Information (SI)

and illustrated in Fig. 1A. Cell lines were pre-stained with CellTracker green CMFDA (Life Technologies), treated with Accutase cell detachment solution (Millipore), and then resuspended in culture medium. The capture and release process of CMx platform was as previously described.¹⁹ Around 200 pre-stained cancer cells were spiked into 2 mL of culture medium or whole blood from healthy individuals, and flowed through the microfluidic device at 1.5 mL/h flow rate. After cell capture, the device was rinsed with 1.5 mL PBS. Captured cancer cells were then released by air foam generated from 5% bovine serum albumin (Millipore) (Fig. 1B). The released cells were contained in 300 μL in foam solution. The cells and foam were spun for 5 s in a microcentrifuge to separate the cells from the air foam. 100 μL of 16% paraformaldehyde (pH = 7.4, Sigma Aldrich) was added, gently mixed with pipetting, and incubated for 10 min at room temperature to fix the cells in suspension. The fixed cells were then transferred to a 10mm diameter filter membrane with 2μm pore size (Millipore). The membrane was mounted on a glass slide and ready for imaging. Images were taken with a Nikon-Ti Eclipse microscope at 100x magnification, and analyzed with NIS-Elements AR Analysis software (Nikon). The cell number was counted for the overall efficiency calculation. The overall efficiency of the CMx platform was defined as: (Number of CellTracker green positive cells on the collection membrane) / (Number of CellTracker green positive cells in the original sample) x 100%.

Staining efficiency

The primary antibodies used for cancer markers are as follows: anti-CK18 anti-CK7, anti-TTF-1, anti-CK20, anti-PSA, anti-PSMA (Abcam), and anti-CDX2 (Cell Marque). The secondary antibody was Alexa Fluor-647 conjugate goat anti-rabbit IgG (Invitrogen). The staining protocol for primary and secondary antibodies is summarized in SI. To evaluate the staining efficiency of each marker on respective cell lines, the buffer/blood containing cancer cell lines that were pre-stained with CellTracker were captured, purified and released on the membrane chip, followed by staining with respective antibody pair (see SI). The staining efficiency is defined as: (Number of Alexa Fluor-647 positive cells) / (Number of CellTracker green-positive cells) × 100%.

Blood sample collection and processing

Blood samples were obtained with consent, and the protocols were approved by the institutional review boards. Healthy volunteers were recruited from Academia Sinica (IRB no: AS-IRB-BM-14026). Blood samples of CRC or prostate cancer were from Chang-Gung Memorial Hospital (AS-IRB-BM-14031 for CRC, AS-IRB-BM-14023 for prostate) and blood samples of NSCLC were from Taipei Veterans General Hospital (AS-IRB-BM-13054). All cancer patients' clinical status were not disclosed until CTC numbers were reported to ensure objective CTC assessments. A total of 12 mL of blood was drawn and preserved at room temperature by adding 3 mL of Streck cell preservative reagent, and processed within 24h using 6 CMx chips. The cell effluent was applied to 6 collection membrane chips for immunofluorescence staining (Fig. 1C). As the

baseline control, a total of 36 mL of blood was drawn from healthy donors to perform triplicate experiments, followed by the same procedures.

Statistical analysis

The results of identified CTC numbers in patient samples were expressed as means and standard deviations. Statistical analyses were performed using multiple t tests with the SPSS statistical software package.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

- Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; 65:87-108; PMID:25651787; <http://dx.doi.org/10.3322/caac.21262>
- Yu M, Stott S, Toner M, Maheswaran S, Haber DA. Circulating tumor cells: approaches to isolation and characterization. *J Cell Biol* 2011; 192:373-82; PMID:21300848; <http://dx.doi.org/10.1083/jcb.201010021>
- Pantel K, Alix-Panabieres C, Riethdorf S. Cancer micrometastases. *Nat Rev Clin Oncol* 2009; 6:339-51; PMID:19399023; <http://dx.doi.org/10.1038/nrclinonc.2009.44>
- Harouaka R, Kang Z, Zheng SY, Cao L. Circulating tumor cells: advances in isolation and analysis, and challenges for clinical applications. *Pharmacol Ther* 2014; 141:209-21; PMID:24134902; <http://dx.doi.org/10.1016/j.pharmthera.2013.10.004>
- Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 2004; 351:781-91; PMID:15317891; <http://dx.doi.org/10.1056/NEJMoa040766>
- Chu PG, Weiss LM. Keratin expression in human tissues and neoplasms. *Histopathology* 2002; 40:403-39; PMID:12010363; <http://dx.doi.org/10.1046/j.1365-2559.2002.01387.x>
- Arrieta O, Pineda B, Muniz-Hernandez S, Flores D, Ordonez G, Borbolla-Escoboza JR, Orta D. Molecular detection and prognostic value of epithelial markers mRNA expression in peripheral blood of advanced non-small cell lung cancer patients. *Cancer Biomarkers* 2014; 14:215-23; PMID:24934364; <http://dx.doi.org/10.3233/CBM-140394>
- Knosel T, Emde V, Schluns K, Schlag PM, Dietel M, Petersen I. Cytokeratin profiles identify diagnostic signatures in colorectal cancer using multiplex analysis of tissue microarrays. *Cell Oncol* 2006; 28:167-75; PMID:16988472; <http://dx.doi.org/10.1155/2006/354295>
- Castillo V, Valenzuela R, Huidobro C, Contreras HR, Castellon EA. Functional characteristics of cancer stem cells and their role in drug resistance of prostate cancer. *Int J Oncol* 2014; 45:985-94; PMID:24990514
- Riesenberg R, Oberneder R, Kriegmair M, Epp M, Bitzer U, Hofstetter A, Braun S, Riethmuller G, Pantel K. Immunocytochemical double staining of cytokeratin and prostate specific antigen in individual prostatic tumour cells. *Histochemistry* 1993; 99:61-6; PMID:7682210; <http://dx.doi.org/10.1007/BF00268022>; <http://dx.doi.org/10.3892/ijo.2014.2529>
- Xi L, Nicastrì DG, El-Hefnawy T, Hughes SJ, Luketich JD, Godfrey TE. Optimal markers for real-time quantitative reverse transcription PCR detection of circulating tumor cells from melanoma, breast, colon, esophageal, head and neck, and lung cancers. *Clinical Chem* 2007; 53:1206-15; PMID:17525108; <http://dx.doi.org/10.1373/clinchem.2006.081828>
- Kirby BJ, Jodari M, Loftus MS, Gakhar G, Pratt ED, Chanel-Vos C, Gleghorn JP, Santana SM, Liu H, Smith JP, et al. Functional characterization of circulating tumor cells with a prostate-cancer-specific microfluidic device. *PloS one* 2012; 7:e35976; PMID:22558290; <http://dx.doi.org/10.1371/journal.pone.0035976>
- Stott SL, Lee RJ, Nagrath S, Yu M, Miyamoto DT, Ulkus L, Inserra EJ, Ulman M, Springer S, Nakamura Z, et al. Isolation and characterization of circulating tumor cells from patients with localized and metastatic prostate cancer. *Science translational medicine* 2010; 2:25ra3; PMID:20424012; <http://dx.doi.org/10.1126/scitranslmed.3000403>
- Wang ZP, Eisenberger MA, Carducci MA, Partin AW, Scher HI, Ts'o PO. Identification and characterization of circulating prostate carcinoma cells. *Cancer* 2000; 88:2787-95; PMID:10870062; [http://dx.doi.org/10.1002/1097-0142\(20000615\)88:12%3c2787::AID-CNCR18%3e3.0.CO;2-2](http://dx.doi.org/10.1002/1097-0142(20000615)88:12%3c2787::AID-CNCR18%3e3.0.CO;2-2)
- Welinder C, Jansson B, Lindell G, Wenner J. Cytokeratin 20 improves the detection of circulating tumor cells in patients with colorectal cancer. *Cancer letters* 2015; 358:43-6; PMID:25528628; <http://dx.doi.org/10.1016/j.canlet.2014.12.024>
- Wong SC, Ng SS, Cheung MT, Luk LY, Chan CM, Cheung AH, Lee VH, Lai PB, Ma BB, Hui EP, et al. Clinical significance of CDX2-positive circulating tumour cells in colorectal cancer patients. *Br J Cancer* 2011; 104:1000-6; PMID:21364588; <http://dx.doi.org/10.1038/bjc.2011.32>
- Chang YL, Lee YC, Liao WY, Wu CT. The utility and limitation of thyroid transcription factor-1 protein in primary and metastatic pulmonary neoplasms. *Lung Cancer* 2004; 44:149-57; PMID:15084379; <http://dx.doi.org/10.1016/j.lungcan.2003.10.008>
- Casavant BP, Strotman LN, Tokar JJ, Thiede SM, Traynor AM, Ferguson JS, Lang JM, Beebe DJ. Paired diagnostic and pharmacodynamic analysis of rare non-small cell lung cancer cells enabled by the VeriFAST platform. *Lab on a chip* 2014; 14:99-105; PMID:24158597; <http://dx.doi.org/10.1039/C3LC50912E>
- Lai JM, Shao HJ, Wu JC, Lu SH, Chang YC. Efficient elution of viable adhesive cells from a microfluidic system by air foam. *Biomicrofluidics* 2014; 8:052001; PMID:25332725; <http://dx.doi.org/10.1063/1.4893348>
- Wu JC, Tseng PY, Tsai WS, Liao MY, Lu SH, Frank CW, Chen JS, Wu HC, Chang YC. Antibody conjugated supported lipid bilayer for capturing and purification of viable tumor cells in blood for subsequent cell culture. *Biomaterials* 2013; 34:5191-9; PMID:23615560; <http://dx.doi.org/10.1016/j.biomaterials.2013.03.096>
- Chan CW, Wong NA, Liu Y, Bicknell D, Turley H, Hollins L, Miller CJ, Wilding JL, Bodmer WF. Gastrointestinal differentiation marker Cytokeratin 20 is regulated by homeobox gene CDX1. *Proc Natl Acad Sci U S A* 2009; 106:1936-41; PMID:19188603; <http://dx.doi.org/10.1073/pnas.0812904106>
- Abe M, Havre PA, Urasaki Y, Ohnuma K, Morimoto C, Dang LH, Dang NH. Mechanisms of confluence-dependent expression of CD26 in colon cancer cell lines. *BMC cancer* 2011; 11:51; PMID:21284881; <http://dx.doi.org/10.1186/1471-2407-11-51>
- Jiang J, Ulbright TM, Younger C, Sanchez K, Bostwick DG, Koch MO, Eble JN, Cheng L. Cytokeratin 7 and cytokeratin 20 in primary urinary bladder carcinoma and matched lymph node metastasis. *Arch Pathol Lab Med* 2001; 125:921-3; PMID:11419977; [http://dx.doi.org/10.0003-9985\(2/0003-9985\(2001\)125<0921:CACIPU>2.0.CO;2](http://dx.doi.org/10.0003-9985(2/0003-9985(2001)125<0921:CACIPU>2.0.CO;2)
- Tot T. Cytokeratins 20 and 7 as biomarkers: usefulness in discriminating primary from metastatic adenocarcinoma. *Eur J Cancer* 2002; 38:758-63; PMID:11937308; [http://dx.doi.org/10.1016/S0959-8049\(02\)00008-4](http://dx.doi.org/10.1016/S0959-8049(02)00008-4)
- Parimi V, Goyal R, Poropatich K, Yang XJ. Neuroendocrine differentiation of prostate cancer: a review. *Am J Clin Exp Urol* 2014; 2:273-85; PMID:25606573
- Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, Yu M, Pely A, Engstrom A, Zhu H, et al. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. *Cell*

- 2014; 158:1110-22; PMID:25171411; <http://dx.doi.org/10.1016/j.cell.2014.07.013>
27. Hou JM, Krebs MG, Lancashire L, Sloane R, Backen A, Swain RK, Priest LJ, Greystoke A, Zhou C, Morris K, et al. Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. *J Clin Oncol* 2012; 30:525-32; PMID:22253462; <http://dx.doi.org/10.1200/JCO.2010.33.3716>
 28. Shin JH, Bae JH, Lee A, Jung CK, Yim HW, Park JS, Lee KY. CK7, CK20, CDX2 and MUC2 Immunohistochemical staining used to distinguish metastatic colorectal carcinoma involving ovary from primary ovarian mucinous adenocarcinoma. *Jpn J Clin Oncol* 2010; 40:208-13; PMID:19926591; <http://dx.doi.org/10.1093/jjco/hyp150>
 29. Takami H, Sentani K, Matsuda M, Oue N, Sakamoto N, Yasui W. Cytokeratin expression profiling in gastric carcinoma: clinicopathologic significance and comparison with tumor-associated molecules. *Pathobiology* 2012; 79:154-61; PMID:22286119; <http://dx.doi.org/10.1159/000335694>
 30. Kaimakhtchiev V, Terracciano L, Tornillo L, Spichtin H, Stoios D, Bundi M, Korcheva V, Mirlacher M, Loda M, Sauter G, et al. The homeobox intestinal differentiation factor CDX2 is selectively expressed in gastrointestinal adenocarcinomas. *Mod Pathol* 2004; 17:1392-9; PMID:15205684; <http://dx.doi.org/10.1038/modpathol.3800205>
 31. Gurda GT, Zhang L, Wang Y, Chen L, Geddes S, Cho WC, Askin F, Gabrielson E, Li QK. Utility of five commonly used immunohistochemical markers TTF-1, Napsin A, CK7, CK5/6 and P63 in primary and metastatic adenocarcinoma and squamous cell carcinoma of the lung: a retrospective study of 246 fine needle aspiration cases. *Clin Translational Med* 2015; 4:16; PMID:25977750; <http://dx.doi.org/10.1186/s40169-015-0057-2>
 32. Tot T. Patterns of distribution of cytokeratins 20 and 7 in special types of invasive breast carcinoma: a study of 123 cases. *Ann Diagn Pathol* 1999; 3:350-6; PMID:10594286; [http://dx.doi.org/10.1016/S1092-9134\(99\)80013-6](http://dx.doi.org/10.1016/S1092-9134(99)80013-6)
 33. Park SY, Kim BH, Kim JH, Lee S, Kang GH. Panels of immunohistochemical markers help determine primary sites of metastatic adenocarcinoma. *Arch Pathol Lab Med* 2007; 131:1561-7; PMID:17922593; [http://dx.doi.org/10.1043/1543-2165\(2007\)131\[1561:POIMHD\]2.0.CO;2](http://dx.doi.org/10.1043/1543-2165(2007)131[1561:POIMHD]2.0.CO;2)
 34. Dettmer M, Kim TE, Jung CK, Jung ES, Lee KY, Kang CS. Thyroid transcription factor-1 expression in colorectal adenocarcinomas. *Pathol Res Pract* 2011; 207:686-90; PMID:22019009; <http://dx.doi.org/10.1016/j.prp.2011.08.009>
 35. Bostwick DG, Pacelli A, Blute M, Roche P, Murphy GP. Prostate specific membrane antigen expression in prostatic intraepithelial neoplasia and adenocarcinoma: a study of 184 cases. *Cancer* 1998; 82:2256-61; PMID:9610707; [http://dx.doi.org/10.1002/\(SICI\)1097-0142\(19980601\)82:11%3c2256::AID-CNCR22%3e3.0.CO;2-S](http://dx.doi.org/10.1002/(SICI)1097-0142(19980601)82:11%3c2256::AID-CNCR22%3e3.0.CO;2-S)
 36. Sweat SD, Pacelli A, Murphy GP, Bostwick DG. Prostate-specific membrane antigen expression is greatest in prostate adenocarcinoma and lymph node metastases. *Urology* 1998; 52:637-40; PMID:9763084; [http://dx.doi.org/10.1016/S0090-4295\(98\)00278-7](http://dx.doi.org/10.1016/S0090-4295(98)00278-7)
 37. Minner S, Wittmer C, Graefen M, Salomon G, Steuber T, Haese A, Huland H, Bokemeyer C, Yekebas E, Dierlamm J, et al. High level PSMA expression is associated with early PSA recurrence in surgically treated prostate cancer. *Prostate* 2011; 71:281-8; PMID:20809553; <http://dx.doi.org/10.1002/pros.21241>
 38. Bernacki KD, Fields KL, Roh MH. The utility of PSMA and PSA immunohistochemistry in the cytologic diagnosis of metastatic prostate carcinoma. *Diagn Cytopathol* 2014; 42:570-5; PMID:24273068; <http://dx.doi.org/10.1002/dc.23075>
 39. Kraus TS, Cohen C, Siddiqui MT. Prostate-specific antigen and hormone receptor expression in male and female breast carcinoma. *Diagn Pathol* 2010; 5:63; PMID:20863373; <http://dx.doi.org/10.1186/1746-1596-5-63>
 40. Kinoshita Y, Kuratsukuri K, Landas S, Imaida K, Rovito PM, Jr., Wang CY, Haas GP. Expression of prostate-specific membrane antigen in normal and malignant human tissues. *World J Surg* 2006; 30:628-36; PMID:16555021; <http://dx.doi.org/10.1007/s00268-005-0544-5>
 41. Babayan A, Hannemann J, Spotter J, Muller V, Pantel K, Joosse SA. Heterogeneity of estrogen receptor expression in circulating tumor cells from metastatic breast cancer patients. *PLoS One* 2013; 8:e75038; PMID:24058649; <http://dx.doi.org/10.1371/journal.pone.0075038>
 42. Ning Y, Hanna DL, Zhang W, Mendez A, Yang D, El-Khoueiry R, Matsusaka S, Sunakawa Y, Stremtizer S, Parekh A, et al. Cytokeratin-20 and Survivin-Expressing Circulating Tumor Cells Predict Survival in Metastatic Colorectal Cancer Patients by a Combined Immunomagnetic qRT-PCR Approach. *Mol Cancer Ther* 2015; 14:2401-8; PMID:26227487; <http://dx.doi.org/10.1158/1535-7163.MCT-15-0359>
 43. Lin HC, Hsu HC, Hsieh CH, Wang HM, Huang CY, Wu MH, Tseng CP. A negative selection system PowerMag for effective leukocyte depletion and enhanced detection of EpCAM positive and negative circulating tumor cells. *Clin Chim Acta* 2013; 419:77-84; PMID:23415697; <http://dx.doi.org/10.1016/j.cca.2013.01.018>
 44. Nel I, Jehn U, Gauler T, Hoffmann AC. Individual profiling of circulating tumor cell composition in patients with non-small cell lung cancer receiving platinum based treatment. *Transl Lung Cancer Res* 2014; 3:100-6; PMID:25806288; <http://dx.doi.org/10.3978/j.issn.2218-6751.2014.03.05>
 45. DeYoung BR, Wick MR. Immunohistologic evaluation of metastatic carcinomas of unknown origin: an algorithmic approach. *Semin Diagn Pathol* 2000; 17:184-93; PMID:10968704
 46. Dennis JL, Hvidsten TR, Wit EC, Komorowski J, Bell AK, Downie I, Mooney J, Verbeke C, Bellamy C, Keith WN, et al. Markers of adenocarcinoma characteristic of the site of origin: development of a diagnostic algorithm. *Clin Cancer Res* 2005; 11:3766-72; PMID:15897574; <http://dx.doi.org/10.1158/1078-0432.CCR-04-2236>