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Circulating tumor cell enumeration for improved screening and disease detection of patients with colorectal cancer

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ABSTRACT

Background: The immunochemical fecal occult blood test (iFOBT) for colorectal cancer (CRC) screening and the serum carcinoembryonic antigen (CEA) assay for disease detection of CRC is associated with a high false-positive rate and a low detection sensitivity, respectively. There is an unmet need to define additional modalities to complement these assays. Different subsets of circulating tumor cells (CTCs) are present in the peripheral blood of cancer patients. Whether or not CTCs testing supplements these clinical assays and is valuable for patients with CRC was investigated.

Methods: CTCs were enriched from pre-operative patients with CRC ($n = 109$) and the non-cancerous controls ($n = 65$). CTCs expressing either epithelial cell adhesion molecule (EpCAM) or podoplanin (PDPN, the marker associated with poor cancer prognosis) were defined by immunofluorescence staining and were analyzed alone or in combination with iFOBT or serum CEA.

Results: Patients with early or advanced stage of CRC can be clearly identified and differentiated from the non-cancerous controls ($p < 0.001$) by EpCAM$^+$-CTC or PDPN$^+$-CTC count. The sensitivity and specificity of EpCAM$^+$-CTCs was 85.3% and 78.5%, respectively, when the cutoff value was 23 EpCAM$^+$-CTCs/mL of blood; and the sensitivity and specificity of

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PDPN^{+}-CTCs was 78.0% and 75.4%, respectively, when the cutoff value was 7 PDPN^{+}-CTCs/mL of blood. Combined analysis of iFOBT with the EpCAM^{+}-CTC and PDPN^{+}-CTC count reduced the false-positive rate of iFOBT from 56.3% to 18.8% and 23.4%, respectively. Combined analysis of serum CEA with the EpCAM^{+}-CTC and PDPN^{+}-CTC count increased the disease detection rate from 30.3% to 89.9% and 86.2%, respectively.

Conclusion: CTC testing could supplement iFOBT to improve CRC screening and supplement serum CEA assay for better disease detection of patients with CRC.

Colorectal cancer (CRC) is among the most common cancers worldwide [1], and is the most common cancer in men and the second most common cancer in women in Taiwan [2,3]. CRC screening and disease monitoring are essential to reduce patient mortality. The immunochemical fecal occult blood test (iFOBT) is commonly used in population screening to identify individuals who have CRC without notice. People who have a positive iFOBT receive colonoscopy examination to confirm the occurrence of advanced stage adenomas and early cancer which if not treated may cause CRC mortality. However, the high false positive rate of iFOBT increases substantially the use and the cost associated with the use of colonoscopy resources [4]. A non-invasive and cost-effective method to supplement iFOBT to define individuals who have false positive iFOBT results should make iFOBT screening more practical.

At a glance of commentary

Scientific background on the subject

Circulating tumor cells (CTCs) is a type of liquid biopsies for monitoring disease status of patients with cancer. Whether or not combined analysis of CTCs with immunochemical fecal occult blood test (iFOBT) or serum carcinoembryonic antigen (CEA) assay improves screening and disease detection of patients with colorectal cancer was investigated.

What this study adds to the field

Combined analysis of CTCs with iFOBT reduced the false positive rate of colorectal cancer screening, while with CEA increased the disease detection rate. This study implies that CTC testing may supplement the current clinical assays for improved screening and disease detection of patients with colorectal cancer.

Carcinoembryonic antigen (CEA), without satisfying accuracy, is now the only biomarker for CRC in the clinical setting. It is used not only for detection of CRC but also for monitoring disease status during treatment. However, elevated serum CEA was found in 72% of cases with un-resectable or metastatic disease and in 3%, 25%, 45% and 65% of patients with Dukes' A, B, C, and D, respectively [5,6]. Multiple imaging techniques including virtual colonoscopy, elastography, and dual-energy spectral computed tomography may supplement CEA results and offer surveillance of patients to avoid missed cases [7]. These procedures either are expensive or require exposure to radiation. Additional methods to supplement CEA assay for disease detection of patients with CRC are worthy to define.

Cancer cells are released into the blood stream even at the early stage of cancer [8]. Circulating tumor cells (CTCs) enumeration has been used to monitor treatment efficacy and the disease status of patients with CRC [9,10]. However, the detection rates of CTCs in different clinico-pathological conditions are various. The application of CTC enumeration in screening for early stage of cancer is also controversial [11–13]. Whether sufficient number of CTCs can be detected by a specific CTC assay mainly depends on the binding affinity of the capture antibody which in most studies targeting on the epithelial cell adhesion molecule (EpCAM) [14,15]. However, cancer cells usually undergo epithelial–mesenchymal transition (EMT) when they are released from the primary tumor site [16]. Phenotypic variation can occur in CTCs [17]. CTCs expressing EMT-related proteins such as N-cadherin, E-cadherin, vimentin, and the 38–40 kDa transmembrane molecule podoplanin (PDPN) have been identified in different cancer types [18–22]. Among these EMT-related proteins, PDPN expression was increased in almost all intestinal tumors (colon, rectum, and small intestine) compared with corresponding normal tissues [23,24]. PDPN is implicated in cancer progression [25–27] and cancer-associated thrombosis [28,29] by increasing cell spreading, collective and single cell migration and invasion [30], induction of RhoA activity, modulation of actin cytoskeleton [30] and inducing platelet aggregation by binding to the platelet C-type lectin-like receptor 2 (CLEC-2) [31,32]. On the other hand, PDPN regulates lymphatic vessel formation [33], cerebrovascular patterning and integrity [34], megakaryocyte proliferation, proplatelet formation [35], wound healing [36] and efficient T-cell priming [37], and maintains the integrity of high endothelial venules in lymph nodes [38] under physiological condition.

Negative selection by depleting CD45^{+}-leukocytes with anti-CD45 antibody provides a better strategy to enrich different subtypes of CTC [39,40]. PDPN-positive CTCs have been enriched by negative selection from patients with head and neck squamous cell carcinoma [18] and papillary thyroid carcinoma (PTC) [41]. Whether PDPN is expressed in the CTCs from patients with CRC has not yet been defined. Whether analysis of EpCAM^{+}-CTC and PDPN^{+}-CTC count supplements iFOBT to reduce the false positive results and when analyzed together with CEA assay to provide better disease monitoring of patients are worthy to further investigation.

In this study, CTCs were enriched from patients at different stages of CRC by using a negative selection system [42]. The number of $EpCAM^+$-CTCs and $PDPN^+$-CTCs in $CD45^+$-depleted cell filtrates was determined. Combined analysis of CTC count with iFOBT or serum CEA was performed and correlated with the clinical status of patients with CRC. The significance of these findings was discussed.

Materials and methods

Study subjects

This study was approved by the Chang Gung Memorial Hospital Institutional Review Board (approval ID: 103–2529B and 1604190001). Written informed consent was given by all patients before inclusion in the study. A total of 109 patients with CRC and 65 non-cancerous controls were enrolled in the Division of Colon and Rectal Surgery, Department of Surgery, Chang Gung Memorial Hospital at Linko. Of the 109 CRC patients, 16 of them were at stage I, 36 at stage II, 47 at stage III, and 10 at stage IV, respectively. The Tumor-Node-Metastasis (TNM) classification of the tumor was based on the American Joint Committee on Cancer, seventh edition. All clinicopathologic parameters of patients were classified according to the chart records. Patients with other known cancers and multiple gastrointestinal tract diseases (such as polyps and ulcerative colitis) were excluded from the analysis.

Non-cancerous controls were divided into two groups based on colonoscopic exams and chart records. The individuals in group 1 (G1) had no previously known cancer and without any benign colorectal diseases at the time of the study. The individuals in group 2 (G2) had benign colorectal diseases such as hyperplastic, adenomatous, or severely dysplastic polyps. This prospective study was double-blinded in terms of both blood draw and CTC enumeration.

Reagents

The Alexa Fluor 488-conjugated donkey anti-mouse antibody, Alexa Fluor 555-conjugated donkey anti-rat antibody, and Hoechst 33342 were purchased from Invitrogen Inc. (Carlsbad, CA). The mouse anti-EpCAM antibody was purchased from Abcam Inc. (Cambridge, England). The anti-PDPN antibody was purchased from Angiobio (San Diego, CA).

Depletion of $CD45^+$-leukocytes by PowerMag system

CTCs were enriched by using a negative selection system PowerMag as previously described [42]. In brief, the red blood cells (RBC) in the fresh whole blood samples (4 ml) were lysed by RBC lysis buffer for 5 min at room temperature. After centrifugation at 400 g and 10 °C for 10 min and washing twice with culture medium, the nucleated cells were resuspended in culture medium (2 ml). CD45 depletion cocktail (StemCell Technologies, Vancouver, BC) was added to the nucleated cell suspension and incubated at room temperature for 15 min for depletion of $CD45^+$-leukocytes by capturing the cells in a PowerMag depletion column. The $CD45^+$-depleted cells were obtained by centrifugation of the cell filtrates at 400 g for 10 min. The cell pellets were subject to immunofluorescence staining for CTC enumeration.

Table 1 Basic information of patients enrolled in this study.

Variables	No. of patient or median years	Interquartile range or percentage
Age	63	54–72
Gender, male/female	67/42	
Tumor location		
Cecum	7	6.4
A-colon	9	8.3
T-colon	13	11.9
Splenic-flex	4	3.7
D-colon	12	11
S-colon	25	22.9
Rectum	39	35.8
Tumor-node-metastasis staging		
Stage Ia	6	5.5
Stage Ib	10	9.2
Stage IIa	31	28.4
Stage IIb	5	4.6
Stage IIIa	29	26.6
Stage IIIb	8	7.3
Stage IIIc	10	9.2
Stage IV	10	9.2
Histological grade		
Well differentiation (Grade I)	9	8.3
Moderate differentiation (Grade II)	88	80.7
Poor differentiation (Grade III-IV)	12	11
T classification		
T0-2	20	18.3
T3-4	89	81.7
N classification		
N0-1	102	93.6
N2-3	7	6.4
M classification		
M0	100	91.7
M1	9	8.3

Immunofluorescence staining and CTC enumeration

For immunofluorescence staining, $CD45^+$-depleted cell filtrates were incubated with the primary antibodies (anti-EpCAM or anti-PDPN) in the presence of the DNA staining dye Hoechst 33342 at room temperature for 1 h in the dark. After washing and centrifugation twice, the cell pellets were resuspended and incubated with the Alexa Fluor 488-conjugated (for EpCAM) or Alexa Fluor 555-conjugated (for PDPN) secondary antibodies in the dark for 30 min. After removing the unbound antibody, the cell aliquots were placed on a slide and the immunofluorescent images were captured by the fluorescence microscopy using automated slide scanning platform (Zeiss Axiovert 200M). Image analysis was performed by using the IN Cell Analyzer 1000 Cellular Imaging and Analysis System (GE Healthcare Life Sciences, Pittsburgh, PA). Hoechst-positive cells that are positive for EpCAM and

Table 2 Basic characteristics and CTC count of the study subjects.

Characteristics	Gender (M/F)	Age (year)[a]	$EpCAM^{+}$ cells/ml[a]	$PDPN^{+}$ cells/ml[a]
Controls (n = 65)	38/27	57 (48–63)	11 (5–23)	5 (3–8)
Healthy individuals (G1, n = 25)	12/13	55 (47–61)	10 (4–21)	5 (2–8)
Benign polyps (G2, n = 40)	26/14	58 (48–64)	13 (7–25)	5 (3–8)
Patients (n = 109)	67/42	63 (54–72)	109 (31–237)	24 (8–56)
Stage I (n = 16)	14/2	70 (54–76)	194 (24–420)	32 (8–72)
Stage II (n = 36)	22/14	65 (52–73)	159 (42–283)	32 (8–69)
Stage III (n = 47)	25/22	62 (55–68)	63 (25–186)	15 (6–43)
Stage IV (n = 10)	6/4	64 (49–66)	118 (48–251)	33 (14–46)
***p*-value**[b]	0.165	0.003	<0.0001	<0.0001

[a] Data represent the median and interquartile range for the indicated parameters.
[b] Statistical analysis was performed for comparing controls (G1 and G2) with patients.

PDPN were defined as $EpCAM^{+}$-CTCs and $PDPN^{+}$-CTCs, respectively.

Serum CEA and iFOBT assays

Serum CEA was determined by using an ADVIA Centaur® Analyzer (WI, USA) with the cutoff set at 5 ng/mL. The iFOBT was determined by using an ADVIA Centaur® Analyzer (WI, USA) with the cutoff set at 100 ng/mL.

Statistical analysis

CTC counts in non-cancerous control and in patients with CRC were compared using the Kruskal–Wallis test for all groups. The Dunn's test was used as the post-hoc test between any two groups. The discrimination ability between any two groups was determined by using the ROC analysis. The correlations of the CTC count to iFOBT, and the CTC count to serum CEA assay were calculated using the Pearson Chi–Square test with cross-tabulation. Statistical analysis was performed using SPSS for Windows (version 18, SPSS, Chicago, IL). A p-value < 0.05 was considered statistically significant.

Results

Basic characteristics of patients and non-cancerous controls

Basic characteristics of patients enrolled in this study are shown in Table 1. The median age was 63 years with the interquartile range from 54 to 72. Rectum (35.8%) was the primary tumor site followed by S-colon (22.9%) and T-colon (11.9%) [Table 1]. The staging status of patients included stage I (14.7%), stage II (33.0%), stage III (43.1%), and stage IV (9.2%). The histological grade of 88 patients (80.7%) was classified as moderate differentiation (grade II). Eighty-nine patients (81.7%) were classified as T3-4 with the tumors invading through the muscularis propria into pericolorectal tissues. The non-cancerous controls were gender-matched with the median age of 57 years and an interquartile range from 48 to 63 [Table 2].

Differentiation of patients with CRC from non-cancerous controls by $EpCAM^{+}$-CTC and $PDPN^{+}$-CTC count

PDPN is a marker for lymphatic vessels and is involved in cancer progression by mediating cancer cell-induced platelet aggregation and cancer-associated thrombosis [25–29]. The fragments per kilobase million files were downloaded from The Cancer Genome Atlas and combined for bioinformatic analysis to demonstrate the expression profile of PDPN in colorectal cancer. PDPN was increased in tumor tissues from patients with CRC when compared to the normal counterpart as determined by the analysis of variance model in the Partek Genomics Suite [$p < 0.001$, see Supplementary Fig. 1].

The negative selection system PowerMag [42] was used to enrich CTCs by depletion of $CD45^{+}$ leukocytes. The $EpCAM^{+}$ and $PDPN^{+}$ cells in the CD45-depleted cell filtrates were defined by immunofluorescence staining [Fig. 1A]. The number of CTCs was determined and correlated with the clinical condition of patients [Table 2, Fig. 1B and C]. The median $EpCAM^{+}$ and $PDPN^{+}$ cell count for G1 control (healthy individuals) was 10 (interquartile range 4–21) and 5 (interquartile range 2–8) cells/ml of blood, respectively. The median $EpCAM^{+}$ and $PDPN^{+}$ cell count for the G2 control (individuals with benign polyps) was 13 (interquartile range 7–25) and 5 (interquartile range 3–8) cells/ml of blood, respectively. $EpCAM^{+}$ and $PDPN^{+}$ cell count was not significantly different between G1 and G2, implying that non-cancerous benign disorders of colorectal tissues do not increase cell release from colorectal tissues into the circulatory system.

The median $EpCAM^{+}$-CTC and $PDPN^{+}$-CTC count for patients with CRC was 109 (interquartile range 31–237) and 24 (interquartile range 8–56) cells/ml, respectively [Table 2]. The number of both CTC subtypes was significantly increased when compared to the individuals who were considered as healthy without cancer history and who had benign colorectal disease [$p < 0.0001$, Table 2, Fig. 1B and C]. The $EpCAM^{+}$-CTC count distinguished patients with CRC from non-cancerous controls (G1 + G2). The area under the curve (AUC) in a receiver operating characteristic (ROC) analysis was 0.8602 [$p < 0.0001$, Fig. 2A]. The sensitivity and specificity of the assay was 85.3% and 78.5%, respectively, when the cutoff was set at 23 $EpCAM^{+}$ cells/ml [Fig. 2A]. $PDPN^{+}$-CTCs distinguished patients with CRC from non-cancerous controls (G1 + G2) with the AUC equivalent to 0.8126 [$p < 0.0001$, Fig. 2B]. The sensitivity and specificity of the assay was 78.0% and 75.4%,

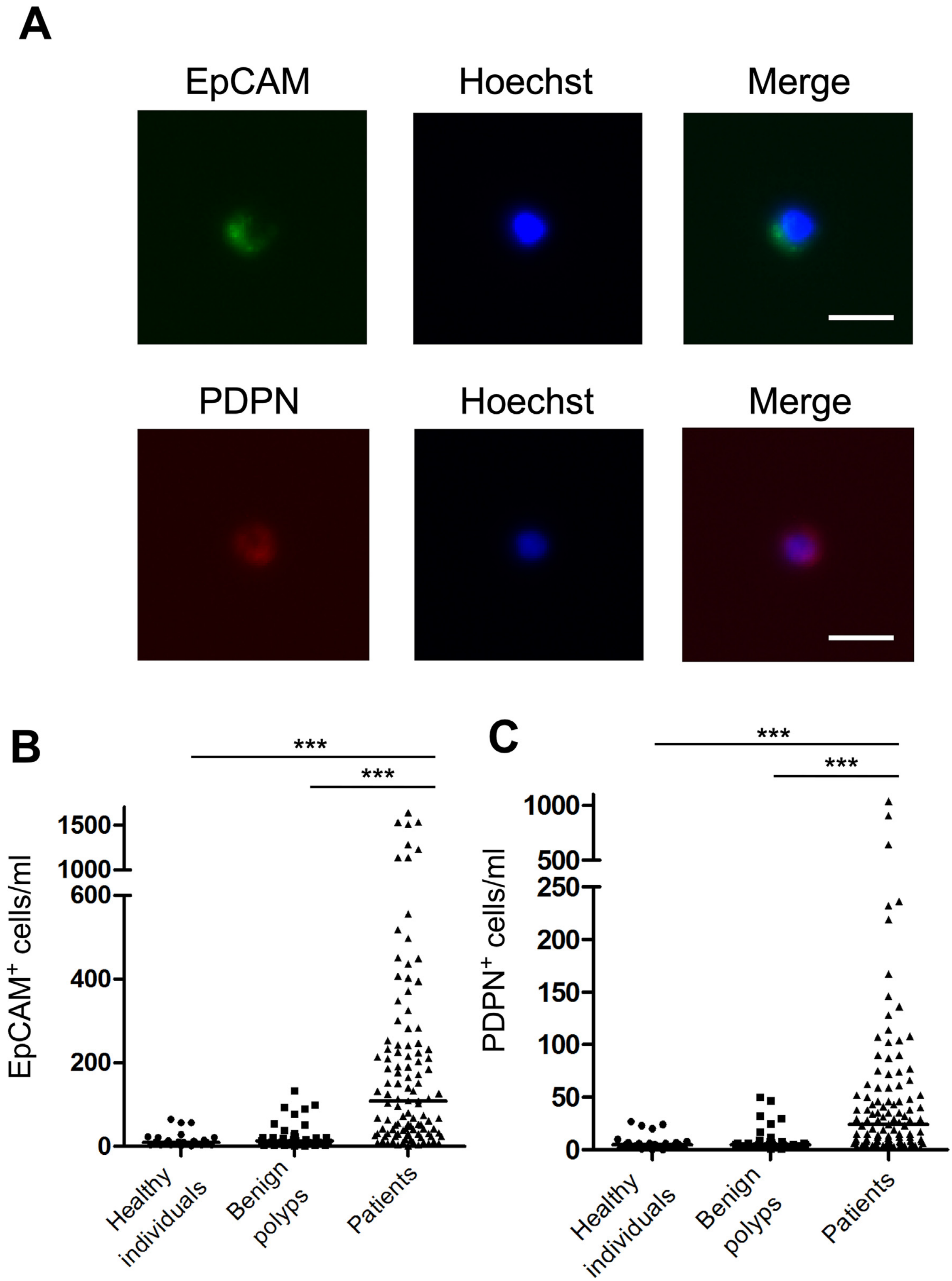
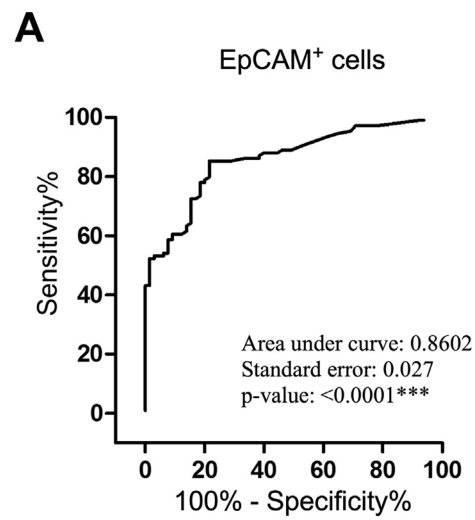
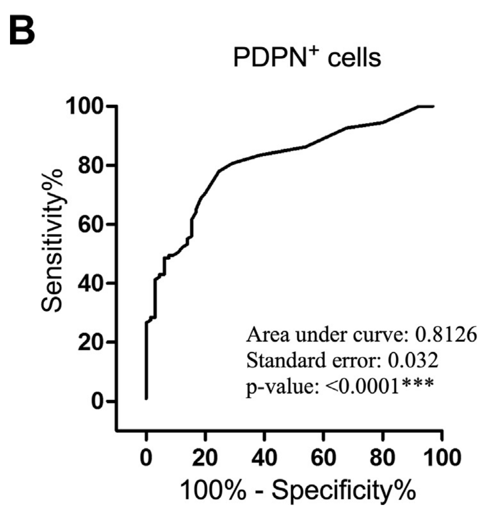


Fig. 1 Fluorescence images of EpCAM+ and PDPN+ cells and the scatter dot plots for the number of EpCAM+ and PDPN+ cells in the indicated groups of individuals. (A) Immunofluorescence staining was performed to define EpCAM+ and PDPN+ cells using the anti-EpCAM and anti-PDPN antibody, respectively. The cells that were positive to Hoechst 33342 staining indicate intact nucleated cells. Bar = 10 μm. (B–C) The scatter dot plots for EpCAM+ (panel B) and PDPN+ (panel C) cell count obtained from individuals without colorectal diseases (healthy individuals, n = 25), with benign colorectal diseases (benign polyps, n = 40), and with CRC (patients, n = 109). Statistical analysis was performed by using the Kruskal–Wallis test. The Dunn's test was used for post-hoc analysis. The median for each group are indicated by horizontal line. ***$p < 0.001$.



Cutoff value	Sensitivity (%)	Specificity (%)
16.5	86.24	61.54
18	86.24	64.62
20	86.24	66.15
21.5	85.32	70.77
22.5	85.32	75.38
23.5	85.32	78.46
24.5	81.65	78.46
25.5	79.82	78.46
27	78.9	80
28.5	77.98	80
29.5	77.98	81.54



Cutoff value	Sensitivity (%)	Specificity (%)
3.5	92.66	32.31
4.5	86.24	46.15
5.5	83.49	61.54
6.5	80.73	70.77
7.5	77.98	75.38
8.5	70.64	80
9.5	68.81	81.54
10.5	65.14	83.08
11.5	64.22	83.08
12.5	61.47	84.62

Fig. 2 ROC analysis of non-cancerous controls and patients with CRC. (A–B) ROC curve with AUC value for EpCAM$^+$ (panel A) and PDPN$^+$ (panel B) cell count and its use in distinguishing non-cancerous controls (group G1 and G2) and patients with CRC. Several cutoff values were listed.

respectively, when the cutoff was set at 7 cells/ml for PDPN$^+$-CTCs [Fig. 2B].

EpCAM$^+$-CTC count was not significantly different among patients at different clinical stages [see Supplementary Table S1]. The median EpCAM$^+$-CTC count was 194 (interquartile range 24–420), 159 (interquartile range 42–283), 63 (interquartile range 25–186) and 118 (interquartile range 48–251) cells/ml for patients at stage I, II, III and IV, respectively [Table 2 and Fig. 3A]. The EpCAM$^+$-CTC count was significantly different between G1 and all stages of patients ($p < 0.0001$), and between G2 and all stages of patients ($p < 0.0001$). PDPN$^+$-CTC count was not significantly different among patients at different clinical stages. The median PDPN$^+$-CTC count was 32 (interquartile range 8–72), 32 (interquartile range 8–69), 15 (interquartile range 6–43) and 33 (interquartile range 14–46) cells/ml for patients at stage I, II, III and IV, respectively [Table 2 and Fig. 3B]. The PDPN$^+$-CTC count was significantly different between G1 and all stages of patients (stage I, $p < 0.001$; stages II, III, and IV, $p < 0.0001$), and between G2 and all stages of patients (stage I, $p < 0.001$; stages II, III, and IV, $p < 0.0001$). These results reveal that either EpCAM$^+$-CTC or PDPN$^+$-CTC counts can differentiate patients with CRC from the individuals with benign polyps or without cancer history.

Combined analysis of CTC count and iFOBT reduced the false positive results of iFOBT

The iFOBT is a cost-effective and non-invasive screening method for CRC with a high false positive rate. This prompted an investigation of whether or not combined analysis of CTC and iFOBT reduced the false positive rate of iFOBT. In the non-cancerous control group (G1 + G2), 24 of the 36 individuals who were iFOBT-positive (66.6%) had an EpCAM$^+$-CTC count below the cutoff set at 23 cells/ml. Twenty-four of the 28 patients who were iFOBT-positive (85.7%) was confirmed by high EpCAM$^+$-CTC count [Table 3]. Similarly, 21 of the 36 non-

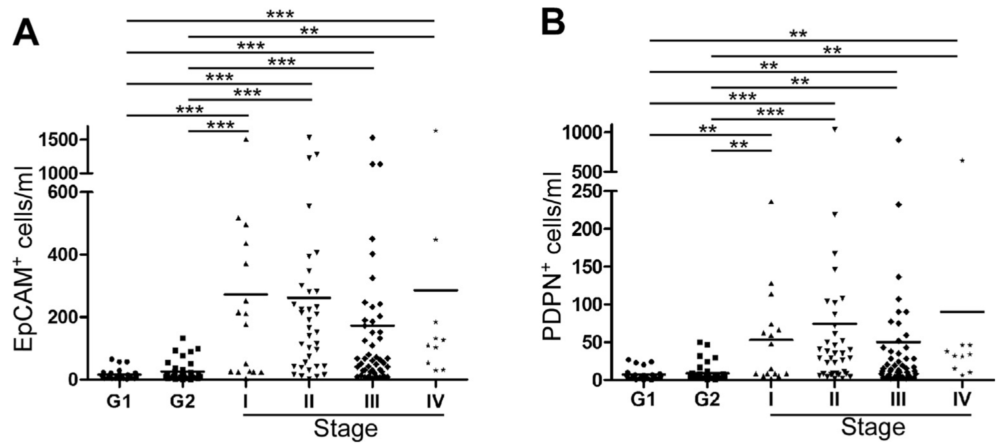


Fig. 3 Scatter dot plots for the number of EpCAM+ and PDPN+ cells from non-cancerous controls and patients with CRC at different clinical stages. (A–B) Scatter dot plots for the number of EpCAM+ (panel A) and PDPN+ (panel B) cells in the peripheral blood of individuals without colorectal diseases (G1), with benign colorectal diseases (G2), and with CRC at different clinical stages. Statistical analysis was performed by using the Kruskal–Wallis test followed by the Dunn's test for post-hoc analysis. The median for each group are indicated by horizontal line. $^{**}p < 0.01$, $^{***}p < 0.001$.

cancer controls who were iFOBT-positive (58.3%) had an PDPN+-CTC count less than the cutoff set at 7 cells/ml. Twenty-two of the 28 patients who were iFOBT-positive (78.6%) was confirmed by high PDPN+-CTC count [Table 3]. These data demonstrate that analysis of EpCAM+-CTC and PDPN+-CTC count could reduce the false positive results of iFOBT.

Combined analysis of CTC count and serum CEA facilitates disease detection of patients with CRC

CEA is the clinical biomarker to indicate the progression or recurrence of CRC. However, serum CEA is increased in only 3–65% of the patients [43,44]. When serum CEA was measured preoperatively, only 33 of the 109 patients (30.3%) had an increase in CEA (≥5 ng/ml) [Table 4]. Of the 76 patients with CEA <5 ng/ml, 64 (84.2%) and 61 (80.3%) patients had EpCAM+-CTCs ≥ 23 cells/ml and PDPN+-CTCs ≥ 7 cells/ml, respectively. Combined analysis of serum CEA with EpCAM+-CTC and PDPN+-CTC count increased the positive detection rate of CRC to 89.0% (97 patients with EpCAM+-CTCs ≥ 23 and/or CEA ≥ 5) and 86.2% (94 patients with PDPN+-CTCs ≥ 7 cells/ml and/or CEA ≥ 5), respectively [Table 4]. Using EpCAM+-CTCs ≥ 23 as the cutoff, the positive detection rate among patients with CEA <5 were 92.8% (n = 13), 83.3% (n = 20), 78.8% (n = 26), 100% (n = 5) in the groups of stage I, II, III, and IV, respectively [Table 4]. When using PDPN+-CTCs ≥ 7 cells/ml as the cutoff, the positive detection rate among patients with CEA < 5 were 78.6% (n = 11), 79.2% (n = 20), 75.8% (n = 25), 100% (n = 5) in the groups of stage I, II, III, and IV, respectively [Table 4]. Regardless the clinical stage of patients, 84.2% and 80.3% of CRC patients with CEA < 5 ng/ml had the EpCAM+-CTC and PDPN+-CTC count above the cutoff, respectively. Combined analysis of CTC count and CEA is superior to CEA assay alone in disease detection of patients with CRC.

Table 3 Combined analysis of CTC count and iFOBT reduced false positive iFOBT results.

iFOBT+	EpCAM+ cells/ml		PDPN+ cells/ml	
	<23	≥23	<7	≥7
	No. of case (%)[a]		No. of case (%)[a]	
Non-cancerous controls (n = 36)	24 (66.6%)	12 (33.4%)	21 (58.3%)	15 (41.7%)
CRC patients (n = 28)	4 (14.3%)	24 (85.7%)	6 (21.4%)	22 (78.6%)
Total	28 (43.8%)	36 (52.2%)	27 (42.2%)	37 (57.8%)
p value[b]	<0.0001		<0.0001	

[a] Data represent the number of cases and the percentage of individuals at the indicated category.
[b] Statistical analysis was performed by Person Chi–Square test.

Table 4 Combined analysis of CTC count and serum CEA facilitates disease monitoring of patients with CRC.

CEA (ng/ml)	EpCAM+-CTCs (cells/ml)		PDPN+-CTCs (cells/ml)	
	<23	≥23	<7	≥7
	No. of cases (%)[a]		No. of cases (%)[a]	
<5 (n = 76)	12 (15.8%)	64 (84.2%)	15 (19.7%)	61 (80.3%)
Stage I (n = 14)	1 (7.2%)	13 (92.8%)	3 (21.4%)	11 (78.6%)
Stage II (n = 24)	4 (16.7%)	20 (83.3%)	4 (20.8%)	20 (79.2%)
Stage III (n = 33)	7 (21.2%)	26 (78.8%)	8 (24.2%)	25 (75.8%)
Stage IV (n = 5)	0 (0.0%)	5 (100%)	0 (0.0%)	5 (100%)
≥5 (n = 33)	4 (12.1%)	29 (87.9%)	6 (18.2%)	27 (81.8%)
Stage I (n = 2)	0 (0.0%)	2 (100%)	0 (0.0%)	2 (100%)
Stage II (n = 12)	1 (8.3%)	11 (91.7%)	1 (8.3%)	11 (91.7%)
Stage III (n = 14)	3 (21.4%)	11 (78.6%)	4 (28.6%)	10 (71.4%)
Stage IV (n = 5)	0 (0%)	5 (100%)	1 (20.0%)	4 (80.0%)
Total (n = 109)	16 (14.7%)	93 (85.3%)	21 (19.3%)	88 (80.7%)

[a] Data represent the number of cases and the percentage of patients with the indicated CEA results or clinical stages.

Discussion

Peripheral blood is an important biological resource for screening and disease detection of patients with cancer. In this study, CTCs expressing EpCAM or PDPN were defined to present in the early and late stages of CRC patients. By CTC testing alone, both $EpCAM^{+}$-CTC and $PDPN^{+}$-CTC count clearly distinguishes patients with CRC from non-cancerous control. CTC enumeration when combined analysis with iFOBT results significantly reduced the false positive rate of iFOBT, while when combined analysis with serum CEA improved the detection rate of patients with CRC. This study thereby paves the way for implementation of CTC testing in screening and disease detection of patients with CRC.

CTCs are composed of heterogeneous populations with distinct cellular properties [17,45,46]. Different subsets of CTCs have distinct impacts on disease progression of patients. For example, the number of CTCs expressing $CD133^{+}CD54^{+}CD44^{+}$ correlates with liver metastasis in patients with CRC [47]. We compared and analyzed CTC subsets with EpCAM or PDPN expression. PDPN is frequently up-regulated in squamous cell carcinoma, thyroid cancer, ovarian cancer, germinal neoplasia, and central nervous system tumors with increased motility and metastasis [48–53]. PDPN causes cancer-associated thrombosis and cancer progression in human and animal models [28,49]. $PDPN^{+}$ cells are present in the peripheral blood of patients with CRC. Not all $PDPN^{+}$ cells were $EpCAM^{+}$ (data not shown). This is consistent with the finding that PDPN is present in both cancer cells and cancer-associated fibroblasts in the stroma surrounding CRC tumor tissue [54–58]. Pericryptal stromal cells expressing PDPN is associated with epithelial tumor development in the colorectum [59]. The biology and the pathological significances related to PDPN expression in the peripheral blood of patients with CRC are worthy to further investigation.

CTCs are detectable not only when cancer cells metastasize [60,61], but also at the early stage of cancer when metastases are not evident [62–64]. CTCs have been found in 69% of patients with the T1/T2 stage of breast cancer [65]. Both early- and late-stage lung cancer CTCs have been reported when using stringent morphologic inclusion criteria for characterizing high definition CTCs (HD-CTCs) [66]. CTCs were detected in the early stage of CRC patients and had prognostic value in non-metastatic CRC [67,68]. The number of CTCs is correlated with tumor mass, with a higher number of CTCs in the late stage of cancer and a lesser number of CTCs in the early stage of cancer [69]. In this study, the median CTC count was not different among CRC patients with disease status of stage I to stage IV. The finding is consistent with a large cohort study of CRC [68]. No difference for the HD-CTCs counts among stages IV, III, and I/II of patients with non-small cell lung cancer has also been reported [66]. Whether the methods to enrich CTCs, the different time points of blood collection, and the differences in clinico-pathological characteristics of the patients account for the different findings among these studies requires further investigation. We noted that the $EpCAM^{+}$-CTCs and $PDPN^{+}$-CTCs counts at stage III is lower when compared to the other stages. This is likely related to the occurrence of EMT during cancer progression [16]. The slight increase in the CTC count at stage IV when compared to stage III may reflect the balance of increased release of CTCs and the occurrence of EMT. The use of additional markers which are related to cancer progression or EMT may further improve late stage cancer detection with CTC counting.

CRC screening is crucial in effective management of cancer patients. Most current methods do not provide sufficient sensitivity. The iFOBT is a relatively simple method for CRC screening. However, it usually associates with high false positive rate [70]. Although colonoscopy is the gold standard in the initial diagnosis of CRC following iFOBT, not all individuals are comfortable with this invasive procedure. In this study, analysis of CTC count and iFOBT data obtained from healthy controls and patients prior to surgical operation revealed that CTC testing is able to reduce the false positive rate of iFOBT. Because CTC testing requires only one blood draw from the individual, implementation of CTCs testing into the surveillance program of CRC for combined analysis with iFOBT most likely leads to better and practical screening program.

Detection of disease status is important for management of patients with CRC. Serum biomarkers such as CEA are commonly used in the clinical setting. With about 30% patients have no elevated serum CEA, there is an unmet need for additional modalities to follow up treatment response and the occurrence of recurrence. CTC enumeration compromises serum thyroglobulin (Tg) testing when the anti-Tg antibody was present in patients with PTC [71]. Combined analysis of CTC count and serum Tg testing is superior to serum Tg testing alone in establishing the disease status of patients [71]. Supplement of standard medical tests by sequential CTC enumeration during therapy can benefit the management of patients who have locally advanced or metastatic HCC but with low AFP expression [72]. This concept was applied in the current study to investigate whether CTC testing can supplement serum CEA assay to increase disease detection rate in patients with CRC. We demonstrated in this study that 85% of CRC patients had elevated CTC count and/or serum CEA, while only 30% of patients in our cohort including 12.5% ($n = 2$) of stage I, 33.3% ($n = 12$) of stage II, 29.9% ($n = 14$) of stage III, and 50% ($n = 5$) of stage IV (Table 4) had CEA ≥ 5. Simultaneous testing of CTC and CEA thereby increases positive disease detection rate for CRC patients who are under or have completed their course of treatment, such as surgery, chemotherapy, radiotherapy and targeted therapy.

Conclusions

CTCs are heterogeneous and distinct subsets of CTCs express PDPN or EpCAM. These cells were found in patients with CRC. CTC testing is an assay that could be more frequently performed when compared to the standard methods for screening and disease detection of patients with CRC. The findings of the current study imply that CTC testing can supplement iFOBT or serum CEA measurement and may benefit clinical management of patients with CRC, in particular for CRC screening and for disease detection of patients without elevated serum CEA.

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Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bj.2020.09.006.

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