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Article *in* Clinica Chimica Acta · June 2018

DOI: 10.1016/j.cca.2018.06.034

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Improvement of sensitive and specific detection of circulating tumor cells using negative enrichment and immunostaining-FISH

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ARTICLE INFO

Keywords:
Circulating tumor cells (CTCs)
Negative enrichment-fluorescence in situ hybridization (NE-FISH)
Esophageal cancer
Lung cancer
Gastric cancer
Breast cancer

ABSTRACT

Background: Circulating tumor cells (CTCs) provide an opportunity to obtain pivotal biological information required for the development of personalized medicine. However, the current assays of CTCs' detection face serious challenges regarding specificity and sensitivity.

Methods: In this study, we developed a novel strategy that combined negative enrichment (NE), immunocytochemistry CD45 staining and fluorescence in situ hybridization (FISH) to identify, enumerate and characterize CTCs. CTCs were identified as DAPI+/CD45-/Chromosome multiploid. The assay was evaluated with different cancer cell lines including lung, breast, esophageal and gastric cancer. And then, the developed assay was applied in cancer patients to explore the possibility of clinical application and whether CTC number was related to clinicopathological factors.

Results: The average recover rate of esophageal cancer cell line Eca-109 using negative enrichment was higher than 80% and the multiploid cells rate of four cancer cell lines were > 96%, which demonstrate the NE-FISH platform is favorable for CTCs detection. CTCs count was significantly higher in lung cancer patients than healthy controls and benign lung disease with an area under ROC curve of 0.905 (95% confidence interval 0.866–0.944, $P < .001$). Using a cutoff value of 2 CTCs, the positive rate of detecting lung, gastric, breast and esophageal cancer patients were 71.33%, 86.21%, 76.77% and 78.35%, respectively. Besides, CTCs could be detected in stage I with the positive rate of 64.15% for lung cancer, 83.33% for gastric cancer, 78.95% for breast cancer and 68.18% for esophageal cancer, which may promote the early diagnose and influence the treatment decision for better management of those cancer in clinic.

Conclusions: Our study showed that CTCs could be detected in diverse cancers using the novel NE-FISH platform with high sensitivity and specificity. Therefore, analysis of CTCs with NE-FISH has a clear potential to improve the management of cancer patients in clinical use.

1. Introduction

With the population growth, ageing and sociodemographic changes, cancer became the leading cause of death and major public health problem in China since 2010 [1]. Lung, breast, gastric and esophageal cancer were commonly diagnosed, and those cancer were identified as leading causes of cancer death. Most cancer-related deaths are caused by metastasis, the dissemination of cancer cells from the primary tumor through the circulatory system to new organ sites [2]. These cells include circulating tumor cells (CTCs) in the bloodstream and disseminated tumor cells in the bone marrow [3].

Even early in the formation and growth of a primary tumor,

Abbreviation: CTCs, circulating tumor cells; NE, negative enrichment; EpCAM, epithelial cell adhesion molecule; CKs, cytokeratins; EMT, epithelial-to-mesenchymal transition; FISH, fluorescence in situ hybridization; SSC, saline sodium citrate; ROC, receiver operating characteristics; CD45, cluster of differentiation 45; DAPI, 4′,6-diamidino −2-phenylindole; CEP, chromosome enumeration probes; AUC, area under the curve; CI, confidence interval

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https://doi.org/10.1016/j.cca.2018.06.034
Received 22 January 2018; Received in revised form 21 June 2018; Accepted 21 June 2018
Available online 22 June 2018
0009-8981/

especially in breast cancer [4, 5], CTCs are released into the bloodstream and lymphatic system to target distant organs and develop metastatic tumors, which cause over 90% of cancer deaths despite surgical resection and adjuvant therapy [6]. The role of CTCs within metastasis biology has been explored, and subgroup of CTCs with tumor-initiating capacity has also been identified [7, 8]. Most significantly, as single cell technologies evolve [9, 10], DNA and RNA profiles of CTCs will allow precise measurements of heterogeneity between individual cells and matched biopsy specimens [10, 11]. Therefore, if we could identify, count and extract information from these CTCs, we might be able to detect the cancer early, determine its aggressiveness, monitor and guide therapy in cancer patients [12].

Because of the discontinuous and heterogeneous shedding of primary tumor cells into the blood stream, CTCs may represent only 1 cell among 10^6 peripheral blood mononuclear cells [13], which poses a serious challenge for any analytical system. These CTCs can be enriched and detected via different technologies that take advantage of their physical properties, such as size, density [14–16], or biologic properties, such as expression of protein markers [17]. However, the current assays of CTCs' detection face serious challenges regarding specificity and sensitivity.

Over the past decade and in most of the current assays, CTCs have been detected through the use of epithelial markers such as epithelial cell adhesion molecule (EpCAM) and cytokeratins (CKs) that are not expressed on the surrounding mesenchymal blood cells [18, 19]. However, epithelial tumor cells may lose both EpCAM and CKs during epithelial-to-mesenchymal transition (EMT) which restrict clinical application of current strategy to detect CTCs from many types of solid tumors [20, 21]. It is therefore imperative to develop a non-EpCAM-based strategy for effective detection of the full spectrum of heterogeneous CTCs. Recently, a detection platform integrated EpCAM independent subtraction and immunostaining-fluorescence in situ hybridization based on a centromere probe for chromosome 8 (CEP 8), as aneuploidy is the most common characteristic of human solid tumors [22, 23], has been developed for detection of CTCs in many cancer types [24–28]. However, the identification of CTCs with multiple centromere of chromosome probes are still needed since using CEP 8 alone would increase the loss of CTCs count [29–31]. Therefore, CTCs' detection derived from this EpCAM-independent technique still need improvement in terms of sensitivity and specificity.

In this study, we developed a novel strategy that combined negative enrichment (NE), immunocytochemistry CD45 staining and fluorescence in situ hybridization (FISH) to increase sensitivity and specificity of CTCs detection. In addition, the developed assay was applied in many cancer patients including lung cancer, gastric cancer, breast cancer and esophageal cancer, and then explored the possibility of clinical application and whether CTC number was related to clinicopathological factors.

2. Materials and methods

2.1. Study patients

712 donors who were treated as in- and outpatients at Liaocheng People's Hospital (Liaocheng, Shandong, China) from January 2016 to May 2018 were enrolled in this study. There were 182 control individuals, including 34 healthy donors and 28 patients with benign lung diseases, 26 healthy donors and 29 patients with benign gastric diseases, 25 healthy donors and 20 patients with benign esophageal diseases, 20 healthy donors for breast cancer. There were 150 patients with lung cancer, 87 patients with gastric cancer, 194 patients with esophageal cancer and 99 patients with breast cancer (the complete information please see in Supplementary material 1). Those control individuals were age and gender-matched to the cancer patients and there were no statistical significances between them (Table S1 in Supplementary material 2). All the cancer were confirmed by histopathological diagnosis. Benign diseases patients were diagnosed by imaging, serum tests and histopathology. All those patients were first-time visitors and received no therapeutic treatment before hospitalization. The written informed consent forms were received from patients prior to inclusion in the study. The study was approved by Liaocheng People's Hospital and was performed according to the Declaration of Helsinki principles.

2.2. Blood sample collection and processing

Peripheral blood samples (3.2 mL) were collected from each patient in a BD Vacutainer tube (Becton, Dickinson and a Company, Franklin, NJ) and kept at room temperature. All samples were processed within 24 h.

3.2 mL of patient blood was washed once with CS1 buffer (Cyttel Biosciences INC., Jiangsu, China), then centrifuged at 650 $\times g$ for 5 min at room temperature. The red blood cells were lysed with CS2 buffer (Cyttel Biosciences INC., Jiangsu, China) for 8 min. After that, the reaction mixture was spun down at 650 g for 5 min. *Re*-suspended the resulting cell pellet in CS1 and incubated with immunomagnetic particles conjugated to anti-leukocytes monoclonal antibodies (anti-CD45, Cyttel) at room temperature for 20 min with gentle shaking. Placed those mixture on top of a special type of gradient centrifugated liquid CS3 (Cyttel) and separated by gradient centrifugation at 300 $\times g$ for 5 min. Sedimented cells were thoroughly mixed with cell fixative and smeared on one slide (Thermo Fisher Scientific, Franklin, 119 MA, USA), fixed and dried for subsequent analysis.

CTCs were fixed on slides with CF1 (Cyttel Biosciences INC. Jiangsu, China). Slides were soaked in 2 × saline sodium citrate (SSC) at 37 °C for 10 min and dehydrated in series of 75%, 85% and 100% ethanol for 2 min for each concentration. Samples were subsequently subjected fluorescence in situ hybridization (FISH) with centromere probe (CEP) 8 (orange, Cyttel) for lung cancer and control group [32, 33], 8 + 7 (orange + green) for esophageal cancer and control group [34], 8 + 17 (orange + green) for breast, gastric cancer and control groups [35–37] (https://www.cgap.nci.nih.gov/Chromosomes/Mitelman) using a ThermoBrite® Slide Hybridization/Denaturation System. Then soaked the slides in formamide for 15 min and incubated with 2 × SSC twice for 5 min each time. Samples were subsequently subjected to immunostaining with Alexa Fluor 594 conjugated anti-human CD45 (Cyttel) for 1 h in the dark, followed by washing and mounting the slides. The slides were mounted with mounting media containing DAPI (Vector Laboratories, Burlingame, CA, USA).

The area of the slides should be entirely scanned along “S” track with a microscope (BX63, Olympus). And image analyses was carried out using an IMSTAR high content screening (HCS) device equipped with the Pathfinder™ software (IMSTAR S.A., Paris, France). CTCs were identified as DAPI + /CD45-/Chromosome multiploid (Fig. 1).

2.3. Spiking study

1, 5, 10, 20 and 30 cells from Eca-109 esophageal cancer cell lines pre-labeled with Mito-Tracker Green (Beyotime Biotechnology, China) were counted under fluorescence microscope and added to 3.2 mL blood of healthy donors. CTCs were then enriched following our negative enrichment method. The recovery rate, presented as the mean values ± the standard deviations obtained from three independent experiments, was calculated as the ratio of recovered cell numbers to spiked cell numbers.

Lung cancer cell line A549, esophageal cancer cell line Eca-109, breast cancer cell line MCF-7 and gastric cancer cell line SGC-7901 were obtained from Cell Bank of the Chinese Academy of Sciences and cultured as previously described [38, 39]. For quantification the multiploid of those cell lines, approximate 200 cells were applied onto the coated CTC slides to have a monolayer, followed by immunocytochemistry CD45 staining and FISH. The sensitivity of FISH

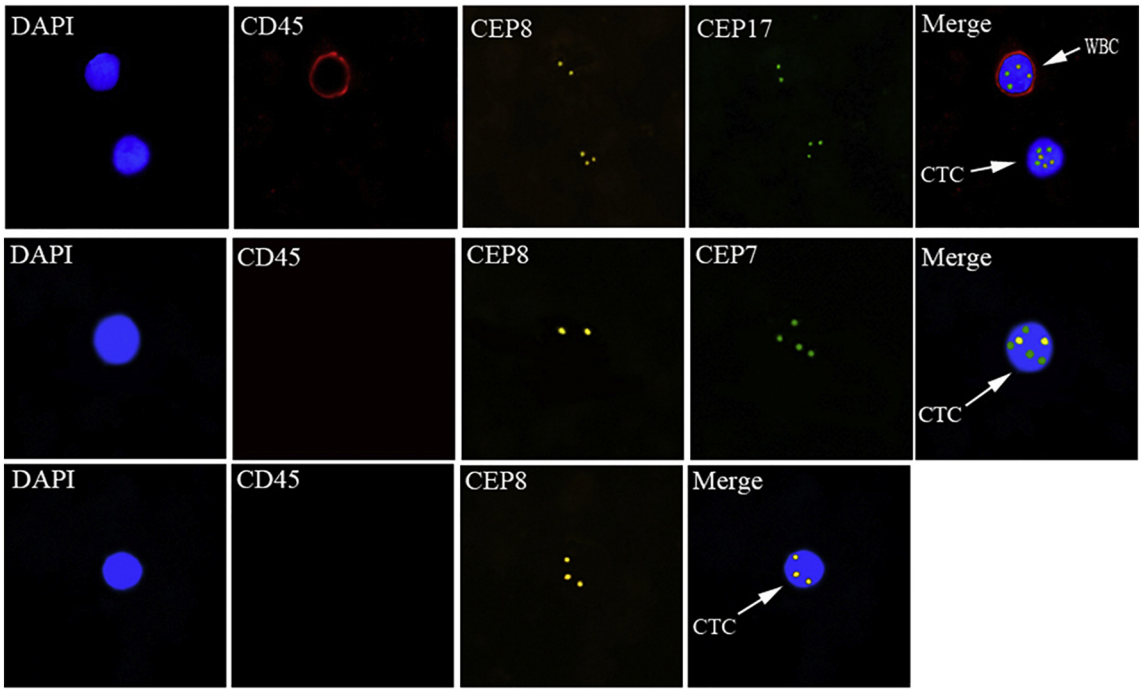

Fig. 1. Identification of CTCs by NE-FISH. CD45: red; DAPI: blue; CEP8: orange; CEP 7 and CEP 17: green. DAPI: (4′, 6-diamidino-2-phenylindole); CD45: cluster of differentiation 45; CEP: Centromere Probe; WBC: White blood cells; CTC: Circulating tumor cell. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was calculated as the rate of tumor cells judged as CTCs according to CTCs' identification criteria by immunocytochemistry CD45 staining and FISH.

2.4. Statistical analyses

The statistical analyses were performed using SPSS for windows (SPSS version 17.0, SPSS Inc., Chicago, IL). The sensitivity and specificity of CTCs in diagnosis of lung cancer were analyzed by the receiver operating characteristics (ROC) curve. Graph was mapped by the GraphPad Prism 5 (GrapPad Software, La Jolla, CA, USA). $P < .05$ was considered statistically significant, and all P values were two-sided.

3. Results

3.1. Spiking study and CTCs' identification

The spiking study showed that the recovery rate of esophageal cancer cell line Eca-109 (1, 5, 10, 20 and 30 cells) enriched by our negative enrichment was > 80% in Table 1. The multiploid cells rate of four cancer cell lines (Eca-1009, A549, MCF-7 and SGC-7901) were > 96% (Fig. 2), which is accordance with the previous studies that aneuploidy is the most common characteristic of human solid tumors [22, 23].

The immunostaining markers used for distinguishing different circulating tumor cells were cluster of differentiation 45 (CD45), 4′,6-diamidino-2-phenylindole (DAPI), chromosome enumeration probes 8 (CEP 8) for lung cancer, chromosome enumeration probes 8 + 7 (CEP 8 + 7) for esophageal cancer, chromosome enumeration probes 8 + 17 (CEP 8 + 17) for breast and gastric cancer. White blood cells were stained with CD45+, DAPI+, but CTCs were stained with CD45-, DAPI + due to the specific marker on the surface of white blood cells. Therefore, CTCs were identified as DAPI+/CD45-/Chromosome multiploid (CEP 8+ and/or CEP7+, CEP17+) (Fig. 1).

Table 1
The recovery rate study of negative enrichment (NE).

Spiked cell number	Recovered	Recovery rate (mean ± SD)[a]
1	1	67% ± 0.58
	0	
	1	
5	5	87% ± 0.12
	4	
	4	
10	9	83% ± 0.06
	8	
	8	
20	19	85% ± 0.09
	16	
	16	
30	23	82% ± 0.05
	25	
	26	
Average	\	81% ± 0.08

[a] Recovery rate = recovered cell number/spiked cell number. All data are expressed as mean ± SD from three separate determinations.

3.2. CTCs in cancer patients and controls

Among lung cancer control group, including 34 healthy donors and 28 patients with benign lung diseases, the number of CTCs was 0–1 cells/3.2 mL blood (median number, 0 cells/3.2 mL). CTC were detected in 8 healthy donors (23.53%, with the median of 0, range from 0 to 1), 10 lung benign disease donors (35.71%, with the median of 0, range from 0 to 1) and 134 lung cancer patients (89.33%, with the median of 2, range from 0 to 59). The difference between lung cancer patients and the control group was statistically significant ($P < .001$, Fig. 3A). There were more CTCs in lung cancer patients than lung benign disease patients and healthy people. In order to discriminate lung cancer patients from healthy and benign disease, receiver operator characteristic (ROC) curve (Fig. 3B) was used to reveal the sensitivity and specificity, which was 71.3% and 100%, respectively. According to

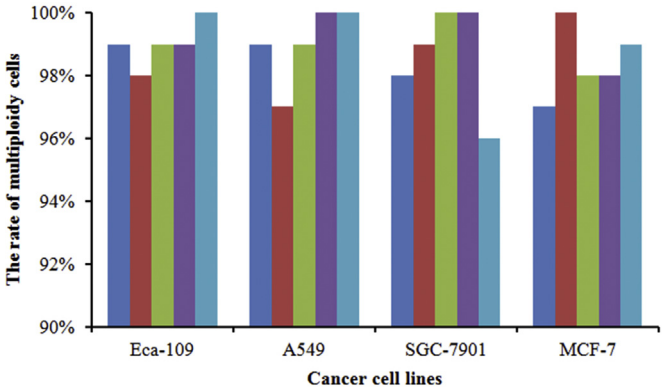

Fig. 2. The rate of multiploid cells recovered by immunocytochemistry CD45 staining and FISH. Cancer cell lines was shown on X-axis, the rate of multiploid cells recovered by immunocytochemistry CD45 staining and FISH was shown on Y-axis.

Yourdon's index, CTCs could be a biomarker to help diagnosing lung cancer when the cut-off value was decided as 1.5 CTCs/3.2 mL (AUC = 0.905, 95% CI 0.866–0.944, $P < .001$) (AUC: Area Under roc Curve; CI: Confidence Interval). Cutoff values of 1 CTCs/3.2 mL and 2 CTCs/3.2 mL yielded sensitivities of 89.33%, 71.33% and specificities of 70.97%, 100%. Therefore, we defined the cutoff values as two CTCs in lung cancer. Use the same method, we also defined two CTCs as the cutoff values in gastric, breast and esophageal cancer (Figs. S1–S3 in Supplementary material 2).

3.3. Correlation of CTCs with clinicopathological characteristics in lung cancer, gastric cancer, esophageal cancer and breast cancer patients

The median number of CTCs detected in lung cancer patients were 2 (range 0–59) and 3 (range 0–30) in stage I-II and stage III-IV, respectively ($P = .605$; Fig. 3C). And ≥2 CTCs per 3.2 mL were detected in 71.33% of patients. Examination of correlation between CTC numbers and various clinicopathological characteristics is summarized in Table 2. Patients with adenocarcinoma and squamous type cancer had a CTC positivity (≥2 per 3.2 mL) of 69.89% and 73.68%, respectively. There were no significant differences in clinicopathological characteristics with different CTC counts. We found that 71.33% of lung cancer patients had detectable CTCs, which was slightly lower than the previous studies [30, 31]. This finding could be related to the early stage I enrolled lung patients and intra-tumor heterogeneity.

The median number of CTCs detected in gastric cancer patients were 3 (range 0–16) and 3 (range 0–66) in stage I-II and stage III-IV, respectively ($P = .560$; Fig. 3D). And ≥2 CTCs per 3.2 mL were detected in 86.21% of patients. Examination of correlation between CTC numbers and various clinicopathological characteristics is summarized in Table 3. Patients with stomach and cardia cancer had a CTC positivity (≥2 per 3.2 mL) of 79.17% and 88.89%, respectively. In our study, we detected CTC count is significant differences to gender ($P = .006$). No obvious correlation was found in age, primary tumor site, distant metastasis, tumor depth, lymph node metastasis and TNM stage.

The median number of CTCs detected in breast cancer patients were 3 (range 0–19) and 3 (range 0–22) in stage I-II and stage III-IV, respectively ($P = .359$; Fig. 3E). And ≥2 CTCs per 3.2 mL were detected in 76.77% of patients. Examination of correlation between CTC

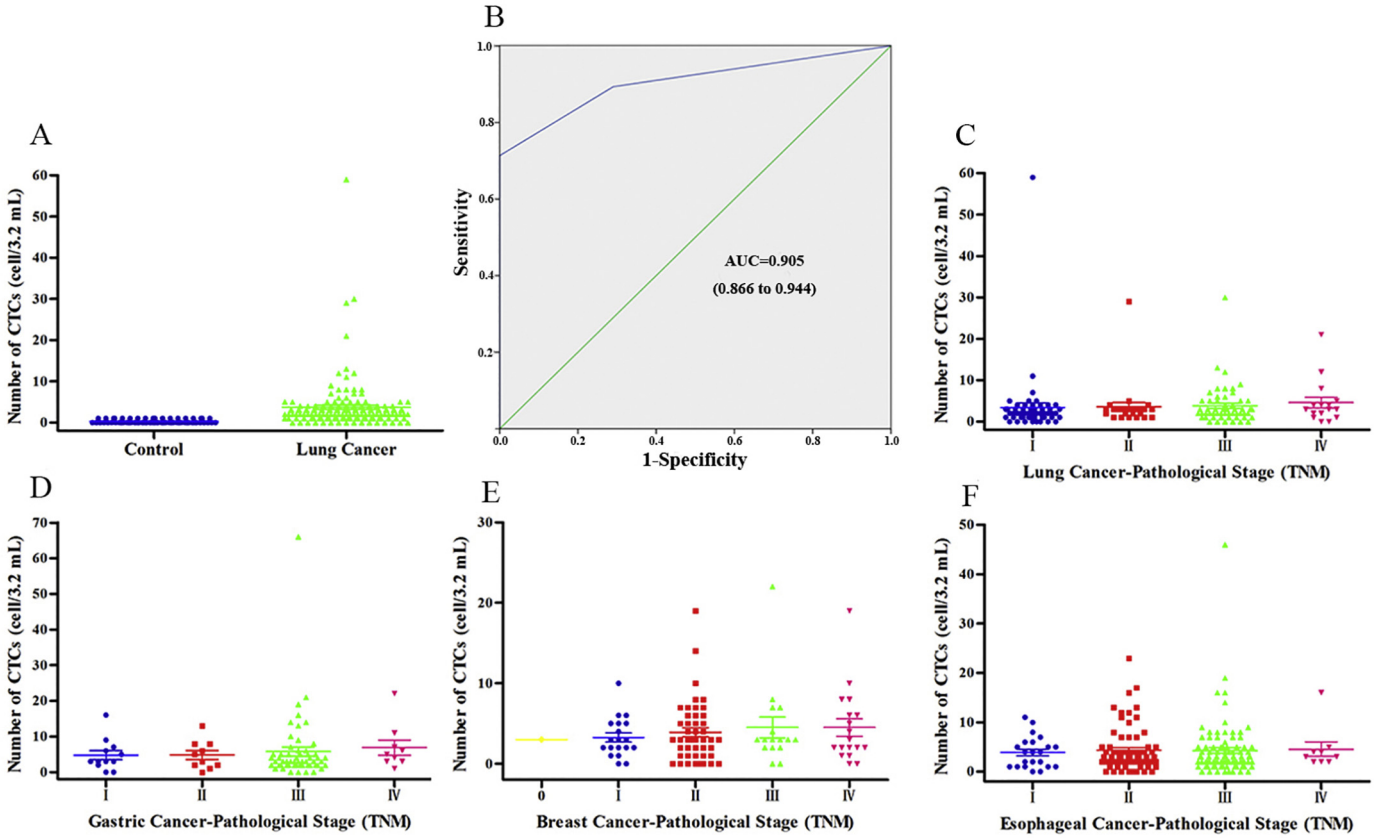

Fig. 3. CTC count in lung, gastric, breast, esophageal cancer patients and controls. (A) Distribution of CTCs in controls and lung cancer. Number of CTCs in 62 controls (including 34 healthy donors and 28 patients with benign lung diseases) and 150 lung cancer patients were recorded. (B) ROC curves for CTCs count to discriminate lung cancer patients from controls and determine the cut-off value of CTCs. (C) Distribution of CTCs in patients with lung cancer according to pathological staging (TNM). (D) Distribution of CTCs in patients with gastric cancer according to pathological staging (TNM). (E) Distribution of CTCs in patients with breast cancer according to pathological staging (TNM). (F) Distribution of CTCs in patients with esophageal cancer according to pathological staging (TNM).

Table 2
Relationship of CTC with patient demographics and clinical characteristics in lung cancer.

Characteristics	*n*	Proportion (%)	CTC < 2		CTC ≥ 2		Z Statistics	*P*
			n	Proportion (%)	*n*	Proportion (%)		
Gender								
Male	101	67.33	27	26.73	74	73.27	0.318	0.751
Female	49	32.67	16	32.65	33	67.35		
Age								
≥60	98	65.33	26	26.53	72	73.47	−0.991	0.323
< 60	52	34.67	17	32.69	35	67.31		
Smoking History								
Yes	75	50	17	22.67	58	77.33	−0.542	0.589
No	75	50	26	34.67	49	65.33		
Histology								
Adenocarcinoma	93	62	28	30.11	65	69.89	−0.898	0.371
Squamous	57	38	15	26.32	42	73.68		
Tumor depth								
Tis-T1	56	37.33	21	37.50	35	62.50	1.43	0.236
T2	64	42.67	17	26.56	47	73.44		
T3	18	12	4	22.22	14	77.78		
T4	12	8	1	8.33	11	91.67		
Lymph node metastasis								
Yes	76	50.67	18	23.68	58	76.32	0.349	0.728
No	74	49.33	25	33.78	49	66.22		
TNM stage (UIUC)								
I	53	35.55	19	35.85	34	64.15	0.154	0.927
II	25	16.67	6	24.00	19	76.00		
III	56	37.33	14	25.00	42	75.00		
IV	16	10.67	4	25.00	12	75.00		

Table 3
Relationship of CTC with patient demographics and clinical characteristics in gastric cancer.

Characteristics	*n*	Proportion (%)	CTC < 2		CTC ≥2		Z Statistics	*P*
			n	Proportion (%)	*n*	Proportion (%)		
Gender								
Male	71	81.61	10	14.08	61	85.92	−2.811	0.006
Female	16	18.39	2	12.50	14	87.50		
Age								
≥60	68	78.16	10	14.71	58	85.29	−1.889	0.062
< 60	19	21.84	2	10.53	17	89.47		
Primary tumor site								
Stomach	24	27.59	5	20.83	19	79.17	0.428	0.670
Gastroesophageal junction	63	72.41	7	11.11	56	88.89		
Distant Metastasis								
M0	79	90.80	11	13.92	68	86.08	0.881	0.381
M1	8	9.20	1	12.50	7	87.50		
Tumor depth								
Tis-T1	3	3.45	0	0	3	100	0.386	0.763
T2	8	9.20	2	25	6	75.00		
T3	39	44.83	5	12.82	34	87.18		
T4	37	42.53	5	13.51	32	86.49		
Lymph node metastasis								
Yes	67	77.01	9	13.43	58	86.57	−0.342	0.733
No	20	22.99	3	15	17	85		
TNM stage (UIUC)								
I	12	13.79	2	16.67	10	83.33	0.159	0.924
II	10	11.49	2	20.00	8	80.00		
III	56	64.37	7	12.50	49	87.50		
IV	9	10.34	1	11.11	8	88.89		

numbers and various clinicopathological characteristics is summarized in Table 4. There were no significant differences in clinicopathological characteristics with different CTC counts.

The median number of CTCs detected in esophageal cancer patients were 3 (range 0–23) and 3 (range 0–46) in stage I-II and stage III-IV, respectively (P = .933; Fig. 3F). And ≥ 2 CTCs per 3.2 mL were detected in 78.35% of patients. Examination of correlation between CTC numbers and various clinicopathological characteristics is summarized in Table 5. There were no significant differences in clinicopathological characteristics with different CTC counts.

4. Discussion

In this study, we employed an improved enrichment strategy which was EpCAM-independent followed by a novel strategy that combined immunocytochemistry CD45 staining and FISH to increase CTCs recovery efficiency. Using this strategy, only 3.2 mL of peripheral blood was used for CTC analysis, while CTC analysis normally used 7.5 mL peripheral blood in the previous studies [17, 24, 26, 28]. In addition, negative enrichment step only takes 2 h and immunostaining-FISH will be done in 4 h. Identification of CTCs were carried out by an IMSTAR

Table 4
Relationship of CTC with patient demographics and clinical characteristics in breast cancer.

Characteristics	*n*	Proportion (%)	CTC < 2		CTC ≥2		Z Statistics	*P*
			n	Proportion (%)	*n*	Proportion (%)		
Gender								
Male	0	0	0	0	0	0		
Female	99	100	23	23.23	76	76.77		
Age								
≥60	11	11.11	1	9.09	10	90.91	1.452	0.150
< 60	88	88.89	22	25.00	66	75.00		
Distant Metastasis								
M0	82	82.83	19	23.17	63	76.83	−0.759	0.450
M1	17	17.17	4	23.53	13	76.37		
Tumor depth								
Tx	3	3.03	1	33.33	2	66.67	1.056	0.390
Tis	3	3.03	1	33.33	2	66.67		
T1	39	39.39	10	25.64	29	74.36		
T2	45	45.45	11	24.44	34	75.56		
T3	3	3.03	0	0	3	100		
T4	6	6.06	0	0	6	100		
Lymph node metastasis								
Yes	55	55.56	11	20	44	80.00	−1.083	0.281
No	44	44.44	12	27.27	32	72.73		
TNM stage (UIUC)								
0	1	1.01	0	0	1	100	0.310	0.871
I	19	19.19	4	21.05	15	78.95		
II	45	45.45	13	28.89	32	71.11		
III	16	16.16	2	12.50	14	87.50		
IV	18	18.18	4	22.22	14	77.78		

HCS device within 1 h for 4 slides.

The developed CTCs' detection assay was validated with spiked in different number of esophageal cancer line Eca-109. The average recover rate was higher than 80% (Table 1), which show negative enrichment strategy is favorable for CTCs enrichment. Except high recover rate of CTCs, negative enrichment can also keep cell viability after recovery. Therefore, CTCs could be isolated via negative enrichment and grown in culture for the establishment of a cell line to examine tumor heterogeneity. Aneuploidy is the most common characteristic of human solid tumors [22, 23] and is mostly caused by chromosomal instability. In our study, we found the rate of multiploid cells in different type of cancer cells were > 96% (Fig. 2), which demonstrate that immunofluorescence and in situ chromosomal hybridization (FISH) to character CTCs is feasible. And then, the developed assay was evaluated in cancer patients. Using a cutoff value of 2 CTCs, the positive rate of detecting lung cancer, gastric cancer, breast cancer and esophageal cancer patients were 71.33%, 86.21%, 76.77% and 78.35% (Tables 2–5), respectively. CTCs count is not related to age, tumor depth, lymph node metastasis and TNM stage. Obviously, the association of CTC enumeration with clinicopathological parameters needs further confirmation in a larger sample size of patients. However, the developed assay can obtain much higher CTCs positive rate than the previous studies in lung cancer [40, 41], breast cancer [17, 42], esophageal cancer [43, 44] and gastric cancer [45, 46] that based on EpCAM-dependent method.

In addition, CTCs could be detected in stageIwith the positive rate of 64.15% for lung cancer, 83.33% for gastric cancer, 78.95% for breast cancer and 68.18% for esophageal cancer, while most imaging modalities failed to detected cancer lesions smaller than 0.5 cm. This may promote the early diagnose and influence the treatment decision for

Table 5
Relationship of CTC with patient demographics and clinical characteristics in esophageal cancer.

Characteristics	*n*	Proportion (%)	CTC < 2		CTC ≥2		Z Statistics	*P*
			n	Proportion (%)	*n*	Proportion (%)		
Gender								
Male	166	85.57	38	22.89	128	77.11	−0.380	0.705
Female	28	14.43	4	14.29	24	85.71		
Age								
≥60	151	77.84	34	22.52	117	77.48	1.543	0.125
< 60	43	22.16	8	18.60	35	81.40		
Tumor depth								
Tis-T1	27	13.92	7	25.93	20	74.07	0.601	0.615
T2	51	26.29	13	25.49	38	74.51		
T3	109	56.19	22	20.18	87	79.82		
T4	7	3.61	0	0	7	100		
Lymph node metastasis								
Yes	106	54.64	21	19.81	85	80.19	−0.102	0.919
No	88	45.36	21	23.86	67	76.14		
TNM stage (UIUC)								
I	22	11.34	7	31.82	15	68.18	0.055	0.983
II	71	36.60	14	19.72	57	80.28		
III	92	47.42	21	22.83	71	77.17		
IV	9	4.64	0	0	9	100		

better management of those cancer in clinic.

In summary, The NE-FISH platform could improve the detection rate of CTCs in lung cancer, gastric cancer, breast cancer and esophageal cancer patients with lower volume of peripheral blood, shorter enrichment and identification time. Although the developed assay need to be evaluated in more patient cohort, the method has the potential to apply in routine clinical test.

5. Conclusions

CTCs could be a valuable, non-invasive surrogate marker to aid in treatment selection for patients. However, the translation of CTCs to a routine clinical use is impeded by the current different technologies for CTC isolation and the lack of validation and qualification of assays. Our study showed that CTCs could be detected in lung, breast, gastric and esophageal cancer using the novel NE-FISH platform with high sensitivity and specificity using the cutoff value of 2 CTCs in 3.2 mL of blood sample. Therefore, analysis of CTCs with NE-FISH in the peripheral blood has a clear potential to improve the management of cancer patients and gives us more insights into understanding of the biology of tumor cell dissemination.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This work was financially supported by the China Postdoctoral Science Foundation (2015M580595), Special Funds for Postdoctoral Innovative Projects of Shandong Province (140222), Shandong Natural Science Foundation of China (ZR2017LH050, ZR2015HQ014 and ZR2016HB73) and National Natural Science Foundation of China (NSFC81602736).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.06.034.

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