Clinica Chimica Acta 485 (2018) 95–102

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

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