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# Highly sensitive detection and mutational analysis of lung cancer circulating tumor cells using integrated combined immunomagnetic beads with a droplet digital PCR chip

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ABSTRACT

Circulating tumor cells (CTCs) have become an important biomarker for liquid biopsy to monitor tumor progression and indicate response to therapies. Many epithelial cellular adhesion molecule (EpCAM) dependent CTC isolation methods have been developed, which have a limitation for low EpCAM expressed tumor cells. In an effort to overcome these drawbacks, we developed combined immunomagnetic beads (EpCAM, Mucin1 and epidermal growth factor receptor) to sensitively isolate CTCs for immunofluorescence analysis and genetic characterization. With this combined immunomagnetic beads, 93.35% H446 cells from spiked blood sample can be recovered. We were able to detect CTCs in 127 among 143 patients included in the study (88.8%). Some CTC clusters were captured with the combined magnetic beads system. In 17 of them, CTCs after chemotherapy significantly decreased compared to that before chemotherapy (4.42 ( ± 3.94) vs. 12 ( ± 7)/mL, $P = 0.002$). For subsequent genetic characterization of CTCs, 2 of 6 samples, using a droplet digital PCR (ddPCR) chip, have detectable EGFR L858R mutation in the cells enriched with the combined immunomagnetic beads. In conclusion, this method integrating the combined immunomagnetic beads and the ddPCR chip for CTCs detection can be of potential application in terms of diagnosis, therapeutic evaluation and personalized medicine in lung cancer.

## 1. Introduction

Metastasis, the spread of tumor cells, is the most reason leading to cancer-related death. During the metastasis, tumor cells, termed as circulating tumor cells (CTCs), have detached from a primary solid tumor to circulate in the bloodstream and reached another organ to form a secondary tumor. Therefore, CTCs are closely associated with tumor size, progress, metastasis, relapse and prognosis [1–4]. Analysis of CTCs in peripheral blood is important in individual treatment and prognosis of solid tumors. However, 1 mL of whole blood (containing around $10^9$ red blood cells and $10^6$ white blood cells) may contain only a few CTCs [5], which thus presents a great challenge for detection of CTCs.

Several CTC detection platforms have been constructed, such as reverse-transcription polymerase chain reaction (RT-PCR) [6–9], immune magnetic bead enrichment, density gradient centrifugation and microfluidic chip-based techniques. Some novel microfluidic chips are relied on the difference of physical properties between tumors cells and white blood cells [10,11]. The purity of the isolated CTCs may be low because of many big size leukocytes, which is a limitation for downstream molecule analysis [12]. While CTCs isolation platforms based on biological properties are specific with tumor cells and have a high purity [13]. These methods are mostly dependent on a common CTC marker, epithelial cell adhesion molecule (EpCAM). However, several reports showed that many malignant tumor cells in blood circulation may not express EpCAM in the process of epithelial to mesenchymal

*Abbreviations:* NSCLC, non-small cell lung cancer; SCC, lung squamous cell carcinoma; SCLC, small cell lung cancer; AC, adenocarcinoma; RECIST, response evaluation criteria in solid tumors

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transition (EMT) [14–16]. Hence, EpCAM based CTC detection may miss EpCAM-negative cells, which lead to low sensitivity. CellSearch platform (Janssen Diagnostics, LLC, USA) based on anti-EpCAM magnetic bead has been used as a tool for clinical utility in metastatic prostate, breast, and colorectal cancer. Several groups attempted to detect CTCs in lung cancers using the CellSearch platform,but found that the sensitivity was low [17–19]. Notably, Krebs et al. reported that two-thirds of non-small cell lung cancer (NSCLC) patients in stage IV had no detectable CTCs, and CTCs were detectable in less than 5% of stage III patients using this platform [19].

To overcome the low sensitivity of anti-EpCAM magnetic beads-based methods for lung cancer, we developed a combined EpCAM, MUC1 and EGFR immuno-magnetic beads method for lung cancer CTC detection. MUC1 serves a protective function by binding to pathogens [20] and also functions in cell signaling, which can prevent cell death [21]. Overexpression of MUC1 is often associated with colon, breast, ovarian, lung and pancreatic cancers [22,23]. MUC1 is currently used as an important biomarker for cancer diagnosis, prognosis and tumor vaccines [24,25]. Furthermore, EGFR overexpression due to gene mutation is ubiquitous in several cancers, especially lung cancer [26]. Therefore, it is also an important biomarker for targeted therapy of lung cancer.

In this context, we have applied this method in detecting three different lung cancer cell lines and CTCs from lung cancer patients. Except for enumeration, the combined immuno-magnetic beads also provide captured CTCs for downstream nucleic acid characterization of specific tumor mutations, which is valuable for guiding proper treatment selection. Digital PCR method has a higher sensitivity for quantitating rare nuclear acids compared with real-time quantitative PCR, which makes this detection method a proper tool for molecular analysis of CTCs [27,28]. We later show that this combined immuno-magnetic beads method integrated a droplet digital PCR (ddPCR) chip [29] can be exploited to detect EGFR mutations in tumor cells and lung cancer patient's CTCs. Furthermore, our data suggest that CTCs can indicate patients' response to chemotherapy efficiently, highlighting the potential clinical utility of this method.

## 2. Materials and methods

### 2.1. Patients and blood processing

One hundred and forty-three lung cancer patients and forty-two healthy participants were enrolled at the Affiliated Hospital of Nantong University from between November 2012 and December 2015. This study protocol was approved by the Ethics Review Committee of the Affiliated Hospital of Nantong University. Informed consents according to ethics board-approved study protocols were offered to all patients and healthy controls. Peripheral blood (2–4 mL) samples from patients were collected in blood collection tubes containing EDTA. Then the blood samples were washed with PBS, followed by lysis of red blood cells. The mixture was centrifuged at 2500 rpm for 10 min. The cell pellets were resuspended in PBS and subsequently detected within 24 h.

### 2.2. Cell culture and spiking

The A549 (derived from lung adenocarcinoma), SK-MES-1 (derived from lung squamous carcinoma) and H446 (derived from small cell lung cancer) cell lines were obtained from cell bank of Chinese Academy of Sciences. Prior to the experiment, cells were collected and resuspended at a known concentration in PBS or healthy human peripheral blood. Cells' concentration was measured by Count star automated cell counter (Inno-Alliance Biotech, USA) following the manufacturer's instructions. In the recovery assay, 200–500 tumor cells were spiked in 2 mL blood samples.

### 2.3. Preparation of immuno-magnetic beads

Magnetic beads (100 mg/mL, Invitrogen, Norway) were modified with anti-EpCAM (Ber-EP4, Invitrogen™), MUC1 (M4H2, 8.2 mg/mL, Life Science, USA), MUC1 (M2C5, 5 mg/mL, Life Science, USA) or EGFR antibody (F4,1 mg/mL, Abcam, Hong Kong) separately according to the manufacturer's protocol. Briefly, the carboxylic acid groups on the surface of magnetic beads (3 mg) were activated by the primary amino groups of 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide Hydrochloride (EDC) (150 μL, 50 mg/mL) and N-hydroxysuccinimide (NHS) (150 μL, 50 mg/mL) on a roller for 30 min at room temperature. After incubation, the activated beads were washed, added with 150 μg antibody and incubated over night at room temperature with slow rotation. The immuno-magnetic beads were finally reconstituted in 240 μL buffer (PBS containing 0.1% BSA and 0.05% Tween20) and stored at 4 °C.

### 2.4. Isolation using the combined immuno-magnetic beads and fluorescence staining of CTCs

2 mL processed blood samples were incubated with 40 μL mixture of 10 μL anti-EpCAM, -MUC1 (M4H2 and M2C5) and -EGFR antibodies modified magnetic beads (12.5 mg/mL) respectively. After 30 min incubation with gentle shaking on ice, the eppendorf tube was placed onto the magnet for 2–3 min. The mixtures of magnetic beads and cells were collected, incubated with 0.2% TritonX100 in PBS at 25 °C for 10 min to permeabilize cell membranes. Cells were further incubated with a mix of anti-cytokeratin-phycoerythrin antibody (CK-PE) ([C-11], Abcam, Hong Kong), anti-CD45-isothiocyanate antibody (CD45-FITC) (BD Biosciences, United States) and 4′6 Diamidino 2 phenylindole (DAPI) (Beyotime Biotechnology, China) at 37 °C for 40 min. Finally, cells were fixed with 4% paraformaldehyde in PBS. Cells were counted and scanned with an Olympus IX51 microscope linked with image analysis software (DP Controller). The cells that stained CK + /CD45 − / DAPI + and met the phenotypic morphological characteristics were scored as CTCs. CD45 + /CK − /DAPI + cells were considered as leukocytes.

### 2.5. Analysis of treatment responses

Seventeen paired before and after chemotherapy blood samples were collected for CTC analysis. Prior to the first cycle of chemotherapy and after the end of the treatment, 2 mL peripheral blood was collected. The treatment responses of patients who had distant metastasis were evaluated according to the response evaluation criteria in solid tumors (RECIST) standard. In a long-term study of individual patients, two additional patients with advanced lung cancer received sequential chemotherapy according to the severity of their respective conditions. Each patient received four courses of standard chemotherapy, and each course lasted for 3 weeks. CTC assessments were made after every course of chemotherapy.

### 2.6. EGFR mutational analysis with a droplet digital PCR chip

CTCs from blood samples were enriched by the combined immunomagnetic beads. Following enrichment, cells were lysed by proteinase K (Thermo Fisher Scientific, USA) at 65 °C for 1 h,and then incubated at 99 °C for 10 min. After that, the lysis and beads were placed onto the magnet for 2–3 min and the supernatants were collected for detecting EGFR mutational gene with a ddPCR chip (Fig. 1). The ddPCR chip shows a high sensitivity for nuclear detection, which is at the level of single molecule resolution. Fabrication and operation of this chip are modified based on the previous reports [28,29]. The details are listed in the Supplementary material.

Primers and TaqMan-MGB probes for EGFR mutational analysis were designed from the EGFR 21 exon gene sequence, using Primer