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A Novel System to Detect Circulating Tumor Cells Using Two Different Size-selective Microfilters

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Abstract. *Background/Aim: Clusters of circulating tumor cells (CTCs) increase metastatic potential compared to single CTC. However, conventional technologies have been unable to generate an accurate analysis of single and cluster CTCs in the peripheral blood. We propose an effective strategy to detect and isolate both single and cluster CTCs using two size-selective microfilters. Materials and Methods: Five ml of whole blood were collected from 10 patients with epidermal growth factor receptor mutation-positive non-small cell lung cancer. Single and cluster CTCs were identified using precision microfiltration membranes with two distinct pore sizes together with anti-EpCAM antibody labeling. Results: Single and cluster CTCs were detected by simultaneously using two size-selective microfilters. The EGFR-L858R mutation was detected in the DNA from cells captured using both microfilters. Conclusion: Our method can be used to detect and isolate single and cluster CTCs in the whole blood and may facilitate the development of a liquid biopsy strategy.*

In the United States alone, 228,150 new lung cancer patients were diagnosed in 2019, with a total of 147,510 deaths due to this disease (1). Furthermore, in 2016, lung cancer ranked first in cancer-associated mortality in Japan (2). The majority of cancer-related deaths result from disease progression due to distant metastases, and hematogenous spread has been identified as one of the major contributors to this complication. Current diagnostic procedures are largely invasive; they include bronchoscopy, computed tomography (CT)- or ultrasound-guided biopsies, and/or open surgical procedures. Secondary biopsies are frequently performed in order to evaluate resistance to drug therapy; all these invasive procedures are associated with complications (3, 4).

Circulating tumor cells (CTCs) are identified as malignant cells that originate from a primary tumor and circulate in the bloodstream; these cells typically migrate to a secondary site which is then seeded and may support growth of one or more metastatic tumors (5-7). The presence and number of CTCs may be directly involved with the intermediate stage of tumor metastasis and as such may have an impact on overall prognosis (8). Efforts have been made to identify and characterize CTCs as they represent a potential source of cells for the diagnosis and treatment of cancer via a "liquid biopsy" (9, 10). In the circulation, CTCs take on various forms, including singlets and cell clusters (11). Recent reports indicated that cluster CTCs are more likely than single CTCs to survive, proliferate, and be the source of metastatic diseases (12). As such, identification and analysis of both single and cluster CTCs may provide us with a more comprehensive picture of the metastatic process and likewise may advance our understanding and improve the management of metastatic disease. The CellSearch system (CellSearch™) is currently the only U.S. Food and Drug

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Administration (FDA)-approved device for detecting CTCs from patient blood samples (13-17). There are several published reports documenting successful monitoring of CTCs using the CellSearch™ system; this includes patients diagnosed with lung cancer (18, 19).

Unfortunately, conventional technologies are limited and are not able to provide a fully accurate analysis of a highly heterogeneous populations of CTCs. First, CTCs overall are very rare and represent a very small minority of cells detected in the peripheral circulation. Cluster CTCs are even rarer than single CTCs; as such, it is overall quite difficult to detect and isolate a statistically significant number of either single cells or clusters. Second, conventional technologies focus primarily on the differences in physical and/or biological properties that differentiate CTCs from non-cancerous cells; these methods are designed to detect single CTCs, but cannot identify cluster CTCs. As such, a strategy that can be used to detect and isolate all CTCs from whole blood will represent a significant advance as it will permit further exploitation of the potential for liquid biopsy. Recently, Matsusaka *et al*. (20) reported a novel approach based on concentrating CTCs using size-selective microfilters. In this study, we present an effective strategy that facilitates detection and isolation of all CTCs using two size-selective microfilters, together with anti-EpCAM antibody labeling. We present a proof-of-principle demonstration of this strategy via a size-based detection and differentiation strategy used to identify both single and cluster CTCs from the whole blood of patients diagnosed with non-small cell lung cancer (NSCLC).

Materials and Methods

Study design. This study was a retrospective analysis based on a prospective study in which EGFR-TKI was used to treat patients with advanced lung cancer; this study was conducted at the Cancer Institute Hospital of the Japanese Foundation for Cancer Research. Patients eligible for inclusion were 20 years or older with histologically confirmed NSCLC who underwent treatment with EGFR-TKI. The standard inclusion criteria applied were as follows: Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1, presence of measurable disease, a life expectancy ≥3 months, and adequate organ function. Ten patients were enrolled who provided whole blood samples for analysis. Study protocols were approved by the Institutional Review Board at the Cancer Institute Hospital of the Japanese Foundation for Cancer Research. All patients provided written informed consent for the evaluation of molecular correlates.

Isolation of single and cluster CTCs. Five ml of whole blood were collected in an ethylenediamine tetraacetic acid (EDTA) collection tube (Venoject II vacuum blood collection tube, Terumo, Tokyo, Japan); blood was aspirated using a 10 ml syringe (SS-10SZ; Terumo) that was attached to an automatic pump (KDS100; KD Scientific, Holliston, MA, USA). The following procedures were performed at a flow rate of 50 ml/h. One filter with a 13 mm diameter and 15±0.5 μm pore size (ϕ15-V-P22d-t5, Optnics Precision, Ashikaga, Japan) and a second filter with a 10±0.5 μm pore size (ϕ10-V-P17d-t5/Ni, Optnics Precision) were mounted on the syringe filter holders (16514, Sartorius, Göttingen, Germany); the two filter units were then connected in tandem to the syringe containing the sample. Blood samples were pumped through the 15 μm pore filter, followed by the 10 μm pore filter; cells were collected at each filter (Figure 1). The filters were washed with 10 ml phosphate-buffered saline (PBS) with 2 mM EDTA. Three ml of 1X lysis buffer (BD Pharm Lyse, 555899, BD Biosciences, San Jose, CA, USA) was used to hemolyze red blood cells remaining on the filter. The filters were washed again with 10 ml of PBS with 2 mM EDTA. The cells were fixed with 2 ml of 0.25% paraformaldehyde (16661-00, Kanto Chemical, Tokyo, Japan) at room temperature for 10 min. Cells were washed once again with 10 ml PBS and then incubated with 2 ml of staining solution (100x-diluted AlexaFlour 647-conjugated mouse anti-EpCAM IgG2b; 9C4, 324212, BioLegend, San Diego, CA, USA, and 2,000x-diluted Hoechst 33342 in PBS, H3570, Invitrogen, Carlsbad, CA, USA) at room temperature for 30 min. The holder was then removed from the syringe and washed with 10 ml of PBS. Since the filter is tightly attached to the holder, periphery was removed and collected using a scalpel (No.23, Feather Safety Razor, Osaka, Japan).

Enumeration of single and cluster CTCs. The collected filters were placed on glass slides (S2112, Matsunami, Osaka, Hapan; 22×22 mm, Matsunami); ~60 ul of PBS was added, and the sample was placed under a cover glass; excess PBS was then removed. The perimeter of the cover glass was sealed using a nail polish (AC quick dry, Do-Best, Tokyo, Japan). Cells were visualized with an automatic fluorescence microscope (IX83, OLYMPUS, Tokyo, Japan); images of the entire area of the filter were obtained with a 10x objective lens, with an excitation wavelength of 649 nm, and emission wavelength of 670 nm and also with an excitation wavelength of 355 nm, and an emission wavelength of 465 nm; the images were then automatically linked, and a single image generated image file. A CTC was defined as a single, intact round oval cell with a visible nucleus (Hoechst 33342 positive) that stains positively with anti-EpCAM. Clusters were defined as aggregates of two or more CTCs (Figure 2). Quantification of single and cluster CTCs were presented as the number of cells and clusters in 5 ml of peripheral blood.

Identification of EGFR mutations. After counting, the nail polish seal was cut using a scalpel, and the filter was recovered. DNA was recovered from the filter and also from the filtrate from the first filter (i.e., the plasma equivalent fraction). DNA was isolated from the filter using NucleoSpin Tissue XS (U0901A, Macherey-NAGEL, Düren, Germany); DNA was purified from plasma using Maxwell RSC ccfDNA Plasma kit (AS1480, Promega, Madison, WI, USA) and recovered in 200 μl of Milli-Q water. The filter was placed in a 1.8 ml plastic tube; DNA recovery was initiated by adding 160 μl of T1 buffer; DNA was recovered in 20 μl of Milli-Q Synthesis A10 water (Merck Millipore, Burlington, MA, USA). One μl of the DNA sample obtained via this method was amplified according to the manufacturer's protocol using the whole genome amplification kit (WG001R, MENARINI Silicon Biosystems, Castel Maggiore, Italy); 1.5 μl of the amplified DNA solution was subjected to TaqMan SNP Genotyping Assays for rare mutation analysis (EGFR6224, Applied Biosystems, Foster, CA, USA) using digital PCR (QuantStudio 3D Digital PCR, Thermo Fisher, Waltham, MA, USA) to search for the presence or absence of EGFR L858R mutation.

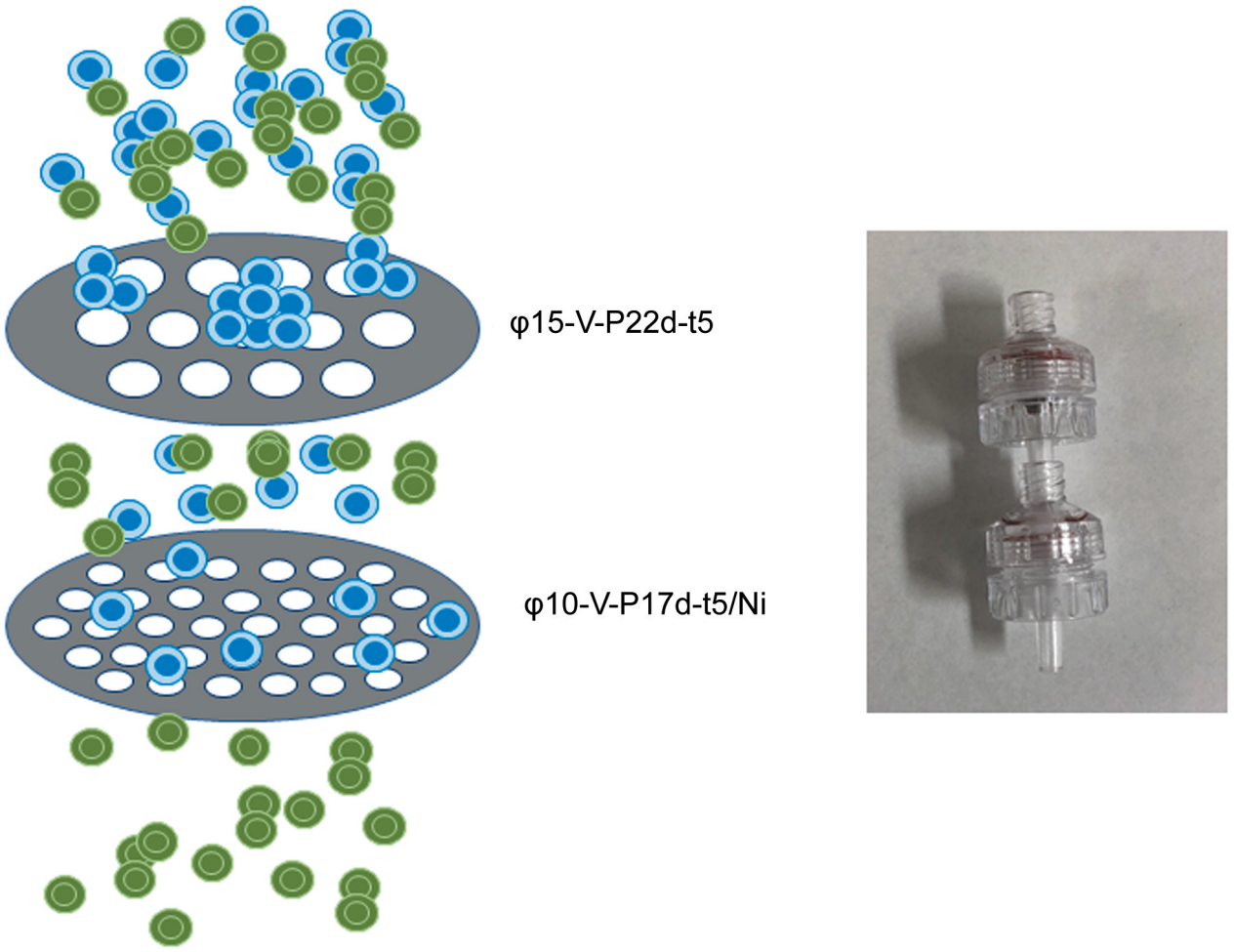

Figure 1. *Tandem size filtration. Two 13-mm-diameter filters, one with a pore size of 15±0.5 μm (φ15-V-P22d-t5, Optnics Precision, Ashikaga, Japan) and another with a 10±0.5 μm pore size (φ10-V-P17d-t5/Ni, Optnics Precision), were mounted on syringe filter holders (16514 Sartorius, Goettingen, Germany) and were connected in tandem to the syringe. Single and cluster circulating tumor cells (CTCs)(blue), which have larger sizes than white blood cells (green), are trapped on the filters.*

Results

Patient and tumor characteristics. Clinicopathological characteristics of all patients are summarized in Table I. The median age was 64.5 years (range=41-81 years); the patient cohort included four men and six women. The ECOG performance status was PS0 for six of the patients and PS1 for the other four. Six of the patients were identified to be non-smokers, and four were former smokers. The histological type was adenocarcinoma in all cases. The degree of differentiation was as follows: high differentiation (3 cases), medium differentiation (2 cases), and low differentiation (2 cases); the degree of differentiation in three of the cases was not known. The EGFR-L858R mutation had been identified in all cases; eight patients had undergone treatment with EGFR-TKI, while two patients had not undergone EGFR-TKI treatment. In six of the cases, EGFR-TKI was administered just prior to blood sampling. At the time of blood collection, two patients had metastatic disease in one organ, another two patients had metastatic disease in two organs, and four had metastases in three organs, one in four organs, and one in five organs. Six patients had distant metastases in the lung and chest wall and/or the brain metastasis; five patients were identified to have bone metastases at the time of blood sampling.

Single and cluster CTC enumeration. The number of single CTCs and cluster CTCs on each filter is shown in Table II. CTCs were fractionated in six cases on the 15 μm filter. The median cell number obtained was 5 (range=2-21); cluster CTCs were fractionated in five cases. Single CTCs were

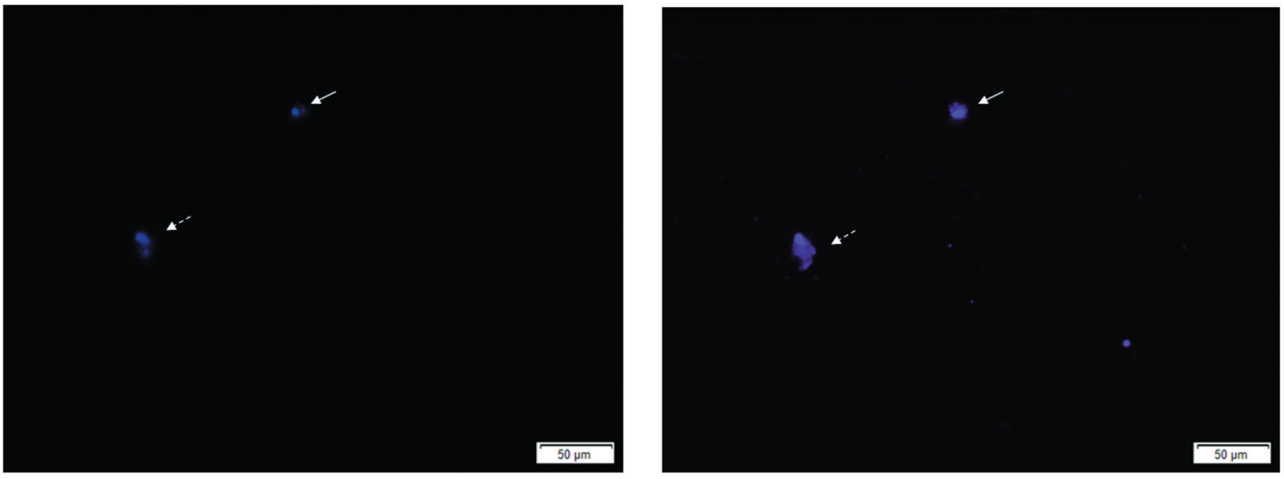

Figure 2. *Representative image of single and cluster circulating tumor cells (CTCs). Trapped cells were stained with anti-EpCAM antibody (purple) and with Hoechst stain (blue) on the filters; the cells detected by both anti-EpCAM and Hoechst stain were identified as CTCs (line arrow). Cluster CTCs (dotted arrow) were defined as aggregates of two or more CTCs.*

Table I. *Patient characteristics.*

Case	Age	Gender	Brinkman index	ECOG -PS	Histology	EGFR mutation	Differentiation degree	Previous courses of systemic chemotherapy	Previous courses of EGFR-TKI therapy	Sites of metastases
1	66	M	0	0	Ad	L858R	w/d	1	0	Brain
2	73	F	60	1	Ad	L858R	Unknown	1	1	Brain/liver/bone
3	73	F	0	1	Ad	L858R	w/d	4	3	Brain/pleura/pulmonary nodule
4	47	M	480	1	Ad	L858R	Unknown	2	2	Adrenal/bone
5	66	F	0	0	Ad	L858R	p/d	3	3	Liver/pulmonary nodule
6	61	F	0	0	Ad	L858R	Unknown	4	3	Brain/lymph nodes/bone/ pericardial fluid/skin
7	41	F	0	0	Ad	L858R	w/d	2	2	Brain/bone/pulmonary nodule
8	71	M	2000	0	Ad	L858R	p/d	3	2	Brain/lymph nodes/pleura/ pulmonary nodule
9	81	F	0	1	Ad	L858R	m/d	0	0	Pulmonary nodule
10	75	M	480	0	Ad	L858R	m/d	2	2	Pleura/pulmonary nodule/bone

Ad, adenocarcinoma; EGFR, epidermal growth factor receptor; PS, performance status; TKI, tyrosine kinase inhibitor; w/d, well-differentiated; m/d, moderately differentiated; p/d, poorly differentiated; ECOG-PS, Eastern Cooperative Oncology Group (ECOG) performance status (PS).

fractionated in all 10 cases using the 10 μm filter. The median cell number obtained was 9 (range=1-152); cluster CTCs were fractionated in three cases.

Analysis of EGFR gene mutation. We identified the EGFR-L858R mutation in sample #6 to confirm the identity of single and cluster CTCs as NSCLC. Among our results, three cluster CTCs and no single CTCs were identified on the 15 μm filter, and no cluster CTCs and 20 single CTCs were identified on the 10 μm filter in an evaluation of sample #6. EGFR-L858R was detected in the DNA extracted from captured cells associated with both filters.

Discussion

In this study, we constructed a size-selective microfilter system in order to identify and isolate both single and cluster CTCs from peripheral blood samples of NSCLC patients. Single and cluster CTCs were detected simultaneously using precision microfiltration membranes with two distinct pore sizes; single and cluster CTCs could be easily separated using this method. Cluster CTCs were captured in 5 of the 10 cases (50%) using 15 μm pore filter, while 3 cases (30%) are determined using 10 μm pore filter. The number of cells involved in the clusters at each filter was analyzed. Most clusters on the 10 μm filter were

Table II. *Number of single and cluster CTCs.*

Case	15 μm filter		10 μm filter	
	Single CTCs	Cluster CTCs	Single CTCs	Cluster CTCs
1	21	4	7	2
2	5	0	1	0
3	6	2	5	0
4	0	0	10	0
5	0	0	6	0
6	0	3	20	0
7	5	8	8	0
8	2	2	152	3
9	0	0	59	0
10	4	0	39	8

CTCs, Circulating tumor cells.

aggregates that included a small number of cells; most cluster CTCs on the 15 μm filter were aggregates of 10 cells or more. Large CTC aggregates might be captured using the 15 μm filter.

Importantly, single and cluster CTCs enriched on the filter were identified as harboring the EGFR L685R gene mutation analysis using PCR; cells with the EGFR L858R mutation were detected on both filters used to evaluate patient number 6. In this patient, three cluster CTCs and no single CTCs were detected on the 15 μm filter; no cluster CTCs and 20 single CTCs were detected on the 10 μm filter. As such, these findings reveal that both cluster and single CTCs are comprised of cancer cells; our results clearly demonstrate that single and cluster CTCs can be recovered using a size-selective microfilter system.

Our study has certain limitations. As this is a retrospective analysis with a very limited sample size, large prospective studies will be needed in order to validate our findings. In addition, we need to determine the sensitivity of the tests used to identify the L858R EGFR gene mutation in both single and cluster CTCs. Nonetheless, our system suggests the possibility of accurately detecting and quantitative evaluating both single and cluster CTCs in a large population of peripheral blood cells; these are findings that may be developed into alternative treatment strategies for lung cancer patients.

Conflicts of Interest

Kiyotaka Shiba and Satoshi Matsusaka received a research grant from Optnics Precision Co., Ltd. No potential conflicts of interest were disclosed by the other Authors.

Authors' Contributions

Conception and design: Sonoda T, Yanagitani N, Nishio M, Matsusaka S. Development of methodology: Shiba K, Matsusaka S. Acquisition of data: Sonoda T, Yanagitani N, Yoshizawa T, Nishikawa S, Kitazono S, Horiike A. Analysis and interpretation of data: Sonoda T, Yanagitani N, Shiba K, Nishio M, Matsusaka S. Writing, review, and/or revision of the manuscripts: Sonoda T, Yanagitani N, Shiba K, Ishizuka T, Nishio M, Matsusaka S. Administrative, technical, or material support: Kanako Suga. Study supervision: Nishio M, Matsusaka S.

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