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Verification of A New Filter for Isolation of Circulating Tumor Cells by Only Blood Filtration

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Abstract. *Background/Aim: Since circulating tumor cells (CTCs) are precursors of metastatic lesions, extracting CTCs from whole blood is useful in obtaining information for cancer treatment. One of the CTC isolation methods is the size selection method; however, since the conventional methods are expensive and cumbersome, we developed an affordable and simple filter, whose usefulness is verified in this study. Materials and Methods: The new filter [hereafter, soft micropore filter (S-MPF)] is made up of a polyethylene film with a thickness of 15 μm and conical pores having a diameter of 8-10 μm, which are opened uniformly (opening rate, 20%). This filter can filter whole blood by free-falling under gravity. The possibilities of the filter's usage for model CTC isolation, immunostaining, short-term cell culture, and gene mutation detection in extracted model CTCs were verified. Results: S-MPF was able to extract model CTCs with an isolation rate of up to 15%. These model CTCs were detected by cytology, immunostaining, and culture by short-term incubation of filtered cells. Furthermore, genetic mutations were identified in the cultured cells. In addition, CTC isolation from the peripheral blood of patients with lung cancer was demonstrated by setting the volume of collected blood to 15 ml to prevent a low recovery rate. Conclusion: The S-MPF can be used to extract model CTCs quickly and easily. Moreover, cytological diagnosis, immunostaining, short-term culture, and gene mutation search are possible with this filter. Given its proven applicability in clinical samples, this filter can be used in clinical settings.*

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Key Words: Circulating tumor cells, filter, Immunostaining, cell culture, gene mutation analysis.

Cancer is one of the leading causes of death in high-income countries (1), and metastasis is the major cause of cancer mortality (2, 3). A cancer lesion gives rise to circulating tumor cells (CTCs), which are precursors of metastasis (4). Therefore, in cancer cases, understanding the status of CTCs is crucial in comprehending treatment outcomes (5, 6) and predicting patients' prognoses (7). There are two CTC identification methods: counting surrogate markers (*e.g.*, marker proteins, nuclear acids, *etc.*) and morphological detection by CTC isolation (8). Examples of CTC isolation methods include immunocapture methods and biophysical property positive selection methods (9). The membrane filtration method is one of the methods that use positive selection of biophysical properties. The main membrane filtration methods include the flexible micro spring array (10), ScreenCell® (micro pore filter and suction) (11), isolation by size of tumor cells (12), separable bilayer microfilter (13), and fluid-assisted separation technology lab-on-a-disc platform (14).

Although membrane filtration techniques are simple to use, suction by negative pressure and/or dedicated instruments are required to implement them. In addition, they can process only

small volumes of diluted blood per time and are inclined to membrane clogging (10-14). Furthermore, they result in high leukocyte contamination, and therefore in low levels of purity (10-14). That is, with no specific way to efficiently remove CTCs from the membrane, a simple washing step can result in a huge loss of CTCs and may not be a viable step for cell recovery (10-14). Although such filtration techniques offer the possibility of cell staining on the filtration membrane, it is exceedingly difficult to create a permanent specimen because the membrane is purged with a volatile solvent. However, if a high leukocyte count can be excluded, such filters can be placed directly into a cell culture dish for cell culture or into a recovery tube for pooled DNA/RNA isolation (9).

We developed a filter that does not purge with a volatile solvent, that can extract CTCs from blood with a low white blood cell count by simply filtering an unlimited amount of non-diluted blood freely falling under gravity without using special procedures. In this study, we verified the feasibility of cancer cell isolation from whole human blood using our filter.

Materials and Methods

Ethical considerations. The study protocol was approved by the Institutional Review Board of Nara Medical University Hospital (No. 1718, No. 2264). Informed consent was obtained from all the study volunteers.

Evaluation outcomes. The primary outcome was identification of extracted tumor cells from whole blood by the newly developed micropore filter.

The secondary outcomes were validation of 1) immunostaining, 2) short-term culture, and 3) gene mutation identification properties.

Soft micropore filter (S-MPF). The new filter contains a polyethylene film with a thickness of 15 μm and conical pores with a diameter of 10-8 μm that are opened uniformly at a rate of 20% (Toray Industries, Inc., Tokyo, Japan). When a filter holder for a 13-mm filter (SX0001300, Merc Millipore, MA, USA) is set, a 10 ml syringe (Terumo-syringe 10 ml SS-10ESZ, manufactured by Terumo Co., Ltd., Tokyo, Japan) is connected to its plunger (Figure 1). The S-MPF can filter whole blood; it uses the principle of free-falling under gravity. The time to filter 5 ml of saline and 5 ml of whole blood is approximately 60 and 240 s, respectively. In addition, this filter has no volume limitation.

The S-MPF CTC recovery procedure is as follows (Figure 1):

1. The filter is set to the Millipore holder.
2. One milliliter of saline is aspirated into the syringe, which is connected to the holder and then poured. The surface tension on the filter hole is removed.
3. The collected blood is injected into the syringe, after which the collected blood is washed with 2 ml of saline that is injected using the syringe; this procedure is to collect CTCs attached to the wall of the syringe.
4. After all the blood has dripped and vascular lavage has been performed, 5 ml of saline is injected into the syringe for the recovery of cells that are attached to the syringe wall.
5. The syringe is pushed upward and passed through the holder; this process is to remove blood and the saline left in the holder).
6. The cells on the film are stained either inside or outside the holder.
7. The filter, if present in the holder, is removed from the holder and put on the slide glass.

Model CTC isolation from the whole blood. Regarding the model CTCs, cells from lung adenocarcinoma cell line HCC827 (exon 19 deletion mutation positive) were mixed with whole blood from healthy volunteers; this blood was maintained in a non-coagulated state using ethylenediaminetetraacetic acid, adjusted to concentrations of 100 cells/ml; model CTCs were extracted from this blood. The follow-through fluid was collected, and the number of cells lost through the filter were counted using Vi-CELL XR (Beckman Coulter Inc., Tokyo, Japan).

Model CTC identification. The model CTCs collected *via* the new filter were counted microscopically and stained with the Giemsa stain (Cyto Quick™ MUTO Pure Chemicals Co., Ltd, Tokyo, Japan) in a dry-fixed specimen on the S-MPF filter outside the holder. In addition, the extracted CTCs in or out of the holder, were stained using the Papanicolaou method.

Immunostaining. Specimen preparation was performed using both dry- and wet-fixed specimens. The dry-fixed specimen was prepared as follows: after passing the cells *via* the filter, they were left to dry for one day. After passing the cells *via* the filter, Cytorop (Alfresa Pharma Co., Ltd., Osaka, Japan), a fixation reagent for cytology testing, was poured into the cells, and the cells were left to dry for one day; this was for preparation of the wet-fixed specimens.

During this process, a sample was stained using Cytokeratin (clone CAM5.2, ×5, BD, San Jose, CA, USA), CD45 (clone 2B11+ PD7/26, ×400, DAKO, Glostrup, Denmark) for the mouse monoclonal primary antibody, and Simple Stain MaxPo (Multi) reagent (Nichirei Bioscience Inc., Tokyo, Japan) for secondary antibody. The following steps were then followed: 0) Wet fixation specimen to remove fixation reagent with alcohol, 1) buffer: (dH_2O 900 ml, 10X Tris Buffered Saline 100 ml, and Tween-20 1 ml), 2) endogenous peroxidase removal, 3) buffer 3 times × 5 min, 4) Primary antibody 15 min, 5) buffer 3 times × 5 min, 6) Secondary antibody 15 min, 7) buffer 3 times × 5 min, 8) 3,3'-diaminobenzidine 1 min, 9) buffer 5 min, 10) nuclear staining 3 min, 11) color out cleaning, 12) drying, and 13) inclusion.

Short-term culture and gene mutation analysis. The samples were maintained at 37˚C in 5% (V/V) CO_2 (SCA-165DRS, ASTEC, Fukuoka, Japan) under humidified conditions. The samples were cultured in RPMI medium (Nakarai tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Inc., St. Louis, MO, USA) and 1% penicillin-streptomycin (Nakarai tesque, Kyoto, Japan). Each processed sample (3 ml of whole blood) was split and seeded in a 35-mm dish containing the film.

A gene mutation analysis was performed using Cobas z 480 analyzer (Cobas® 4800 system; Roche Diagnostics, Tokyo, Japan) for detection of epidermal growth factor receptor (*EGFR*) gene mutation using proliferated tumor cells. DNA extraction was performed using QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). Ex19-deletion detection was performed using Cobas® EGFR Mutation Test v2 (Roche Diagnostics, Mannheim, Germany).

Clinical evaluation. Fifteen milliliters of peripheral arterial blood were collected from the patients with non-small cell lung cancer (NSCLC)

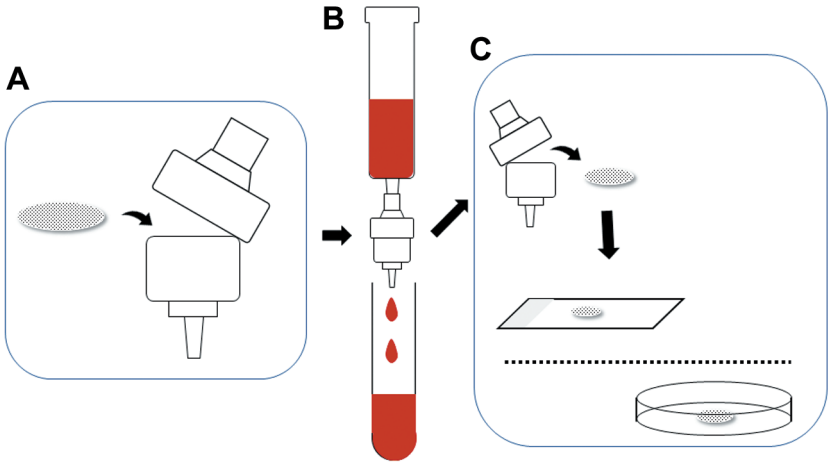

Figure 1. *Circulating tumor cell isolation method. A filter is put in a filter holder with a 13-mm filter (SX0001300, Merck Millipore, MA, USA) (A). The holder with a filter is set, and a 10 ml syringe (Terumo-syringe 10 ml SS-20ESZ, manufactured by Terumo Co., Ltd. Tokyo, Japan) with the plunger removed is connected; the blood sample falls freely under gravity and leaving tumor cells (B). The cells on the filter are stained outside the holder using the Giemsa stain and inside the holder using the Papanicolaou method. The filtrate is removed from the holder and available for either microscopic examination or cell culture (C).*

Table I. *Status of cell isolation from whole blood.*

Sample No.	Dilution[a]	Filtration excess (ml)	Model CTC count	Count of cells captured *via* the filter (%)	Count of model CTCs in flow-through liquid
1	No	3	300	14 (4.7%)	0
2	No	3	300	13 (4.3%)	0
3	No	3	300	3 (1.0%)	0
4	3×	3	100	10 (10%)	0
5	3×	3	100	9 (9.0%)	0
6	3×	3	100	8 (8.0%)	0
7	5×	3	60	9 (15.0%)	0
8	5×	3	60	7 (11.7%)	0
9	5×	3	60	5 (8.3%)	0

[a]3 ml of whole blood containing 300 cells was diluted using normal saline. CTCs: Circulating tumor cells.

preoperatively. Tumor cells were recovered using the procedure described in the Materials and Methods. The collected cells were stained using the Giemsa stain and observed microscopically.

Results

Model CTC isolation from whole blood. Three-milliliter whole blood samples containing model CTCs, 3-ml blood samples diluted 3X using normal saline, and 3-ml blood diluted 5X using normal saline were analyzed; the isolation rate was 15%. However, there were no model CTCs in the flow-through liquids (Table I).

Model CTC identification. The model CTCs collected on a new filter were observed under a microscope both as a dry-fixed specimen stained using the Giemsa stain (Figure 2A) and as a wet-fixed specimen stained using the Papanicolaou method (Figure 2B).

Immunostaining. Regarding the dry fixation method, it was possible to identify model CTCs by Giemsa staining (Figure 3A). However, it was difficult to confirm the form by immunostaining and to identify the cells (Figures 3B, C). On the other hand, regarding the wet fixation method, CAM5.2-positive cells (Figure 3E) were identified in the same form (like when Papanicolaou staining was performed; Figure 3D). CD45-negative cells (Figure 3F) were also identified.

Short-term culture and gene mutation analysis. Model CTCs extracted from the blood formed tumor islets (Figure 4),

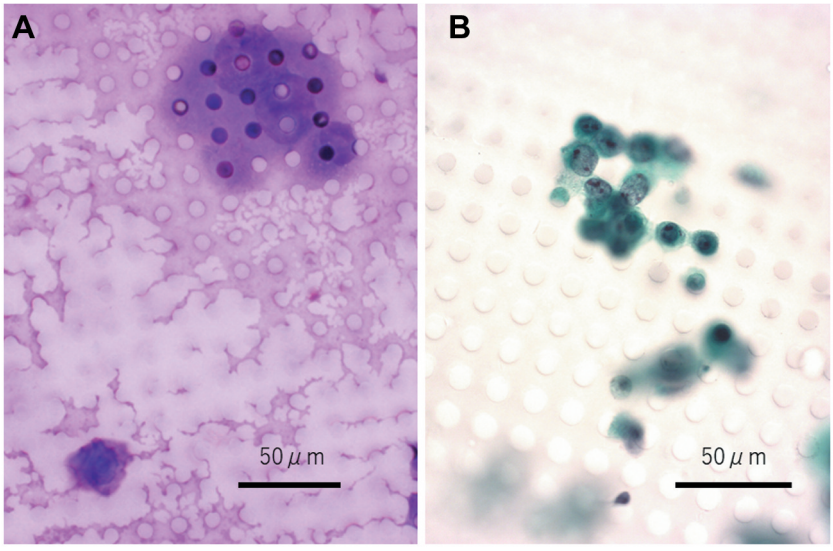

Figure 2. *Model circulating tumor cells (CTCs) extracted using the soft micropore filter. Dry-fixed specimen stained using the Giemsa stain (A). Wet-fixed specimen stained using the Papanicolaou method (B).*

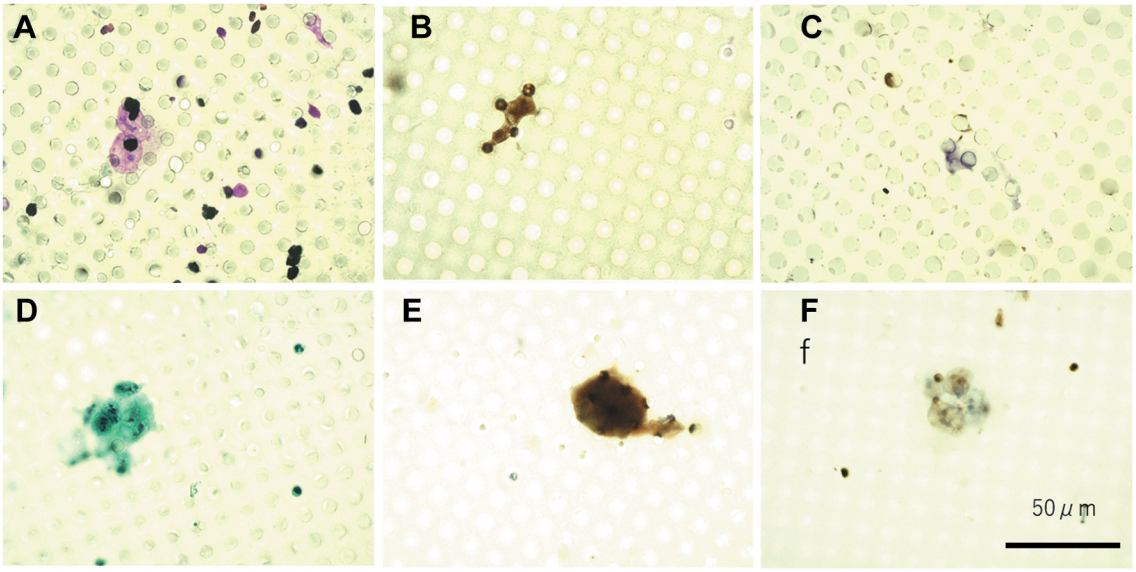

Figure 3. *Immunostaining of model circulating tumor cells (CTCs) extracted via the soft micropore filter. Using the dry fixation method, it was possible to identify the model CTCs by Giemsa staining (A); however, it was difficult to confirm the form by immunostaining and to identify the cells (B, C). On the other hand, using the wet fixation method involving Papanicolaou staining (D), CAM5.2-positive cells (like with Papanicolaou staining) (E) and CD45-negative cells (F) are observed.*

suggesting the presence of the *EGFR* mutation EX19Del, which is the most common gene mutation in lung cancer.

Clinical verification. Samples from two patients with NSCLC were also analyzed in this study. The patients provided informed consent. One of them was a 72-year-old man with Stage IIIA (T2aN2M0) adenocarcinoma, revealing clustered CTCs; this finding was similar to the findings of the touch smear cytology of the tumor region (Figure 5). The other patient was a 61-year-old woman with Stage IB (T2aN0M0) adenocarcinoma; she had similar CTC findings.

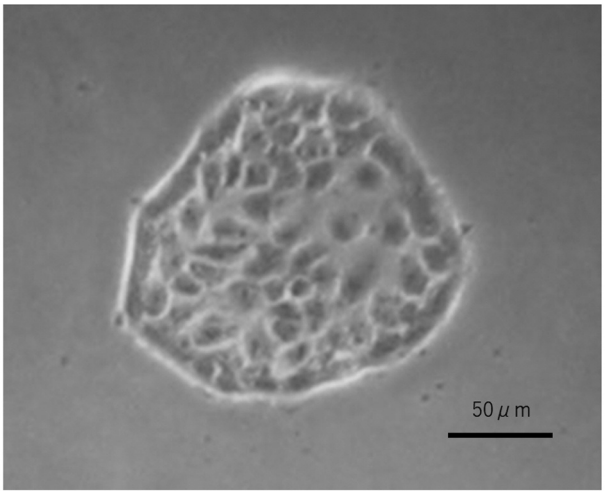

Figure 4. *Cultured model circulating tumor cells. The model CTCs extracted from the blood formed tumor islets, suggesting the EGFR mutation EX19Del.*

Discussion

In this experiment, we observed the following: the S-MPF was able to extract model CTCs freely falling under gravity, and these model CTCs were diagnosed by cytology, immunostaining, and short-term culture of filtered cells. Moreover, genetic mutations were identified from the cultured cells. In addition, CTCs were extracted from the peripheral blood of patients with lung cancer in a clinical setting; 15 ml of blood was used to prevent a low recovery rate.

CTCs are cells that are separated from primary tumor lesions and are disseminated to other sites *via* the bloodstream, resulting in cancer metastasis. CTCs are found in peripheral blood of patients with cancer. However, unlike blood cells, the number of CTCs in the blood is very small and they are difficult to detect. There are two CTC detection methods: one uses surrogate markers and the other a separation based on morphological features (9). The conventional CTC detection methods have problems such as the high cost, complexity, need for dedicated machines, and identification of surrogate markers (8-14). However, since it was demonstrated that the S-MPF counteracts these problems and was suitable for liquid-based cytology of model CTCs, the S-MPF is expected to be used in clinical practice for processes such as gene mutation detection, short-term culture, and immunostaining.

This study has several limitations. First, only one cell line was used, which limits its applicability to other cell lines. However, identification of extracted CTCs was shown in actual clinical samples; therefore, its use in other clinical settings might be possible. Second, the sample size was limited, suggesting that its feasibility in actual clinical practice is

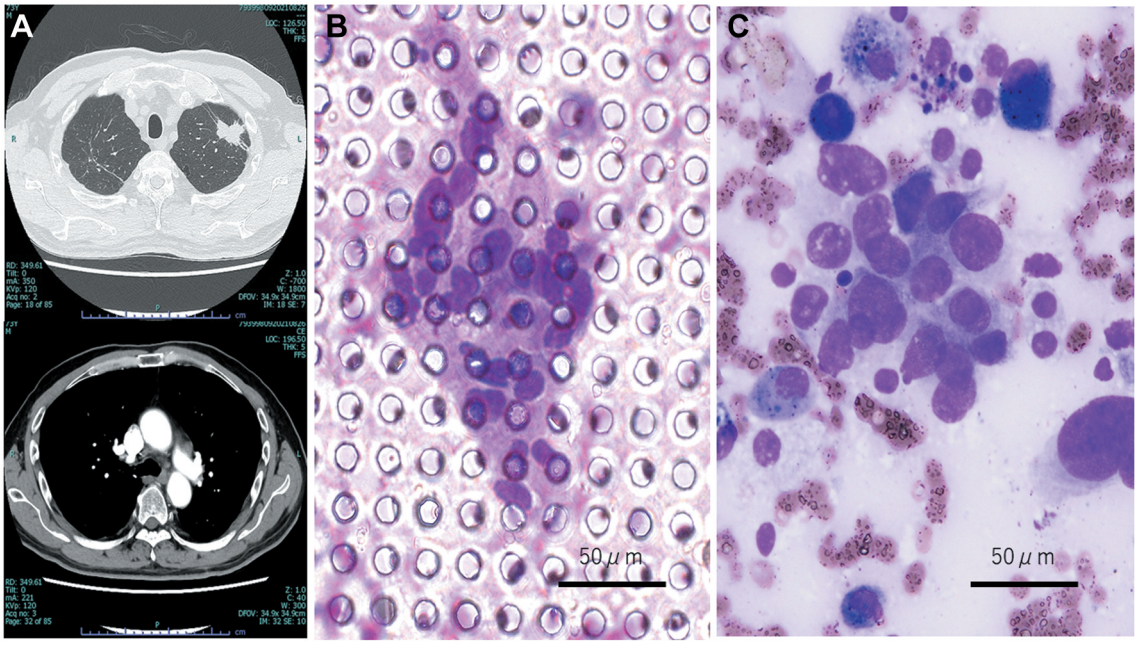

Figure 5. *In vivo circulating tumor cells (CTCs) extracted using the soft micropore filter. A 72-year-old man with Stage IIIA (T2aN2M0) adenocarcinoma (A), revealing clustered CTCs (B) which are comparable to those observed during touch smear cytological examination of the tumor region (C).*

unknown. However, a prospective feasibility study is ongoing (UMIN0000473648) (15); it has been registered and the provisional results are promising.

Conclusion

The newly developed filter (S-MPF) can extract model CTCs quickly and easily, and cytological diagnosis, immunostaining, short-term culture, and gene mutation search are possible. CTC detection from clinical samples was successful; therefore, it can be used in clinical settings.

Conflicts of Interest

S-MPF was created through commissioned research from Toray Co., Ltd; however, there are no payoffs for Authors. In addition, there are no conflicts of interest to be declared.

Authors' Contributions

Conceptualization: Kohei Morita, Noriyoshi Sawabata, Toru Arakane. Methodology: Kohei Morita, Tomomi Fujii, Shigenobu Tatsumi, Takashi Nishikawa. Software and formal analysis: Kohei Morita, Noriyoshi Sawabata. Validation: All Authors. Investigation: Kohei Morita, Tomomi Fujii, Yoshiaki Tominaga, Noriko Ouji-Sageshima, Shigenobu Tatsumi, Takashi Nishikawa. Resources: All Authors. Data curation: All Authors. Writing–original draft: Kohei Morita, Tomomi Fujii, Takashi Nishikawa, Noriyoshi Sawabata. Writing–review and editing: All Authors. Visualization: All Authors. Supervision: Chiho Obayashi, Toshihiro Ito.

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