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High-purity Isolation for Genotyping Rare Cancer Cells from Blood Using a Microfluidic Chip Cell Sorter

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Abstract. *Background/Aim: A multistep sorting method for enrichment of rare cells, such as circulating tumor cells, in the blood without cumbersome pretreatments required by most flow cytometry-based methods, which lead to high cost and decreased detection efficiency, was developed. Materials and Methods: After only hemolysis and cell staining, cancer cells are enriched by repetitive sorting (3×) based on nuclear-positive, cytokeratin-positive, and CD45-negative expression. Results: Experiments using spikes of PC-9 cells showed a mean recovery of 65% and mean purity of 83%, which was retained up to 72 hours after blood draw using preservative tubes. Significant differences in expression level of programmed death-ligand 1 or vimentin were observed between high- and low-expressing cell lines, concurrently with enrichment. Next-generation sequencing analysis of recovered PC-9, A549, and MDA-MB231 cells successfully detected all known mutations. Conclusion: This novel isolation method applicable for preserved samples with sufficient recovery and purity may be substantially beneficial for recovering cells for subsequent molecular analysis.*

The remarkable advancements in cancer treatment using targeted molecular therapies have enhanced therapeutic strategies and promoted precision medicine (1-3). To select optimized treatments for individual patients, detection of genetic abnormalities and protein expression of targeted molecules on primary tumor cells is needed. In addition, real-time monitoring of responses to treatment is critical for adjusting treatment over time. Such needs for diagnosis and treatment monitoring often require invasive tumor biopsy and re-biopsy. Circulating tumor cells (CTCs) are cancer cells circulating in the peripheral blood that have detached from a solid tumor and entered the bloodstream. Thus, CTCs likely mirror characteristics of primary tumor cells, such as genetic alterations and expression of therapeutic targeted molecules (4-6). Such properties of CTCs allow detecting the presence of therapeutic targets and monitoring of response to treatment (7, 8). Therefore, enrichment and analysis of CTCs from peripheral blood are potentially useful for diagnoses with minimal invasiveness. Programmed death-ligand 1 (PD-L1) expression in tumor tissues is a validated biomarker for programmed death 1 (PD-1) pathway blockade in non-small cell lung cancer (NSCLC) (9, 10). PD-L1 expression can be detected on CTCs and might be useful as a predictor of efficacy (11-14). Also, it is thought that the cancer cells that have transitioned from epithelial to mesenchymal transition (EMT) signal a poor prognosis (15). Thus, evaluation of markers for EMT, such as vimentin and AXL, on CTCs is useful in determining prognosis (16).

However, enriching CTCs from whole blood with high recovery, purity, and reproducibility, is challenging despite a decade of technological development. Many approaches for CTC isolation have been developed, such as immunoaffinity based methods, size-based separation, and dielectrophoresis (17, 18). Only one CTC enumeration system, CellSearch, is approved by the US Food and Drug Administration. This method has demonstrated clinically significant CTC enumeration for several cancers (19-21). However, considerable numbers of non-target leukocytes are also captured by this process, making further analysis for molecular and genetic characteristics of CTCs difficult. Previously, a protocol for CTC enumeration and sorting was reported that uses On-chip Sort (On-chip Biotechnologies,

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Tokyo, Japan). This system is newly developed for cell sorting and is equipped with a disposable microfluidic device. The system can detect and isolate CTCs for subsequent molecular analysis using the expression of marker proteins (7, 22, 23). However, this method requires pretreatment with immunomagnetic depletion of CD45-positive white blood cells for CTC isolation. However, CTCs in leukocyte clusters are also removed by the immunomagnetic depletion treatment. CTCs in neutrophil clusters have been reported to cause a very poor prognosis in cancer patients (24). Therefore, it is important to count leukocyte clusters containing CTCs after enrichment of CTCs without the depletion treatment of leukocytes. In the case of sorting CTCs without the pretreatment for removing leukocytes, the sorting time using a normal FACS cell sorter is 10 hours or more due to the increase in the sample volume by the dilution according to 10^8 cells. Therefore, it is difficult to apply a normal cell sorter to CTC enrichment.

In this study, we have developed a method that uses an exchange-type microchannel chip-type cell sorter (On-chip Sort) to achieve a high concentration without using pretreatment for removing leukocytes. The approach was used to characterize isolated cells. The method provides a simpler and more stable cell separation with high purity and reduces separation times to within 3 hours. The principle of the procedure is to remove leukocytes by passing cells through the instrument at 100,000 cells or more per second and recovering cancer cells that are detected at 1 cell or less per second. Thus, the number of leukocytes isolated with cancer cells is far less than the number of leukocytes that flow through. Captured leukocytes mixed with cancer cells are removed during a subsequent sorting step and cancer cell counts are maintained through multiple sorting steps. By repeating the process, it is possible to achieve sufficient purity of cancer cells for subsequent analysis, while maintaining a reasonable recovery. On-chip Sort is equipped with multiple excitation lasers and detection channels and can analyze surface markers on individual cells concurrently with the enrichment of cancer cells. The study indicates that multistep sorting using On-chip Sort is sufficient for rare cancer cell segregation with purity suitable for subsequent genomic analysis. It is shown to be adequate for evaluating expression of PD-L1 and vimentin on isolated cells.

Materials and Methods

Cell lines and culture. The human NSCLC cell line A549 and the human breast cancer cell line MDA-MB231 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The human NSCLC cell line PC-9 was kindly provided by Dr. Fumiaki Koizumi (Tokyo Metropolitan Komagome Hospital). The PC-9 and A549 cell lines were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA) at 37˚C with 5% CO_2 supplementation. The MDA-MB231 cell line was cultured in Dulbecco's modified Eagle medium (Thermo Fisher Scientific) containing 10% fetal bovine serum at 37˚C with 5% CO_2 supplementation.

Cell spike-in experiments. Cells were harvested with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific) at 37˚C and then washed and resuspended in T-buffer composed of 0.5% bovine serum albumin (BSA) (Nacalai Tesque Inc., Kyoto, Japan), 2 mM EDTA (Sigma- Aldrich), and 0.5% Through Path Plus (On-Chip Biotechnologies, Tokyo, Japan) in phosphate-buffered saline (PBS) (Invitrogen) to obtain a final concentration of 1 or 5×10^3 cells/ml. For establishment of the cancer cell detection method, 100 cells of PC-9, a high cytokeratin-expressing cell line, were spiked into 4 ml of peripheral whole blood in blood collection tubes containing EDTA (Becton Dickinson and Company, Franklin Lakes, NJ, USA) from healthy donors who provided informed consent. This study was approved by the institutional review board of Wakayama Medical University Hospital (reference number 2334). For validation of detection rates and next-generation sequencing (NGS) analysis using preserved samples, 0, 10, 100, or 500 cells from the PC-9 cell line were spiked into 20 ml of peripheral whole blood in preservative tubes (Streck, Omaha, NE, USA) Blood was collected from healthy donors at Wakayama Medical University who consented to donations in writing. Similarly, for validation of NGS analysis after cell isolation, 10 or 500 cells of A549 cell line were spiked into 20 ml of peripheral whole blood from healthy donors in preservative tubes, or 300 cells of MDA-MB231 cell line were spiked into 10 ml of that. For evaluation of protein expression on isolated cells, 100 or 300 cells of PC-9 cell line were spiked into 10 or 20 ml of peripheral whole blood from healthy donor in preservative tubes, 100 cells of A549 cell line were spiked into 20 ml of that, or 300 cells of MDA-MB231 cell line were spiked into 10 ml of that. The number of spiked cells was counted visually. Spiked samples were processed as described below and sorted between 24 and 72 h after blood draw. For validation of stability of spiked cells in preservative tubes, 100 cells of PC-9 cell line were spiked into 10 ml of peripheral whole blood from healthy donor in preservative tube within 1 hour after blood draw. Spiked samples were evaluated at the time points of 24, 48, and 72 h after blood draw.

Red blood cell lysis and sample staining. Red blood cell lysis, cell fixation, and permeabilization were performed using Red Blood Cell (RBC) Lysis Buffer and True-Nuclear Transcription Factor Buffer Set (BioLegend, San Diego, CA, USA) utilizing recommended methods with slight modification. Briefly, whole blood samples were added to four times the volume of a 1× working solution of RBC Lysis Buffer, mixed gently by pipetting, and then incubated for 15 min at room temperature. After incubation, samples were centrifuged at 350 × *g* for 10 min at room temperature. After supernatants were carefully removed, cell pellet was suspended in 1 ml of 1× working solution of Fix Concentrate for fixation and incubated for 10 min at room temperature. Then, 1 ml of 1× working solution of Perm Buffer was added for permeabilization, mixed gently by pipetting and incubated for 20 min at room temperature. Samples were mixed with 5 ml of T-buffer containing 0.1% BSA and centrifuged at 350 × *g* for 10 min at room temperature. For immunostaining, cell pellets were suspended in 100 μl of staining solution containing 5 μl of Human TruStain

FcX™ (BioLegend), the fluorescein isothiocyanate (FITC)-conjugated anti-cytokeratin Mouse mAb [CK3-6H5] (1:25 dilution, 130-090-866, Miltenyi Biotec, Bergisch-Gladbach, Germany) and 100 μg/ml of Hoechst33342 (ab145597, abcam, Cambridge, UK) in T-buffer containing 0.1% BSA, and incubated for 30 min at room temperature in the dark. After the incubation, samples were washed with 5 ml of T-buffer containing 0.1% BSA and cell pellets resuspended in 5 ml of T-buffer containing 0.1% BSA and stored at 4°C in the dark until the first sorting step. Just before the first sorting, samples were filtered through a cell strainer with 40 μm pores (Corning, NY, USA) followed by centrifugation and resuspension of cell pellet in 1 ml of T-buffer containing 0.1% BSA for 20 ml whole blood sample or 350 μl of this buffer for 4 ml whole blood sample. After the first sorting, recovered samples were stained with Alexa Fluor 647 conjugated anti-PD-L1 Rabbit mAb [28-8] (1:25 dilution, ab209960, abcam), Phycoerythrin (PE) conjugated Anti-Vimentin Mouse mAb [VI-RE/1] (1:25 dilution, ab49918, abcam), and PE-Cy7 conjugated anti-CD45 Mouse mAb [HI30] (1:50 dilution, ab155340, abcam), then incubated for 30 min at room temperature in the dark. After incubation, samples were transferred to new sample reservoirs and sample volumes were increased up to 350 μl.

Flow cytometry. Flow cytometry was performed using an On-Chip Sort system (On-Chip Biotechnologies). Details of this system were described previously (22, 23) and on the manufacturer's web site (25). Briefly, On-chip sort is a bench-top cell sorter, which uses a disposable microfluidic chip-based flow cytometer that allows absolute contamination-free measurement, whole volume measurement, and sample collectivity after measurement. Thus, the system provides suitable conditions for the capture of cancer cells from blood in a clinical setting. The On-chip Sort system used in the study employs three excitation lasers (violet; 405 nm, blue; 488 nm, and red; 638 nm) and five detection channels. The wavelength range of the five detection channels (FL1, 2, 3, 5, and 6) was modified for cancer cell detection. Ranges used were 435-455 nm for FL1, 532-554 nm for FL2, 570-613 nm for FL3, 658-695 nm for FL5, and 752-798 nm for FL6. Signals for Hoechst33342, FITC, PE, Alexa Fluor 647, and PE-Cy7 were collected via these detection channels. Voltages of light sensor modules were optimized for cancer cell detection (FSC: gain 0.10 V, SSC: 0.24 V, FL1: 0.35 V, FL2: 0.30 V, FL3 0.30 V, FL5: 0.28 V, and FL6: 0.35 V). Data analysis was performed using On-chip Sort software version 1.9.9 (On-Chip Biotechnologies). The analysis of intensity of vimentin expression was performed using FlowJo software version 10.6.1 (BD Biosciences, San Jose, CA, USA) and Microsoft Excel 2016 software (Microsoft, Redmond, WA, USA).

Enrichment and sorting procedures. Enumeration and sorting of cancer cells were performed with an On-chip Sort system according to the manufacturer's instructions. Briefly, the flow path was pre-washed with the 1X Through Path Plus (On-Chip Biotechnologies) and On-chip sample buffer (1X Through Path Plus with 1.5% polyvinylpyrrolidone, On-Chip Biotechnologies). Maximum volume per sorting is 350 μl, therefore if the volume of stained sample obtained from whole blood after RBC lysis was 1 ml, the sample was divided into three approximately 350 μl portions and sorted using the same disposable microfluidic chip for the first sorting. Sorted cells gated into the nuclei- and cytokeratin-positive channels were collected. After the first sorting, 20 μl of T-buffer containing 0.1% BSA was added to the used microfluidic chip and targeted cells were sorted and collected again. After staining with PD-L1, vimentin, and CD45 antibody, 350 μl of stained samples was sorted using a new microfluidic chip and targeted cells were collected by gating into cytokeratin-positive and CD45-negative channels. Then, 20 μl of T-buffer containing 0.1% BSA was added to the used chip and targeted cells sorted and collected once again. For the third sorting, the collected sample was transferred to a new chip, and sample volume was increased to 350 μl followed by sorting. Targeted cells were collected by gating into cytokeratin-positive and CD45-negative channels.

Whole-genome amplification. Sorted cells were transferred from the collection reservoir to 200 μl polymerase chain reaction tubes and centrifuged at 600 × *g* for 10 min at room temperature. After discarding the supernatant, leaving less than 2 μl, which was the starting volume of the whole-genome amplification (WGA). WGA was performed using the Ampli1 WGA kit (Silicon Biosystems, Bologna, Italy) according to the manufacturer's protocol. DNA concentrations were determined using a Qubit 2.0 Fluorometer (ThermoFisher Scientific) and quality control checks of products were performed using the Ampli1 QC Kit (Silicon Biosystems).

Next-generation sequencing. The library for NGS analysis was prepared from 100 ng of amplified genomic DNA products according to the manufacturer's protocol using Ion AmpliSeq Library kit 2.0, Ion Xpress barcode adapters, and Ion AmpliSeq Cancer Hotspot Panel v2 kit that covers 270 amplicons of 50 cancer-related genes (ThermoFisher Scientific). The library was purified with Agent court AMPure XP (Beckman Coulter, Brea, CA, USA) and quantified with Ion Library TaqMan Quantitation Kit (ThermoFisher Scientific) with the StepOne Plus system (Applied Biosystem, Foster City, CA, USA). Quality control checks of libraries were performed using Agilent 2200 TapeStation and High Sensitivity D1000 ScreenTape (Agilent Technologies, CA, USA). Template preparation was performed with the Ion Chef System and Ion PGM Hi-Q View Chef kit. Finally, sequencing was performed on the Ion Torrent PGM system using the Ion PGM Hi-Q View Sequencing kit (Life Technologies) with the Ion 318 chip v2 and a set of 500 flows.

NGS data analysis. All PGM sequences were analyzed using Ion reporter software 5.10, aligning all reads to human reference hg19. Variant calling was performed running the Torrent Variant Caller plugin. Data were annotated using CLC Genomics Workbench software version 9.5.3 (CLCbio, Aarhus, Denmark).

Immunocytochemistry for observation by fluorescence microscopy. Cells were seeded onto an 8-well chamber slide (Thermo Fisher Scientific) and left for more than 24 h to reach 50% confluency. Cells were fixed for 20 min with 4% paraformaldehyde (Sigma-Aldrich) at room temperature and washed three times with PBS for 5 min each. Cells were permeabilized for 5 min with 0.5% Triton X-100 at room temperature. After washing with PBS, the cells were incubated with 2% BSA in PBS for 30 min for blocking, then incubated with a 1: 500 dilution of anti-pan-cytokeratin mouse mAb [C11] (SC-8018, Santa Cruz Biotechnology, Dallas, TX, USA) in 2% BSA or a 1: 500 dilution of anti-vimentin mouse mAb [V9] (SC-6260, Santa Cruz Biotechnology) in 2% BSA for 1 h at room temperature. From then on, the cells were washed three times with PBS for 5 min between

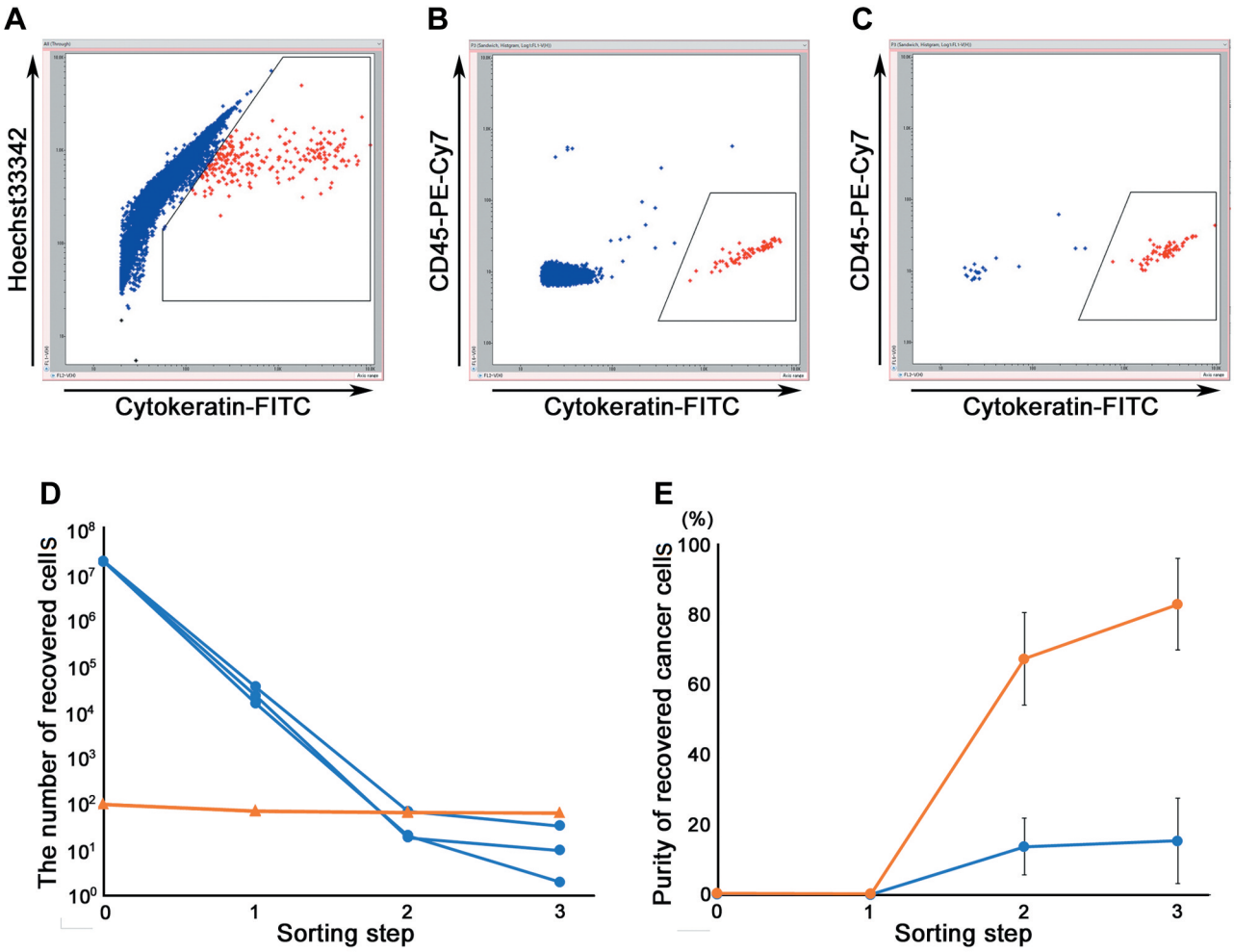

Figure 1. *Enrichment of PC-9 cells spiked into 4 ml of peripheral whole blood by multistep sorting. Cancer cells defined as nuclei-positive, cytokeratin-positive, and CD-45-negative were sorted according to gate settings. Nuclei-positive and cytokeratin-positive cells were identified using Hoechst33342 and plotted against FITC density (A). Nuclei-positive cells were distinguished as CD45-negative using PE-Cy7 and plotted against FITC density (B), (C). (D) The change in the number of white blood cells (blue lines) and PC-9 cells (orange lines) by multistep sorting. Peripheral blood samples (4 ml) were spiked with 100 PC-9 cells. The experiment was carried out three times. (E) The mean purity of recovered PC-9 cells from three replicated experiments. Blue line represents multistep sorting using the same chip, and orange line represents multistep sorting performed using a new chip for every sort.*

operations. Subsequently, cells were incubated with 1:500 diluted goat-anti-mouse Alexa Fluor 488 (A-11001, Thermo Fisher Scientific) in 2% BSA for 30 min at room temperature. Next, cells were incubated with anti-PD-L1 Rabbit mAb [28-8] (ab205921, abcam) in 2% BSA for 1 h at room temperature, and then incubated with a 1:500 diluted goat-anti-rabbit Alexa Fluor 647 (A-21245, Thermo Fisher Scientific) in 2% BSA for 30 min at room temperature. Finally, the slide was mounted using VECTASHIELD with 4',6-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories, Burlingame, CA, USA) as a mounting medium. Images were obtained using a fluorescence microscope, Axio Imager M2m and a digital camera, Axio Cam 503 mono (Carl Zeiss, Oberkochen, Germany). Vimentin or PD-L1 pixel intensity of each cell was calculated by subtracting the average pixel intensity of local background for each cell from the average pixel intensity of the area of the entire cell using the ZEN2 software (Carl Zeiss).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). A *p*-value less than 0.05 was considered statistically significant.

Results

Enrichment of rare cells with high cytokeratin-expression by multiple sorting. As an initial validation of multistep sorting, enrichment of 100 PC-9 cells added to 4 ml of peripheral blood in blood collection tubes containing EDTA was examined. PC-

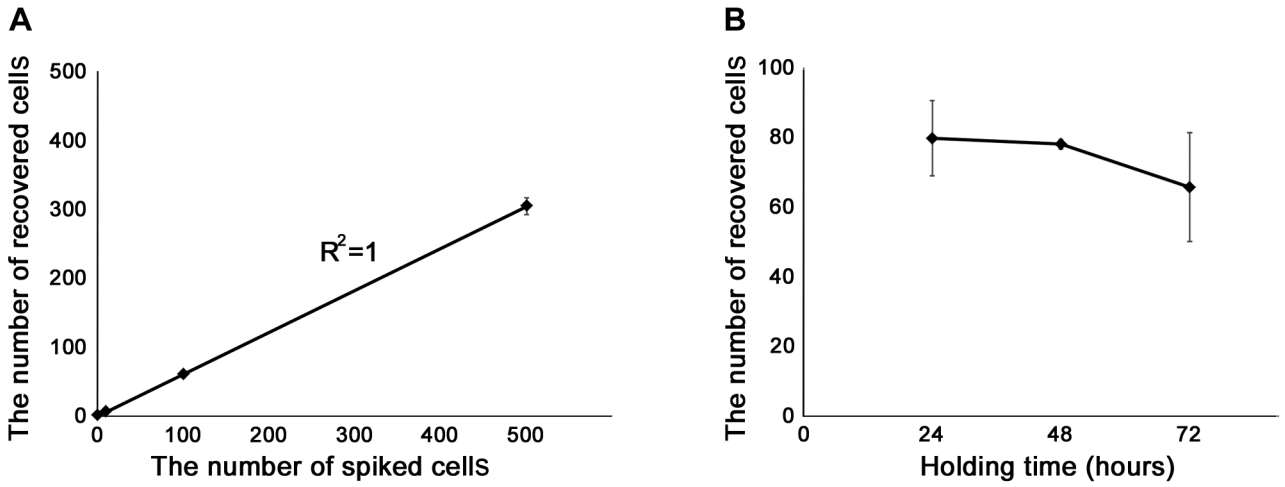

Figure 2. *Linearity of recovery and stability of spiked PC-9 cells into peripheral whole blood in a preservative tube. (A) Linearity of the recovered cell number of spiked PC-9 cells (0, 10, 100, 500 cells) into 20 ml of peripheral whole blood. The linearity was demonstrated by a correlation coefficient (R^2) of 1. Experiment was carried out three times. (B) Stability of 100 PC-9 cells spiked into 10 ml of peripheral whole blood in preservative tubes at 24, 48, and 72 h after blood draw.*

9 cells were identified by an On-chip Sort system using nuclei stained with Hoechst33342. Results were plotted against FITC density (Figure 1A). Cytokeratin-positive cells were distinguished as CD45-negative using PE-Cy7 and results were plotted against FITC density in the second and third steps (Figure 1B and C). Nuclei and cytokeratin-positive, and CD45-negative cells were defined as cancer cells. Figure 1D shows the change in the number of white blood cells and PC-9 cells at each sorting step. White blood cells decreased from approximately 2×10^7 to several dozen when disposable microfluidic chips were replaced with new chips at each sorting step. The number of PC-9 cells remained with a mean recovery of three replicate experiments of 65% (range=63-66%) after three sorting cycles. Purity of the samples sorted using the same microfluidic chip at all steps was 15% on average (range=3-32%). High purity of 83% on average (range=66-97%) of three replicates was achieved after the third sorting when the microfluidic chip was changed at each step (Figure 1E).

Rare cancer cell detection and stability using preservative tubes. Using preserved blood samples, mean recovery of PC-9 cells from three replicates was 61% (range=50-70%). Also, a good correlation was observed between recovered and expected number of cells [correlation coefficient (R_2) of 1 (Figure 2A)]. Stability of PC-9 cells at 24, 48, and 72 h after blood draw was stably recovered with means of 80, 78, and 66%, respectively (Figure 2B). Although variation in recovery was large and mean recovery was somewhat reduced at 72 h, recovery of all experiments was more than 50%. These data suggest that the multistep sorting method is robust for isolating CTCs using preservative tubes.

Confirmation of cancer cells using next-generation sequencing analysis. To evaluate suitability of genome sequencing using recovered cancer cells, recovered PC-9, A549, or MDA-MB231 cells were subjected to NGS after WGA. All known mutations for each cell line using the Ion AmpliSeq Cancer Hotspot Panel v2 kit were successfully detected with sufficient mutant allele frequencies (Table I). Mutations successfully identified included epidermal growth factor receptor (*EGFR*) exon 19 deletion, TP53 single nucleotide variant (SNV), and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) SNV in PC-9 cells; *KRAS* SNV in A549 cells, and *BRAF* SNV, *KRAS* SNV, and *TP53* SNV in MDA-MB231 cells. The level of cytokeratin expression in MDA-MB231 cells was relatively low due to EMT (data not shown), yet all known mutations were successfully detected from recovered MDA-MB231 cells. This result suggests wide applicability of multistep sorting for EMT-like cancer cells despite depending on cytokeratin expression for cell isolation.

Comparison of the levels of PD-L1 and vimentin expression on recovered cancer cells. The intensity of PD-L1 expression on PC-9 or A549 cells, or the intensity of vimentin expression on PC-9 or MDA-MB231 cells demonstrated the suitability of sorted cells for analysis of surface biomarkers. Expression level of PD-L1 was higher on PC-9 than on A549 cells by immunocytochemistry under fluorescent microscopy (Figure 3A and B). Likewise, the intensity of PD-L1 expression of each A549 cell recovered by multistep sorting tended to be lower than individual recovered PC-9 cells (Figure 3C). Further, a significant difference was observed between mean intensity of PD-L1 in samples spiked with

Table I. *Mutation analysis of recovered cancer cells by multistep sorting.*

Cell line	Spiked cell number	Mean depth	Gene	Type	Allele frequency (%)
PC-9	10	1689	*EGFR*	Deletion	70.13
			CDKN2A	SNV	3.18
			TP53	SNV	26.83
	100	1780	*EGFR*	Deletion	97.62
			CDKN2A	SNV	22.73
			TP53	SNV	49.38
	500	1687	*EGFR*	Deletion	100
			CDKN2A	SNV	46.15
			TP53	SNV	74.63
A549	10	1687	*KRAS*	SNV	8.57
	500	2076	*KRAS*	SNV	95.45
MDA-MB231	317	3149	*BRAF*	SNV	36
			KRAS	SNV	28.41
			TP53	SNV	44.58

100 cells of PC-9 or A549 after sorting (Figure 3D). Similarly, consistent with the results of comparing the expression levels of vimentin, a marker for EMT, in PC-9 and MDA-MB231 cells by immunocytochemistry under fluorescent microscopy, the intensity of vimentin expression of each PC-9 cell recovered by multistep sorting was lower than that of individually recovered MDA-MB231 cells after sorting of cells spiked with 300 cells (Figure 4A-D).

Discussion

In this study, a multistep sorting method was developed for cancer cell isolation from up to 20 ml of peripheral whole blood using an On-chip Sort system. Spiking experiments using PC-9 cells showed that the method achieved a mean detection of 65% and mean purity of 83% (Figure 1A and B). Moreover, the method is amenable to a clinical setting since mean recovery from specimens in preservative tubes remained above 60% after 72 h (Figure 2B). In subsequent analyses, known gene mutations in three cancer cell lines were successfully detected in recovered cells by this method without an additional step before WGA (Table I). Moreover, gene mutations can be detected with sufficient allele frequency from the samples that spiked with only 10 cancer cells and from samples spiked with MDA-MB231 cells, which express relatively low levels of cytokeratin. These results suggest that detection of rare cancer cells by multistep sorting is applicable for the specimens with a small number of CTCs with low cytokeratin expression, such as cells from tumors undergoing EMT.

Liquid biopsy is a promising and minimally invasive approach for diagnosis and prognosis. This procedure can be used as an alternative to tissue biopsy, which is highly invasive and difficult or impossible to use for repeated acquisition of specimens for monitoring therapy. Gene mutation analysis using circulating tumor-derived DNA (ctDNA) as a type of liquid biopsy has already been approved for *EGFR* mutation testing (26). ctDNA is an important tool for genomic analysis that reflects the overall gene mutation profile of patients, while CTCs separated cell by cell can be used for analysis of intrapatient heterogeneity (27). Moreover, CTC has the advantage over ctDNA of detection of targeted protein expression for monitoring treatment efficacy. Analysis of CTCs is not only for enumeration of prognostic prediction but also for mutation analysis and expression analysis of targeted protein for prediction of efficacy of treatment and monitoring over time (6). In this study, significant differences in the level of PD-L1 expression between PC-9 and A549 cells were observed after repeated sorting using On-chip Sort (Figure 3C and D). Measurement of PD-L1 on CTC isolated by multistep sorting method may assist in choosing treatment strategies and predicting treatment efficacy. Moreover, differences in the levels of vimentin expression were also observed on PC-9 and MDA-MB231 cells (Figure 4C and D). CTCs from tumors undergoing EMT suggest a poor prognosis for patients with solid tumors (28-31). Also, EMT has been associated with resistance to many kinds of cancer therapy including traditional chemotherapy and targeted therapies (16, 32).

Conventional flow cytometry is a well-established, rapid, and sensitive cell capture procedure that has been used to isolate CTCs from whole blood. However, such capture requires pre-enrichment by density gradient and/or immunomagnetic separation (33-37). Time-consuming pretreatment with multiple steps causes loss of CTCs due to handling and also leads to loss of CTC quality. In addition, immunomagnetic separation, which is frequently used for pretreatment is variable and highly dependent on reagent selection and affinity for antigens (38). On the other hand,

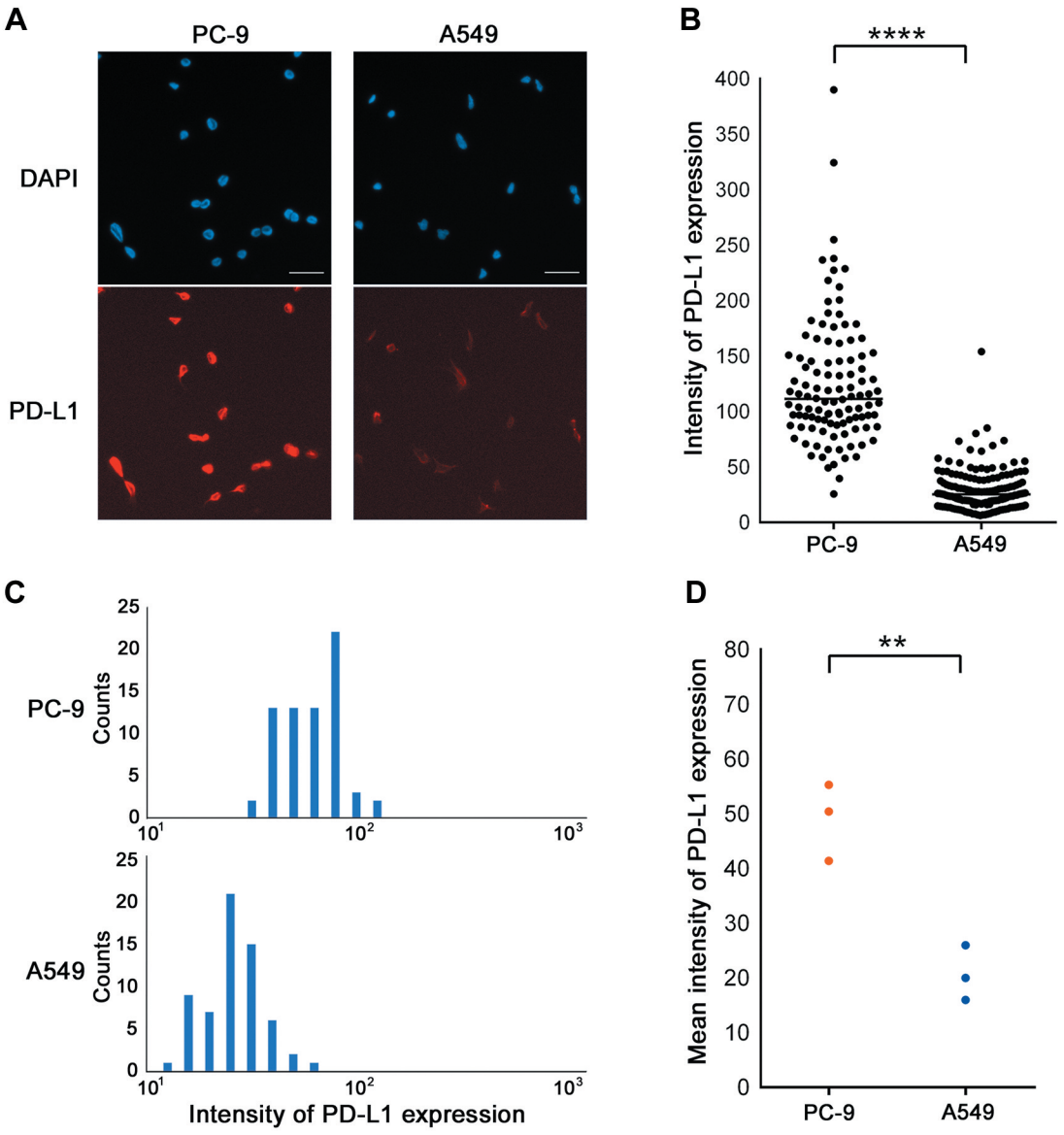

Figure 3. *Intensity of PD-L1 expression between PC-9 and A549 cells. (A) Immunostaining images of DAPI and PD-L1 on PC-9 or A549 cell lines cultured on glass slides. Scale bar represents 50 μm. (B) Comparison of signal intensity of PD-L1 expression between PC-9 and A549 cells observed by immunocytochemistry under fluorescent microscopy. ****p<0.0001. (C) Signal intensity of PD-L1 expression of 100 cells of spiked PC-9 or A549 detected by the On-chip sort system. (D) Comparison of mean signal intensity of PD-L1 expression between 100 cells of spiked PC-9 and A549 cells. Spike-in experiment was carried three times for each cell line and each dot represents the mean signal intensity of recovered cells from each experiment. **p<0.01.*

flow cytometry has been used to purify CTCs captured by CellSearch, followed by WGA from purified CTCs (27, 39). However, purification of CTC after CellSearch is often accompanied by the loss of cells during multiple purification steps and by storage times before purification (40, 41). The proposed multistep sorting method using an On-chip Sort system requires only hemolysis and immunostaining before sorting. The entire process from blood draw to cell isolation is completed within 3 h. The quality of isolated cells should be maintained. Moreover, isolated cells are directly available for subsequent analysis, and losses during handling are minimized. In addition, although most flow cytometry methods are limited to a blood volume from 1 to 7.5 ml, multistep sorting can process 20 ml of whole blood. Recently,

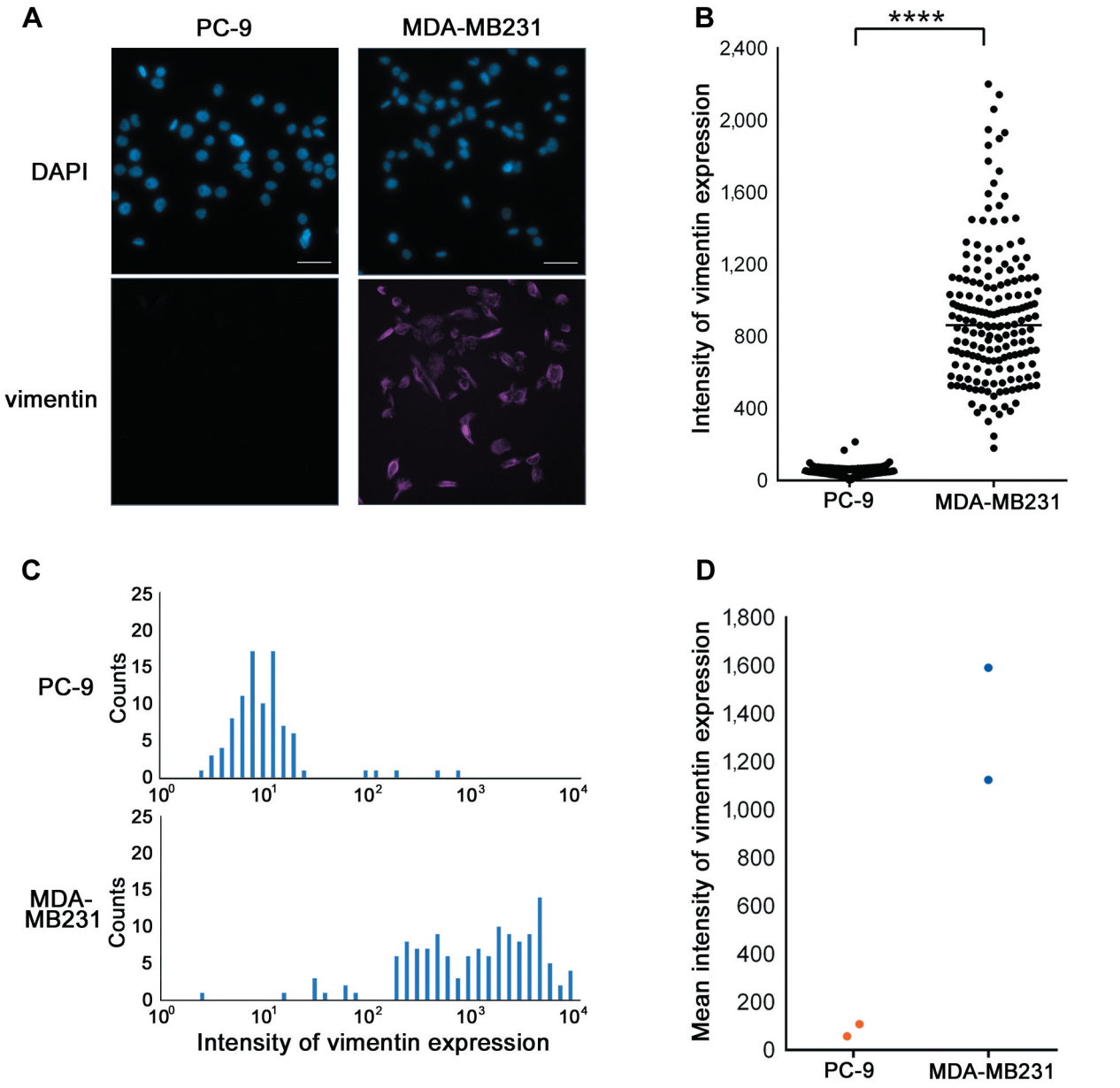

Figure 4. *Intensity of vimentin expression between PC-9 and MDA-MB231 cells. (A) Immunostaining images of DAPI and vimentin on PC-9 or MDA-MB231 cell lines cultured on glass slides. Scale bar represents 50 μm. (B) Comparison of signal intensity of vimentin expression between PC-9 and MDA-MB231 cells observed by immunocytochemistry under fluorescent microscopy. ****p<0.0001. (C) Signal intensity of vimentin expression of 300 cells of spiked PC-9 or MDA-MB231 detected by the On-chip sort system. (D) Comparison of mean signal intensity of vimentin expression between 300 cells of spiked PC-9 and MDA-MB231 cells. Spike-in experiment was carried out two times for each cell line and each dot represents the mean signal intensity of recovered cells from each experiment.*

Lopresti *et al*. reported a rapid CTC isolation method using flow cytometry that is complete within 1 h with minimal procedure and mean recovery of spiked with 10 cells into blood were 86% (range=50-100%) (42); however applicable blood volume for their method was only 1 ml. Since CTCs are extremely rare in whole blood, an effective and simpler way for incremental increases in CTC detection from whole blood is increasing sample volume. Lalmahomed *et al*., compared the number of detected CTCs in 7.5 ml and 30 ml of whole blood from the same patient using CellSearch for a group of 15 patients with colorectal liver metastases, and CTCs were detected in 67% of patient samples with a median

number of one CTC (range=0-4) from 7.5 ml of blood, while CTCs were detected in significantly more (87%) patient samples with a median number of two CTCs (range=0-9) from 30 ml of blood (43). Although approximately 60% of mean recovery of CTCs by the proposed method was not high, a strategy of increasing the volume of blood sample is being considered to raise the number of CTCs detected.

Recent criteria for CTC detection depend on an epithelial marker, cytokeratin, therefore CTCs without cytokeratin expression would not be recovered. To resolve this issue, incorporating expression of vimentin or other EMT-related proteins, such as N-cadherin or CD133, as additional markers is planned. The detection channels of On-chip Sort can expand to 6 channels, another marker can be used with existing equipment for CTC detection and evaluation of therapeutic targets.

In summary, a novel flow cytometry method for CTC detection using an On-chip Sort system was developed that displays recovery and purity of spiked cancer cells sufficient for subsequent NGS analysis. Further, expression levels of PD-L1 and vimentin, predictors of treatment efficacy or prognosis, were evaluated concurrently with CTC isolation. This multistep sorting method is useful and applicable for preserved whole blood samples within 72 h of blood draw and can process a large amount of blood in a shorter time compared to other methods using conventional flow cytometry. This method may be particularly valuable in clinical settings.

Conflicts of Interest

Yasuhiro Koh received research funding from On-chip Biotechnologies Co., Ltd., Masayuki Ishige, Yuu Fujimura, and Kazuo Takeda are employees of On-chip Biotechnologies Co., Ltd., and Kazuo Takeda holds of stock of On-chip Biotechnologies Co., Ltd. The other Authors have no conflicts of interest or financial ties related to this study.

Authors' Contributions

Yasuhiro Koh and Kazuo Takeda designed the study; Mio Ikeda, Jun Oyanagi, Masayuki Ishige and Yuu Fujimura performed experiments and analyzed the data; Mio Ikeda, Yasuhiro Koh and Kazuo Takeda wrote or revised the manuscript; Shunsuke Teraoka, Nahomi Tokudome, Yuichi Ozawa Hiroki Ueda and Nobuyuki Yamamoto critically reviewed and discussed the content of this study; Yasuhiro Koh and Nobuyuki Yamamoto supervised the study.

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