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Isolation and molecular analysis of circulating tumor cells from lung cancer patients using a microfluidic chip type cell sorter

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Circulating tumor cells (CTCs) are a tumor-derived material utilized for liquid-based biopsy; however, capturing rare CTCs for further molecular analysis remains technically challenging, especially in non-small-cell lung cancer. Here, we report the results of a clinical evaluation of On-chip Sort, a disposable microfluidic chip-based cell sorter, for capture and molecular analysis of CTCs from patients with lung adenocarcinoma. Peripheral blood was collected from 30 metastatic lung adenocarcinoma patients to enumerate CTCs using both On-chip Sort and CellSearch in a blind manner. Captured cells by On-chip Sort were subjected to further molecular analysis. Peripheral blood samples were also used for detection of *EGFR* mutations in plasma using droplet digital PCR. Significantly more CTCs were detected by On-chip Sort (22/30; median 5; range, 0–18 cells/5 mL blood) than by CellSearch (9/30; median, 0; range, 0–12 cells/7.5 mL) ($P < 0.01$). Thirteen of 30 patients who had a negative CTC count by CellSearch had a positive CTC count by On-chip Sort. *EGFR* mutations in CTCs captured by On-chip Sort were observed in 40.0% (8/20) of patients with *EGFR*-mutated primary tumor. *EGFR* mutations were often observed in 53.3% (8/15) of patients detected in plasma DNA. Expressions of EGFR and vimentin protein on CTCs were also successfully assessed using On-chip Sort. These results suggest that On-chip Sort is an efficient method to detect and capture rare CTCs from patients with lung adenocarcinoma that are undetectable with CellSearch. Mutation detection using isolated CTCs remains to be further tackled (UMIN000012488).

KEYWORDS
cell sorter, circulating tumor cell, EGFR, liquid biopsy, lung cancer

1 | INTRODUCTION

Lung cancer is the most common cause of cancer-related deaths, and approximately 84% of new lung cancer cases are classified as non-small-cell lung carcinomas (NSCLC), and 15% as small-cell carcinomas,[1] with the majority of patients being diagnosed at an advanced stage (56%).[2] Recent advances in molecularly targeted cancer therapy have offered a wide variety of therapeutic strategies for patients with NSCLC. For example, identification of *EGFR*-activating mutations in patients with NSCLC is required prior to starting treatments with

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epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs).[3] However, kinase inhibition frequently leads to the appearance of drug resistance mutations within the target kinase itself, such as the *EGFR* T790M mutation.[4] In addition to identifying gene mutations, there is also a need for the detection of protein expression and gene amplification of targeted molecules on primary tumor cells, for further stratification of patients.[5] To optimize treatment, real-time monitoring of tumors over the course of the treatment, especially at the point of treatment failure, is necessary. However, the classic biopsy approach does not allow monitoring of primary tumor evolution over time, and sampling of metastatic sites is not always possible for practical reasons.

Through a simple blood draw, circulating tumor cells (CTCs) could potentially serve as an alternative to the tumor tissue as a source of material for the detection of genetic alterations, an approach that is termed "liquid biopsy"[6] owing to its minimal invasiveness. To date, the CellSearch system (Veridex, Raritan, NJ, USA) is the only US FDA-approved CTC enumeration system for the provision of prognostic information regarding survival.[7–11] However, CTCs are very rare and make up a small minority of cells circulating in blood, so their molecular analysis beyond enumeration is technically very challenging.[6,12] Various methods to overcome this issue have been under development and evaluation.[13–17]

The potential of single cell sorting by FACS and whole-genome amplification of CTCs after CellSearch was described previously.[18,19] The main limitation of both of these approaches is that only epithelial cell adhesion molecule (EpCAM)-positive epithelial cells can be isolated and analyzed, because this is the isolation system used by CellSearch. Thus, invasive phenotypes of CTCs that undergo epithelial–mesenchymal transition (EMT) cannot be analyzed.[20]

Recently, we have established a protocol for rare CTC enumeration and sorting using a newly developed cell sorting system.[21,22] This cell sorting system, called On-chip Sort (On-chip Biotechnologies, Tokyo, Japan), is a novel benchtop cell sorter equipped with a disposable microfluidic device, allowing the detection and isolation of rare tumor cells for subsequent molecular analyses.[21] This protocol also enables a detection of EpCAM-negative/cytokeratin (CK)-negative cells using the incorporation of an EMT marker.[22] These results indicate that our system is a precise system for the detection and capture of tumor cells within whole blood. To confirm our previous findings, we compared the capacity and efficiency of our On-chip Sort system and the current gold standard CellSearch system in undertaking CTC detection and enumeration in whole blood samples drawn from a cohort of patients with lung adenocarcinoma. Also, we carried out molecular characterization of the sorted CTCs and analysis of circulating tumor DNA using droplet digital PCR in paired blood samples.

2 | MATERIALS AND METHODS

2.1 | Study design and ethics statement

This prospective study was carried out to evaluate CTC analysis using the CellSearch system and the On-chip Sort system in patients with advanced lung cancer in a blinded experiment (UMIN clinical trial registry no. UMIN000012488). The presence of CTCs was assessed individually according to their criteria before other results were known. The study inclusion criteria were age 20 years or older when giving informed consent, histologically or cytologically confirmed advanced NSCLC, and enrollment at the Shizuoka Cancer Center (Shizuoka, Japan). The institutional review boards of the Shizuoka Cancer Center approved the study protocol, and all patients and healthy volunteers provided written informed consent. Blood was collected from each of the 30 patients and 10 healthy volunteers (5–15 mL) in EDTA tubes for CTC capture by the On-chip Sort system in our laboratory (Shizuoka Cancer Center), 5 mL was collected in EDTA tubes for genotyping plasma DNA using digital PCR, and 10 mL was collected in CellSave collection tubes (Menarini Silicon Biosystems Inc, PA) (San Jose, CA, USA) for CTC enumeration by the CellSearch system in the laboratory of SRL (Tokyo, Japan).

2.2 | Circulating tumor cell enumeration and capture using the On-chip Sort system

Human blood samples were collected in a collection tube with EDTA to prevent coagulation and used within 2 h. Blood from each lung cancer patient (5–15 mL) and healthy volunteer (5 mL) was subjected to CTC capture.

Immunomagnetic enrichment was carried out as described previously.[21,22] Briefly, 5 mL of each blood sample was lysed by lysing solution (BD Biosciences, San Jose, CA, USA), and then was negatively enriched using Dynabeads coated with anti-CD45 mAb (Thermo Fisher Scientific Inc., Waltham, MA, USA) to remove white blood cells, followed by fixation and labeling with the FITC-conjugated anti-CK mAb CK3-6H5 (1:25 dilution; Miltenyi Biotec, Bergisch-Gladbach, Germany), the phycoerythrin-conjugated anti-vimentin mAb D21H3 (1:50 dilution; Cell Signaling Technology, Danvers, MA, USA), and the Alexa Fluor 700-conjugated anti-CD45 mAb F10-89-4 (1:20 dilution; AbD Serotec, Oxford, UK). For some patient samples from whom >10 mL of blood was obtained, additional labeling with Alexa Fluor 647-conjugated anti-EGFR mAb D38B1 was carried out (1:50 dilution; Cell Signaling Technology). Samples were incubated overnight at 4°C in the dark.

Enumeration and sorting of cells were carried out by On-chip Sort according to the manufacturer's instructions and as described previously.[22] Briefly, the flow path was prewashed with 1× Through Path Plus (On-chip Biotechnologies) and the On-chip sample buffer (1× Through Path Plus with 1.5% polyvinylpyrrolidone; On-chip Biotechnologies). Stained samples were dissolved in 25 μL On-chip sample buffer and then sorted with up to 400 events/s flow rate (approximately 1 μL/min = 1.8 kPa in the On-chip Sort setting). Total events were approximately 1×10^5 to 10^6 events per sample. The sorting time required for all the samples was approximately 30 min to 2 h.

The cells gated into the CK- and/or vimentin-positive and CD45-negative channels (Fig. S1A) were collected into the collection reservoir and then observed under a fluorescence microscope (Biorevo

BZ-9000; Keyence, Osaka, Japan) to confirm that the cells were CK- and/or vimentin-positive and CD45-negative. All steps were carried out at room temperature.

In clinical trials, an entire FACS dataset had been obtained using an On-chip Sort. Subsequently, FACS data analysis had been obtained using FlowJo software version 7.6.5 (BD Biosciences) and objects that satisfied the predetermined criteria as described below had been counted. A typical example for the full gating strategy is shown in Figure S1(B). Gating of the CTCs by On-chip Sort was carried out using the CK-FITC staining *versus* vimentin–phycoerythrin staining density plot. The lower limit of the gate that discriminates CTC signals from WBCs autofluorescence as well as from debris was determined by several runs of non-spike experiments using healthy donor control bloods. The CK- and/or vimentin-positive events were then subjected to CD45-negative gating to distinguish tumor cells from WBCs and/or debris. Finally, cell debris was removed using the forward scatter *versus* side scatter density plot.

2.3 | Circulating tumor cell enumeration using the CellSearch system

Whole blood samples were maintained at room temperature, mailed overnight to the laboratory of SRL, and processed within 72 h of collection. All CTC evaluations were carried out without knowledge of patient clinical status, and the results were reported quantitatively as the number of CTCs/7.5 mL blood. Circulating tumor cells were defined as EpCAM-isolated intact cells showing positive staining for cytokeratin and negative staining for CD45. In accordance with previous evaluations of the CellSearch system,[23] a patient was considered CTC-positive if >1 CTC/7.5 mL blood was detected in the patient's sample.

2.4 | Primary tissue DNA sequencing

Tumor biopsy samples from patients before treatment were outsourced for detection of the *EGFR* mutation using the Scorpion-ARMS method and *EML4-ALK* fusion analysis immunohistochemistry confirmed by FISH by an independent clinical laboratory (SRL). The *KRAS* mutation was evaluated in another clinical study.[24]

2.5 | Mutation analysis of CTCs sorted by On-chip Sort

Sorted cells from all sample tubes (Tube 1, 2, and EGFR) were transferred from the collection reservoir to a 200-μL PCR tube, and the collection reservoir was rinsed with sheath solution twice. After centrifugation (600*g* for 10 min), the supernatant was carefully removed to leave ~1 μL, which was the starting volume of the whole-genome amplification (WGA) procedure. Whole-genome amplification was undertaken using the *Ampli*1 WGA kit (Silicon Biosystems, Bologna, Italy) following the manufacturer's protocol and as described previously.[22] Two microliters of amplified DNA product was subjected to mutation analysis using pyrosequencing to detect the *EGFR* L858R and T790M mutations, which have been previously developed,[24] and using an *Ampli*1 EGFR Seq Kit (Silicon Biosystems) for *EGFR* exon 19 deletion followed by fragment analysis following the manufacturer's protocol.

2.6 | Mutation analysis of plasma DNA

Five milliliters of whole blood was centrifuged at 1500*g* for 10 min at 4°C. The plasma supernatant was transferred to 50-mL conical tubes (Falcon; BD Biosciences) and stored at −80°C until use. Plasma DNA was isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was eluted in AVE buffer (30 μL). DNA concentration was measured with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc.).

Droplet digital PCR (ddPCR) for the common *EGFR* mutations (L858R, exon 19 deletion, and T790M) was carried out in separate reactions for each mutation-specific probe on the RainDrop digital PCR system (RainDance Technologies, Billerica, MA, USA) following the manufacturer's instructions (see also Doc. S1). Eight microliters of plasma DNA was used for each assay. The amplification primers and probes for mutations in *EGFR* are described in Table S1. Results are reported as percentage of mutant allele, as done by prior investigators.[25]

2.7 | Statistical analysis

GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses. Statistical significance of difference was determined using the Wilcoxon test.

3 | RESULTS

3.1 | Sensitivity of the On-chip Sort system in CTC detection

In our previous study, varying numbers of various tumor cell lines were spiked into blood, and tumor cell isolation was evaluated using our On-chip Sort system.[22] The calculated detection efficiency was constant and >70% when 5–25 tumor cells were present per 4 mL blood, and there was a 100% success rate in the detection of *EGFR*, *KRAS*, and *BRAF* mutations from captured tumor cells.[22] The limit of blank of the On-chip Sort system was determined as a finite number of false-positive events of CTCs detected per assay. According to 10 healthy volunteer samples, the number of false-positive events was two events/5 mL blood (95% confidence interval of the Poisson model fit; Fig. S2). Therefore, a patient was considered CTC-positive if >2 CTCs/5 mL blood were detected by the On-chip Sort system.

3.2 | Patients and tumor tissue-derived genotypes

Patient characteristics are shown in Table 1. Tumor specimens with corresponding clinical and pathological information were collected

from all patients. The scorpion-ARMS method sequencing of these 30 primary tumor specimens identified 13 *EGFR* exon 19 deletion mutations, six L858R mutations, and one deletion with *EGFR* T790M mutation yielding a frequency of 66.7% (20 of 30 tumors; Table 1). Two patients (7%) had *KRAS* mutation, one (3%) had *EML4-ALK* gene fusion, and four (13%) had no major driver mutations. Blood samples were collected at the time of pretreatment (one patient), in the middle of first-line treatment (eight patients), and after first-line treatment (21 patients).

3.3 | Enumeration of CTCs using the On-chip Sort and CellSearch systems

To carry out blind comparisons of the detection sensitivity of the On-chip Sort and CellSearch systems, blood samples were collected from 30 patients with advanced NSCLC between December 2013 and March 2014. As a result, 22 of 30 (73.3%) patients were identified as CTC-positive using the On-chip Sort system, but only 9 of 30 (30%) patients using the CellSearch system (Fig. 1, Table S2). Of these patients, the nine identified as CTC-positive by CellSearch were confirmed as CTC-positive by the On-chip Sort system. The On-chip Sort system identified 13 additional CTC-positive patients. These results revealed that more CTCs were detected by the On-chip Sort system (median cell count, 6.5; range, 0–27 cells/7.5 mL blood; Fig. 1) than by the CellSearch system (median cell count, 0; range, 0–12 cells/7.5 mL blood; Fig. 1), suggesting the statistical superiority of the On-chip Sort system in CTC enumeration ($P < 0.01$, Wilcoxon test; Fig. 1A), and that CTC counts obtained with both methods were not correlated ($R^2 = 0.0148$) (Fig. 1B). There was no correlation between CTC counts and treatment line ($R^2 = 0.0478$; Fig. S3). The correlation between the measured CTC numbers in two subsequent tubes was moderately correlated with a correlation coefficient of 0.6737 (Fig. S4).

TABLE 1 Characteristics of patients with non-small-cell lung carcinoma ($n = 30$)

No. of patients	$n = 30$
Gender	
Male	14
Female	16
Median age, years	66
Range	38-83
Smoking	
Never smoker	13
Smoker	17
Mutation	
EGFR exon 19 deletion	13
EGFR exon 19 deletion + *EGFR* T790M	1
EGFR L858R	6
EGFR G719X	3
KRAS	1
EML4-LK	1
No mutation	4
Mutation	
Pretreatment	1
Ongoing first line	8
After first line	21

3.4 | Morphological features and protein expression of CTCs sorted using the On-chip Sort

Sorted CTCs in the sorting reservoir chamber were observed under a fluorescent microscope, and then identified as being CK- and/or vimentin-positive, and CD45 negative, on the basis of analysis of fluorescent images. As can be observed in Figure 2, which shows a representative gallery of CTCs identified by image analysis, CTCs expressed cytokeratin alone (CTC #1), vimentin alone (CTC #2), or both (CTC #3). No clustered CTCs (circulating tumor microembolus) were observed in this cohort.

We assessed the EGFR protein expression and vimentin, EMT marker, expression on detected CTCs. We assessed CTCs from 22 patients for vimentin protein, and found them to be differently expressed between CTCs from the same patients, ranging, for example, from negative to strongly positive (Fig. 3). We also assessed CTCs from 20 patients for EGFR protein expression; EGFR-positive CTCs (>200 intensity of EGFR-Alexa647) could be observed in 12 of 20 (60.0%) patients, with only 3 of 20 patients (15.0%) possessing strongly EGFR-positive CTCs (patients #10, #11, and #20) (Fig. 3A). The fluorescent signal from EGFR-conjugated Alexa647 was observed in the peripheral region of the cell (Fig. 3B). All CTC events detected in healthy volunteers were EGFR-negative (data not shown).

3.5 | *EGFR* mutations detected in isolated CTCs

Given the challenge with obtaining liquid biopsy from lung cancer patients, we sought to determine therapeutically variable mutations in *EGFR* from isolated lung adenocarcinoma CTCs. Pooled CTCs sorted by On-chip Sort were subjected to WGA, and WGA products were successfully amplified in all samples with enough genetic material for genotyping (data not shown). Amplified DNA samples were analyzed for *EGFR* L858R and T790M using pyrosequencing, and exon 19 deletion using fragment analysis for detection. We have previously shown the detection of rare alleles down to 10% frequency, even in a few target cells.[22]

In CTCs from patients, the common EGFR-activating mutations were detected in 9 of 30 cases, with the remaining 21 cases sequenced as wild-type at these nucleotide positions. Among 20 patients with tumor biopsy-derived EGFR-activating mutation, 8 (40%) were concordant for activation mutation status (Table 2). *EGFR* T790M mutation was also identified in the CTCs of two patients (#8 and #23). One of them (#23) was not detected in primary tumors; in this patient, CTCs were collected after first-line EGFR-TKI therapy (Table 2).

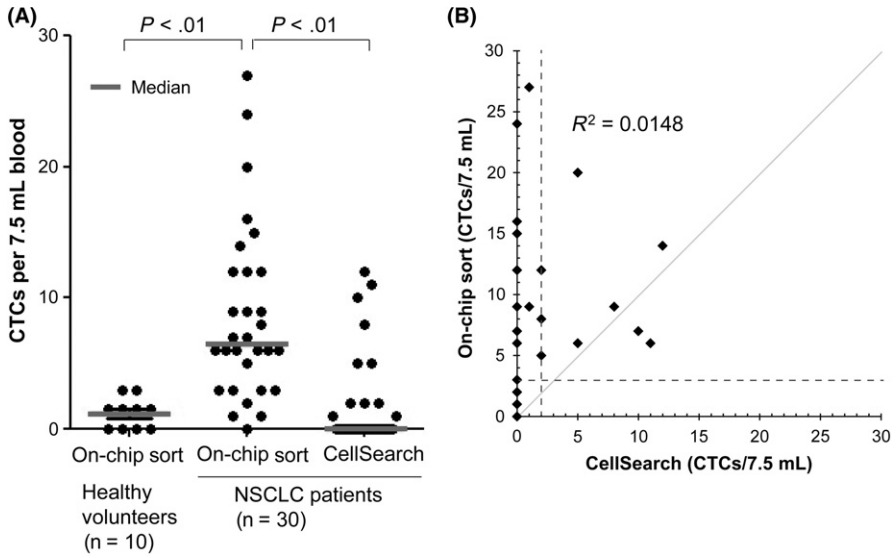

FIGURE 1 Circulating tumor cell (CTC) count using the On-chip Sort system compared with the CellSearch system. (A) CTC count/7.5 mL blood is shown for 10 healthy donors and 30 patients with non-small-cell lung carcinoma (NSCLC). Paired blood samples were analyzed by CellSearch according to the manufacturer's protocol and On-chip Sort by immunolabeling analysis. (B) Direct comparison of CTC counts between CellSearch and On-chip Sort. Gray line represents the theoretical perfect correlation. The cut-off levels (3 CTCs for On-chip Sort and 2 CTCs for CellSearch per sample) are indicated by the dashed lines

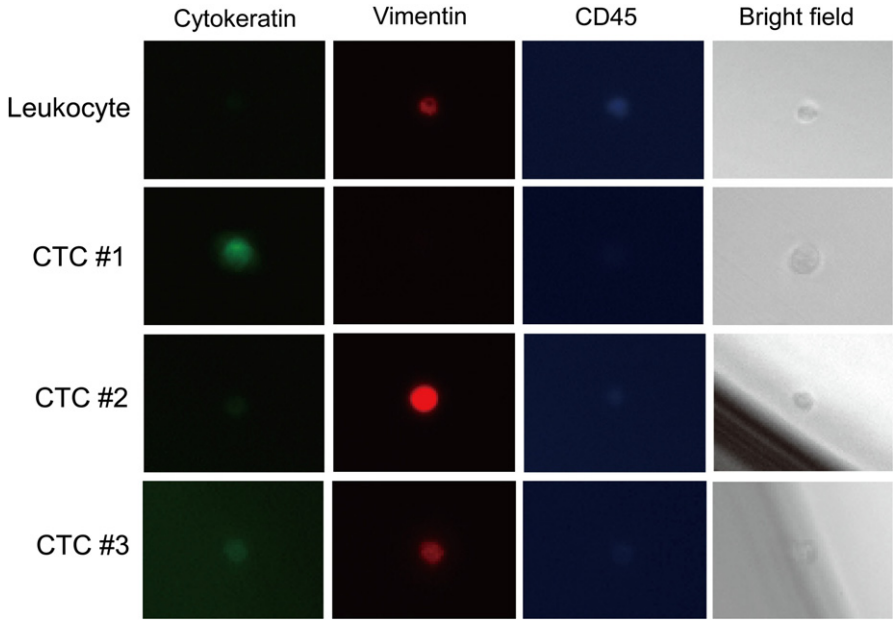

FIGURE 2 Gallery of cells captured by On-chip Sort from patients with non-small-cell lung carcinoma. Cells were stained with FITC-labeled anti-cytokeratin antibody, phycoerythrin-labeled anti-vimentin, and Alexa700-labeled anti-CD45 antibody. CTC, circulating tumor cell

3.6 | *EGFR* mutations detected in plasma DNA

Because genotyping in tumor biopsy was not undertaken concurrently with the study blood collection, matched plasma samples were collected for all patients as a reference. The amount of total plasma DNA varied among samples, ranging from 6.0 to 56.1 ng (Table S3), which is consistent with prior reports.[25–28] These plasma samples were analyzed for three common *EGFR* mutations as described above using ddPCR for detection, which is known to be ultrasensitive.[29,30] The limit of blank of the ddPCR assay was determined as a finite number of false events per assay using mutant and wild-type plasmids. The number of false-positive droplet events for each of the three EGFR assays is six for L858R, four for exon 19 deletion, and three for T790M (Table S4).

Droplet digital PCR detected 14 patients with common EGFR-activating mutations and nine patients were also identified to have

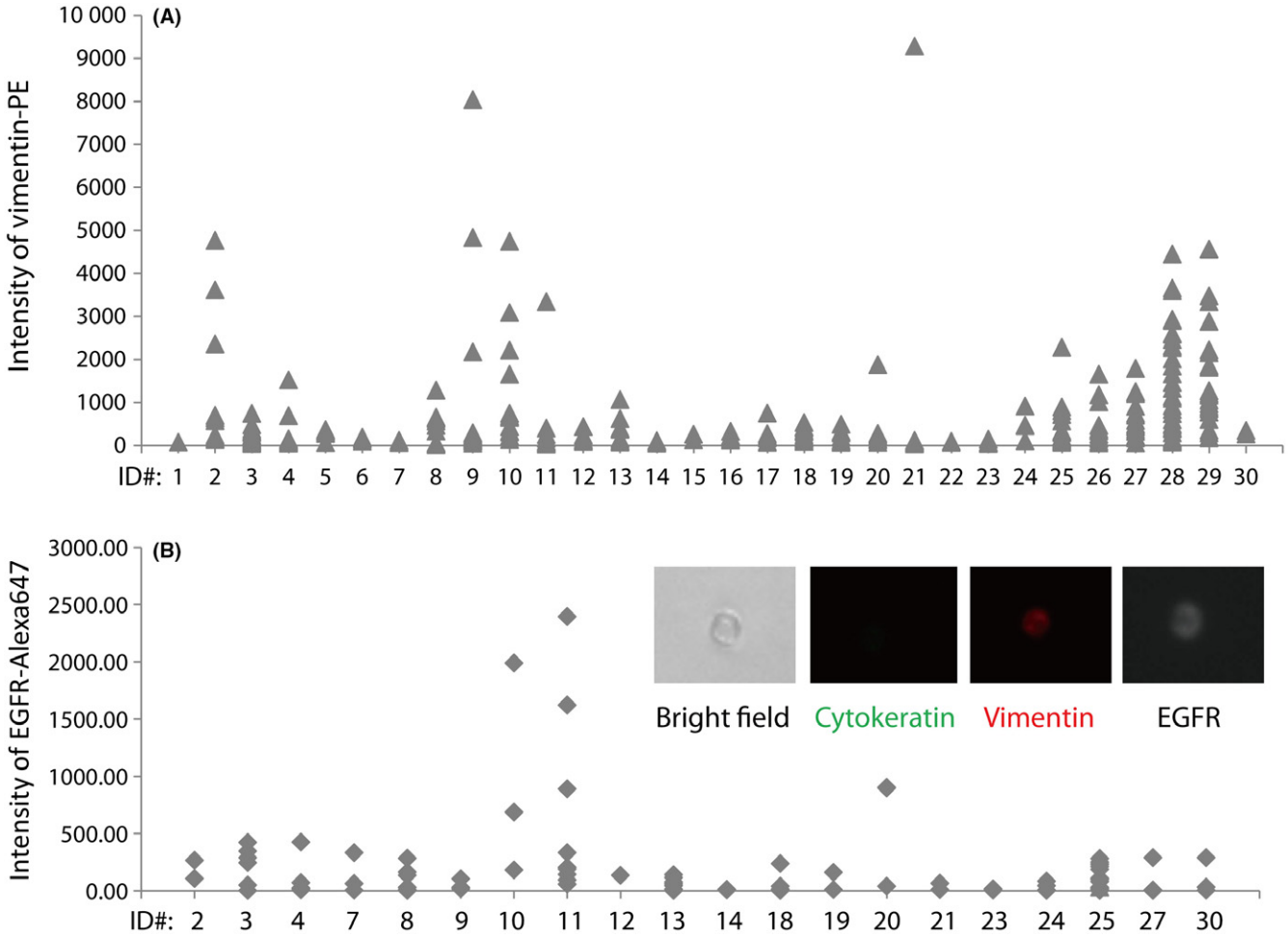

FIGURE 3 Assessment of protein expression in circulating tumor cells (CTCs). Vimentin (A) and epidermal growth factor receptor (EGFR) (B) expressions were assessed by On-chip Sort during sorting. Intensity of fluorescent-labeled protein expression on CTCs was analyzed by FlowJo software 7.6.5. Cell images of EGFR expression on CTCs are embedded in (B). PE, phycoerythrin

an additional *EGFR* T790M mutation (Table 2). One patient (#20) had only the *EGFR* T790M mutation. Four patients (patient #2, #4, #14, and #30) had all three mutations. A comparison of *EGFR* mutation status of CTCs with the corresponding ddPCR plasma DNA results indicated that *EGFR* mutations could be identified in the CTCs of eight patients whose plasma DNA had mutated *EGFR*. Fifteen patients with *EGFR* wild-type plasma DNA had no detectable *EGFR* mutation in CTC (Table 2). These data show a sensitivity of 53.3% and specificity of 100.0% of mutation detection for mutant *EGFR* in CTCs (Table S5).

4 | DISCUSSION

In the present study, CTCs from metastatic NSCLC patients isolated on the On-chip Sort were successfully stained with fluorescent-labeled antibodies that target tumor cell markers. Tube-to-tube variation in CTC counts was confirmed by regression analysis with a correlation coefficient (R^2) of 0.6737, suggesting that our CTC counts are a reproducible and independent with any range of counts.

The On-chip Sort system was found to possess higher detection sensitivity than the CellSearch system in lung adenocarcinoma CTC enumeration, suggesting the superiority of non-EpCAM-based isolation techniques compared to EpCAM-based techniques. The low EpCAM expression in lung adenocarcinoma CTCs has been previously reported. The CTC positivity rates were recorded between 67% and 84% using the EpCAM-independent system,[31–34] whereas EpCAM-dependent systems detected between 12% and 32% of patients.[32–35] In line with these results, in this study, CTCs were detected in 73.3% of patients using the On-chip Sort system and in 30% of patients using the CellSearch system. Our result confirmed that EpCAM-independent approaches are effective to detect CTCs from patients with metastatic NSCLC.

The present study was devised to evaluate the correlation between *EGFR* mutation status in basal tumor biopsies and matching CTCs of patients with NSCLC. Peripheral blood specimens were subjected to CTC preparation by the On-chip Sort system as well as investigated for *EGFR* mutations by pyrosequencing. Of 20 patients with *EGFR* mutations in the original diagnosis biopsy, 19 samples had >1 CTC and 40% (8/20) were concordant between the CTC

TABLE 2 Analysis of *EGFR* mutations in primary tumors, circulating tumor cells (CTCs), and plasma from patients with non-small-cell lung carcinoma

Sample ID#	CTC count	*EGFR* mutations in CTCs (allele frequency %)	*EGFR* mutation in plasma (allele frequency %)	*EGFR* mutations in primary tissue	Time point
1	0	Wild type	L858R (67.4%), T790M (32.8%)	L858R	Post-gemcitabine as 4th line
2	6	Ex19 del	L858R (6.4%), Ex19 del (10.3%), T790M (6.4%)	Ex19 del	On palliative RT
3	13	Ex19 del	Ex19 del (12.4%)	Ex19 del	On erlotinib as 3rd line
4	5	Ex19 del	L858R (9.7%), Ex19 del (25.6%), T790M (11.5%)	Ex19 del	On gefitinib as 1st line
5	2	Wild type	Wild type	Wild type (*EML4-ALK*)	On ALK inhibitor as 4th line
6	2	Wild type	L858R (9.2%)	L858R	Post-docetaxel as 2nd line
7	2	Wild type	Wild type	Ex18 G719X	On amrubicin as 5th line
8	6	Ex19 del, T790M (21%)	Ex19 del (24.8%), T790M (22.4%)	Ex19 del, T790M	Post-carboplatin, paclitaxel, and bevacizumab as 3rd line
9	11	Wild type	Wild type	L858R	On erlotinib as 4th line
10	8	Wild type	Wild type	Wild type (KRAS Q61H)	On gemcitabine as 3rd line
11	9	Wild type	Wild type	Wild type	Post-pemetrexed as 4th line
12	3	Wild type	Wild type	Ex19 del	On carboplatin, paclitaxel, and bevacizumab as 3rd line
13	4	Wild type	Wild type	Ex19 del	On carboplatin, paclitaxel, and bevacizumab as 2nd line
14	4	Wild type	L858R (1.1%), Ex19 del (8.5%), T790M (1.9%)	L858R	On erlotinib as 3rd line
15	1	Wild type	Wild type	Ex19 del	On cisplatin and pemetrexed as 2nd line
16	8	Wild type	Wild type	Wild type	On carboplatin, paclitaxel, and bevacizumab as 1st line
17	4	Wild type	L858R (1.0%)	L858R	On carboplatin, paclitaxel, and bevacizumab as 3rd line
18	6	Ex19 del	Ex19 del (34.0%)	Ex19 del	On gefitinib as 1st line
19	4	Wild type	Wild type	Wild type (KRAS Q61H)	On gemcitabine as 7th line
20	4	Wild type	T790M (4.1%)	Ex18 G719X	On gefitinib as 1st line
21	5	Wild type	Wild type	Ex19 del	On erlotinib as 4th line
22	1	Ex19 del	Ex19 del (51.0%), T790M (13.0%)	Ex19 del	On gefitinib as 1st line
23	4	L858R (10%), T790M (17%)	L858R (21.2%), T790M (6.9%)	L858R	Post-gefitinib as 1st line
24	2	Wild type	Wild type	Wild type	Post-cisplatin and pemetrexed as 1st line
25	16	Ex19 del	L858R (64.2%)	Wild type	Pretreatment
26	5	Wild type	Wild type	Ex19 del	On carboplatin, paclitaxel, and bevacizumab as 7th line
27	8	Wild type	Wild type	Ex18 G719X	Post-chemoradiotherapy
28	18	Wild type	Wild type	Ex19 del	Post-cisplatin and pemetrexed as 2nd line
29	10	Wild type	Ex19 del (17.9%), T790M (1.4%)	Ex19 del	Post-gefitinib as 1st line
30	1	Ex19 del	L858R (6.0%), Ex19 del (14.6%), T790M (5.2%)	Ex19 del	Post-pemetrexed as 4th line

ALK, anaplastic lymphoma kinase; Ex19 del, exon 19 deletion; RT, radiotherapy.

analysis and tissue biopsy analysis. Interestingly, *EGFR* mutations were detected in samples that had detected only one CTC (#22 and #30), consistent with the previous study in a spike-in model.[22] This sensitivity is slightly lower than those in previous studies.[36,37] The primary reason for this lower sensitivity could be due to the timing of biopsy. Whereas tissue and liquid biopsies were obtained at the

same time in previous studies,[36,37] only one sample (#25) was obtained simultaneously in our study. In fact, when matched plasma DNA was used for the reference of *EGFR* mutations, concordance was increased (53.3%).

The ddPCR assay on cell free DNA was much more sensitive at detecting *EGFR* mutations than mutation detection using CTC. This result is in line with those reported previously.[38,39] Mutation detection using isolated CTCs by the On-Chip Sort assay might not be as sensitive as plasma assay in NSCLC, according to the results. However, CTCs offer several unique advantages over the ability to undertake cytomorphological analysis, immunocytochemistry, or FISH assay.[12] Most importantly, CTCs can be used for evaluating protein expression, which can be a target of cancer therapeutics. The application of protein staining using the FL5 channel of the On-chip Sort enables the classification of vimentin expression as surrogate of the EMT and EGFR protein expression levels on single CTCs. These applications might be utilized as a treatment choice for patients with NSCLC. The patients with NSCLC whose tumors have detectable EGFR protein could benefit from the addition of necitumumab to chemotherapy.[40] The AXL protein, associated with EMT, could be a promising therapeutic target for acquired resistance to EGFR TKIs.[41]

Recently, immune checkpoint blockade with antiprogrammed death 1 (PD-1)/PD-ligand 1 (PD-L1) antibodies was reported to be efficacious for lung cancer patients,[42] and PD-L1 expression on tumor tissues could be a predictive biomarker of clinical benefit in patients with NSCLC.[43,44] However, it is still challenging to obtain a tumor biopsy from patients with lung cancer, and tumor heterogeneity is still issue for an PD-L1 immunohistochemistry.[45] We believe that CTCs can be used as an alternative material, instead of tumor tissues, for detecting PD-L1 expression. Preliminary data have been shown by several groups.[46–48]

There are some challenges to be addressed with the On-chip Sort System. To capture the most CTCs, the sorting gate was expanded to the maximum. This might lead to the reduction in the ratio of CTCs in background normal blood cells. The optimization of this sorting gate should increase the detection rate of mutations. The prognostic and predictable value of CTCs detected by the On-chip Sort system needs to be evaluated. Lindsay *et al.* recently showed the prognostic value of CTCs counted by the CellSearch system in patients with treatment-naïve stage IIIb/IV NSCLC.[49] Punnoose *et al.*[50] reported that higher baseline CTC counts were associated with response to treatment and decreased CTC counts following treatment were associated with longer progression-free survival. The prognostic and predictive value of CTCs counted by our system will be evaluated in future clinical studies with optimization of timing of blood collection.

In conclusion, we have evaluated the performance of our On-chip Sort system in detecting CTCs in whole blood from patients with lung cancer. We have also evaluated whether CTC preparations obtained by the On-Chip Sort system represent a suitable source of tumor DNA for efficient detection of *EGFR* mutations. The results were promising and suggest that the On-Chip Sort system has clinical potential for CTC diagnosis in lung cancer. Further prospective investigation with scheduled biopsies on a larger scale is warranted.

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DISCLOSURE STATEMENT

The authors have no conflict of interest.

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