



Enumeration and molecular characterization of circulating tumor cells enriched by microcavity array from stage III non-small cell lung cancer patients

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Background: Various methods of liquid biopsy through the sampling of blood in cancer patients allow access to minuscule amounts of tumor that can easily be sampled repeatedly throughout therapy. Circulating tumor cells (CTCs) represent shed tumor cells that can be characterized by imaging or molecular techniques using an amenable enrichment platform. Here we validate the Hitachi Chemical Micro Cavity Array (MCA) for the enrichment of CTCs from the blood of patients diagnosed with stage III non-small cell lung cancer (NSCLC). MCA is a semi-automated filtration system that enriches CTCs on the basis of size and membrane deformability rather than a biased selection of surface antigens.

Methods: CTCs were enriched from the peripheral blood of 38 patients diagnosed with stage III NSCLC at the start of chemoradiation. Two tubes of EDTA blood were collected from each patient and processed through MCA in parallel. In the first tube, CTCs were identified as pan-cytokeratin (CK)+ CD45- nucleated cells and enumerated. The second tube was depleted of leukocytes using CD45 antibody-coated magnetic microbeads before enrichment by MCA, followed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) to interrogate CTC-enriched lysates for expression of 16 target mRNAs from a panel of epithelial, mesenchymal, stem-like, and cancer signaling-related genes. CTC-enriched lysates from similarly prepared peripheral blood samples from 18 healthy donors were used to define positive gene expression.

Results: CTCs were identified by imaging in 30 of 38 patient samples (79%). At least 1 target gene was positively expressed in 23 of 25 (92%) patient samples that was subjected to molecular characterization. A CTC count of ≥ 7 was associated with poor progression-free survival (PFS) [hazard ratio (HR) 4.24, 95% confidence interval (CI), 1.73–10.40, $P=0.020$] and poor overall survival (HR 8.17, 95% CI, 2.87–23.26, $P<0.001$). Expression of BCL2 by MCA-enriched CTCs was associated with poor PFS (HR 3.11, 95% CI, 1.18–8.22, $P=0.022$). Individually, CTC count and expression of BCL2 each remained statistically significant predictors of disease progression and overall survival in multivariate analysis.

Conclusions: This is the first demonstration that lysates of MCA-enriched CTCs are amenable to molecular characterization. CTCs enriched by MCA are an independent prognostic marker in NSCLC.

Keywords: Blood; biomarkers, tumor; liquid biopsy; neoplastic cells; circulating

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Introduction

Circulating tumor cells (CTCs) are rare cells shed by solid tumors into the peripheral blood. Accurate quantification of CTCs may enable minimally invasive monitoring of disease status in a patient. Many approaches have been developed to capture and characterize CTCs in the blood. Currently, there are 2 general approaches to CTC enrichment and identification, exemplified by 2 of the longest-approved technologies: CELLSEARCH (Menarini Silicon Biosystems, Bologna, Italy) and AdnaTest (Qiagen, Hilden, Germany). The CELLSEARCH system is the first and only clinically validated U.S. Food and Drug Administration (FDA)-cleared blood test for enumerating CTCs in patients with metastatic breast, colorectal, or prostate cancer (1). This system is predictive of progression-free survival (PFS) and overall survival (OS) in patients with metastatic breast (2), colorectal (3), or prostate cancer (4). However, this method only captures CTCs of epithelial phenotype [leukocyte common antigen (CD45)⁻, EPCAM⁺, and cytokeratin 8⁺, cytokeratin 18⁺, and/or cytokeratin 19⁺]. The AdnaTest captures tumor cells using magnetic beads coated with a cocktail of antibodies against multiple cell surface receptors (5). In contrast to CELLSEARCH, mRNA is then extracted from the enriched cell populations and subjected to polymerase chain reaction (PCR) to detect gene transcripts for epithelial tumor cells, such as EPCAM, and tumor cells undergoing epithelial-to-mesenchymal transition (EMT), such as TWIST1 and ALDH1A1 (6). Therefore, AdnaTest cannot provide enumeration, and CELLSEARCH is not validated for molecular analyses. Integration of these 2 approaches into a single platform would allow both enumeration and downstream molecular characterization of enriched CTCs.

The Micro Cavity Array (MCA) system (Hitachi Chemical Co., Tokyo, Japan) is an integrated microfluidic device that enriches tumor cells on the basis of differences in size and membrane deformability between tumor cells and cells of hematopoietic origin (7-11). Owing to the agnostic nature of enrichment that is not reliant on cell capture based on surface protein expression, the MCA system has the potential to capture more CTCs than the EPCAM-based CELLSEARCH system (7) and may be able to provide both enumeration and molecular analysis of the captured cells.

Herein, we used a preclinical tumor cell spike-in model with healthy donor blood (HDB) to evaluate the capture efficiency of the MCA system. We then validated the MCA technology for CTC enumeration and downstream targeted gene expression profiling in patients with stage

III non-small cell lung cancer (NSCLC) and demonstrated the ability of this technology to predict patient outcomes. We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/tlcr-20-841>).

Methods

Overview of MCA system

A succinct overview of the MCA system (Hitachi Chemical Co., Ltd., Ibaraki, Japan) was recently provided by Yagi and colleagues (9). The MCA is a semi-automated filtration method with metal filters coated in nickel and gold. The filters consist of 10,000 fabricated 8 μm \times 100 μm rectangular pores that have been optimized to trap tumor cells while permitting blood cells to flow through the microcavities during whole-blood filtration. Protocols for priming, washing, fixing, permeabilization, and staining are all automated with reagents added to cells captured in the filtration cartridge *in situ*.

Cell culture

Human lung cancer cell lines H358 and A549 were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 10% penicillin-streptomycin in an incubator at 37 °C with a 5% CO₂ atmosphere. Cells were harvested with 0.25% trypsin-EDTA when they reached 70% confluence, and cell viability was determined by trypan blue exclusion.

Tumor cell spiking by micromanipulation

A micromanipulation system (TransferMan 4r, Eppendorf, Hamburg, Germany) mounted on an inverted phase contrast microscope (Nikon, Tokyo, Japan) was used to obtain a known number of tumor cells (0, 10, 50, or 100 cells from each cell line), which were then deposited onto a 20- μL drop of tissue culture medium on the flat surface of a tissue culture dish. The culture medium containing a known number of tumor cells was transferred to 9.5 mL of normal donor blood. A sham spike condition was included as a control.

Study cohorts

This study was conducted at The University of Texas MD Anderson Cancer Center, was approved by the Institutional Review Board as Lab09-0307, and conforms

Table 1 Patient characteristics

Variable	Number of patients	Proportion
Age: median (range), years	67 [53–77]	
Gender		
Female	16	42%
Male	22	58%
Race		
African American	1	3%
Hispanic	3	8%
Caucasian	34	89%
Smoking history		
Pack year ≥ 30	26	68%
Pack year < 30	8	21%
Never smoker	4	11%
Histology		
Adenocarcinoma	18	47%
Squamous cell	16	42%
Non-small cell	4	11%
Grade		
Unknown	4	11%
G1	2	5%
G2	5	13%
G3	27	71%
ECOG		
Unknown	1	3%
0	3	8%
1	33	87%
2	1	3%
Stage		
IIIA	21	55%
IIIB	17	45%

ECOG, Eastern Cooperative Oncology Group status.

to the provisions of the Declaration of Helsinki as revised in 2013. Study outcomes did not affect the clinical management of the enrolled patients. During November 2014 through October 2017, stage III NSCLC patients undergoing comprehensive treatment at MD Anderson were prospectively recruited for blood collection, and

written informed consent from the recruits was obtained (Table 1). Peripheral blood was collected at the start of chemoradiation therapy. Since the study period preceded the FDA approval of durvalumab, no patients received durvalumab after chemoradiation therapy. Sex-matched individuals self-declared as cancer-free were recruited as healthy blood donors. Written informed consent was obtained from all healthy donors according to an Institutional Review Board-approved protocol (PA14-0063). HDB was collected in BD Vacutainer EDTA tubes (BD Biosciences, San Jose, CA).

CTC capture

Enrichment procedures for enumeration and molecular characterization were performed in parallel on separate aliquots of the same blood sample. For HDB, contrived samples comprising known quantities of tumor cells in HDB, and NSCLC patient samples, ~9.5 mL of blood was loaded onto the MCA system for each aliquot. A flow rate of 200 $\mu\text{L}/\text{min}$ was maintained with negative pressure through the MCA cartridge to capture tumor cells.

In situ staining and enumeration of CTCs

For imaging, the automated staining protocol of the MCA system sequentially applied BD FACS Lyse solution (BD Biosciences), BD FACS Permeabilizing Solution 2 (BD Biosciences), and an antibody cocktail, with intermittent and final automated washing steps. The antibody cocktail consisted of anti-pan-CK (AE1/AE3) conjugated with Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA); anti-pan-CK (CK3-6H5) conjugated to fluorescein isothiocyanate (Miltenyi Biotec, Bergisch Gladbach, Germany); anti-CD45 antibody conjugated with Alexa Fluor 594 (BioLegend, San Diego, CA); and 4',6-diamidino-2-phenylindole (DAPI). The MCA cartridges containing captured tumor cells were imaged using an Olympus IX81-DSU system (Olympus, Tokyo, Japan; The Flow Cytometry and Cellular Imaging Core Facility at MD Anderson NCI Cancer Center Support Grant P30CA16672). Captured 10 \times images, along with bright-field morphology, were used to identify CTCs, defined as nucleated cells (DAPI+) that were CK+ and CD45-.

Leukocyte depletion and CTC capture for downstream molecular studies

To diminish the leukocyte background for samples

subjected to downstream molecular studies, it was necessary to significantly deplete leukocytes from peripheral blood samples. To generate this leukocyte-poor fraction, we incubated peripheral blood samples with StraightFrom Whole Blood CD45 MicroBeads at 50 μ L per mL of blood passed through a magnetic column (Miltenyi Biotec) before enrichment by the MCA system.

Following cell capture, samples were cleared of residual red blood cells using BD FACS Lyse solution (BD Biosciences). Enriched cells were harvested by applying a phenol/guanidine thiocyanate solution (QIAzol, Qiagen/Thermo Fisher Scientific, Inc.) to the open face of the capture filter to lyse the captured cells, and the lysate was archived at -80°C for subsequent batch processing. RNA was extracted in batches with chloroform extraction and column enrichment (RNeasy mini, Qiagen). cDNA was prepared using random primers (ABI High-Capacity cDNA Reverse Transcription kit, Thermo Fisher Scientific).

Relative gene expression of captured CTCs

Gene expression profiling was performed by quantitative reverse transcription PCR (qRT-PCR) using customized Bio-Rad SYBR Green PrimePCR plates according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). In short, primers were designed following the principles established via the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (12) and wet lab-validated by the manufacturer. Each PCR array plate was designed to accommodate 6 samples in triplicate and contained primers for 16 genes of interest and 3 housekeeping genes as well as quality control wells for RNA quality, reverse transcription quality, PCR quality, and DNA contamination for each sample. Cq analysis was performed by Bio-Rad CFX Manager software with a constant baseline adjustment of the relative fluorescent units for all array runs to allow accurate comparisons across samples. Data were normalized against the *TBP*, *GAPDH*, and *HPRT* housekeeping genes using the $\Delta\Delta\text{Cq}$ method. Samples were considered positive based on the distribution of leukocyte-depleted enriched HDB samples ($n=18$) after removing HDB outliers with the ROUT method in GraphPad Prism Version 7 (GraphPad, San Diego, CA). A cut-off for positive expression was established at 2.5 standard deviations above the HDB median.

The panel of genes investigated included genes related to epithelial characteristics (*EPCAM*, *HER2*, *EGFR*), EMT

(*TWIST1*, *SNAI1*), stem-like tumor cells (*ALDH1A1*, *CD44*), and signaling pathways commonly perturbed in NSCLC [*ALK*, *BCL2*, *CD274* (PD-L1), *FGFR1*, *KRAS*, *MET*, *RAD50*, *TERT* and *TP53*]. *PTPRC* (CD45) was also included as a white blood cell control.

Statistical analysis

For time-to-event variables, the Kaplan-Meier method was used to estimate the survival functions. OS was defined as the time from the initiation of chemoradiation till death or most recent contact date. PFS was defined as the time from the initiation of chemoradiation until progression, death, or last follow-up. The log-rank test was used to compare survival times between groups. The Cox proportional hazards regression model was used to evaluate the effects of potential prognostic factors on OS or PFS, P values of <0.05 were considered statistically significant. Variables included in the multivariate survival models were selected using a backward model selection algorithm where CTC count (as a continuous variable) and *BCL2* expression (considered positive if normalized expression was above the range of HDB) were pre-determined to be included in the final model. Other candidate variables (chosen on the basis of either the significance of their association with survival on univariate analysis or their clinical importance) were assessed in the initial model before selection. Owing to the limited sample size, covariates with $P<0.4$ were maintained in the model. S-PLUS software (TIBCO Software Inc., Palo Alto, CA), SAS 9.4 software (SAS Institute, Cary, NC), and R version 3.5 (R Foundation for Statistical Computing, Vienna, Austria) were used for statistical analysis.

Results

The performance of CTC enrichment by the MCA system was validated using precise numbers of cells from human lung cancer cell lines spiked into HDB as a model. This validation showed that the MCA cartridge is amenable to direct *in situ* imaging of captured cells. Using commercially available fluorophore-conjugated antibodies, CTCs defined as pan-CK+/DAPI+/CD45- cells in *Figure 1A*) were identified against a background of CD45+/DAPI+/pan-CK-leukocytes (*Figure 1A*). Plasma depletion before enrichment had minimal effects on CTC recovery (*Figure S1*). Likewise, storage of peripheral blood overnight with and without plasma did not make a significant difference in the recovery of CTCs (*Figure S2*). CTC recovery levels were also similar

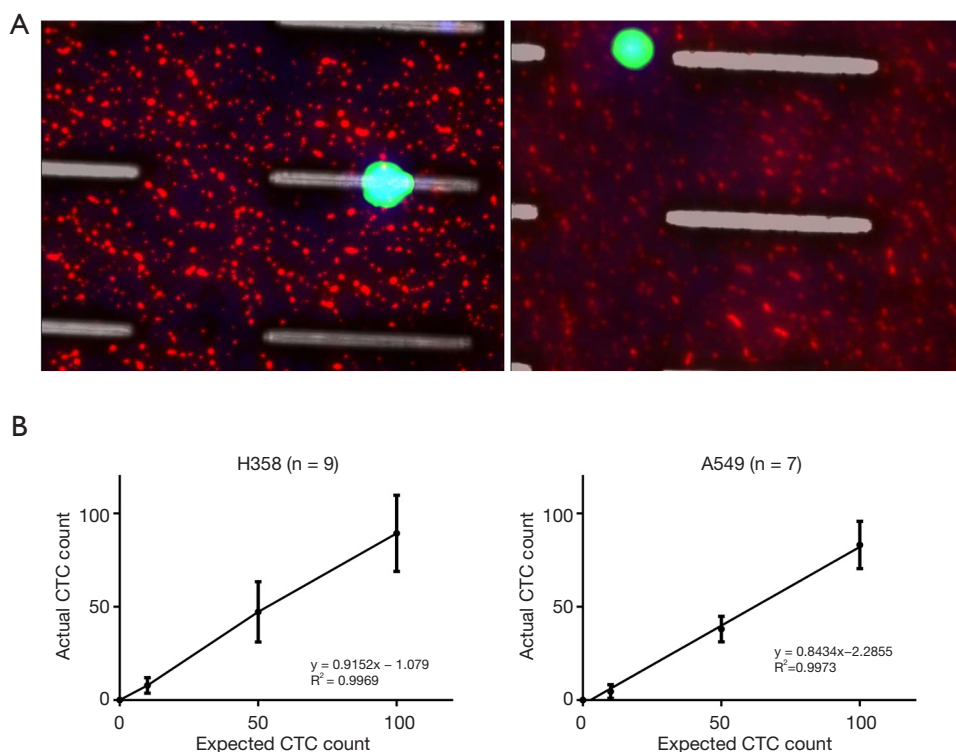


Figure 1 Contrived samples in HDB. (A) Representative on-chip immunofluorescence image of cells captured with the cartridge of the Hitachi MCA system. Red: CD45; blue: DAPI; green: cytokeratin, white: brightfield image of $8\ \mu\text{m} \times 100\ \mu\text{m}$ microcavity, imaged at $100\times$. CTCs are defined as cells that lack CD45 but have a defined DAPI nuclear stain and cytokeratin. (B) Recovery of tumor cell lines spiked into healthy donor blood. The recovery rates of spiked cell lines were 91.5% for H358 and 84.3% for A549. HDB, healthy donor blood; MCA, Micro Cavity Array.

with and without prior leukocyte depletion (Figure S3), which greatly enhanced CTC purity (Figure S4) before molecular characterization. The CTC yield of the MCA system was assessed in HDB ($n=13$) spiked with 10, 50, or 100 individual cells from 2 lung cancer cell lines (H358 and A549). The recovery rates were 91.5% and 84.3% for the H358 and A549 cell lines, with a linearity of R^2 of 0.9969 and 0.9973, respectively (Figure 1B).

CTC enumeration in stage III NSCLC patients by manual counting

Samples from 38 patients with NSCLC were analyzed for CTCs after enrichment by MCA. CTC counts ranged from 0 to 33 CTCs per $\sim 9.5\text{-mL}$ sample (Figure 2A). At least 1 CTC was detected in 30 of the 38 samples (79%), and the median was 2 CTCs (Figure 2B). There was no significant difference in CTC count by sex, stage, histology, or smoking status (data not shown).

Gene expression profile in stage III NSCLC patients by qRT-PCR

Following CTC enrichment by MCA, samples from 25 NSCLC patients that underwent molecular characterization had significantly higher expression of BCL2 (Student's t -test $P=0.0240$) and KRAS ($P=0.0398$) compared with samples from HDB (Figure 2C). Furthermore, samples with CTCs detectable by imaging had higher expression of BCL2 and EPCAM ($P=0.046$ and $P=0.019$, respectively, Figure 2D). Additional genes related to survival as described below are also presented in Figure 2. HDB similarly processed by MCA after leukocyte depletion ($n=18$) was used to establish cut-offs for positive gene expression of CTC-related genes as described in the Methods. Roughly half (52%) of the NSCLC patients' samples were positive for the epithelial marker EPCAM. In contrast, 23 of 25 patients (92%) were positive for at least 1 gene from the full panel of 16 genes (Figure 2E). Furthermore, 11 patients with at least 1 CTC by imaging and 4 patients with at least 5 CTCs by imaging

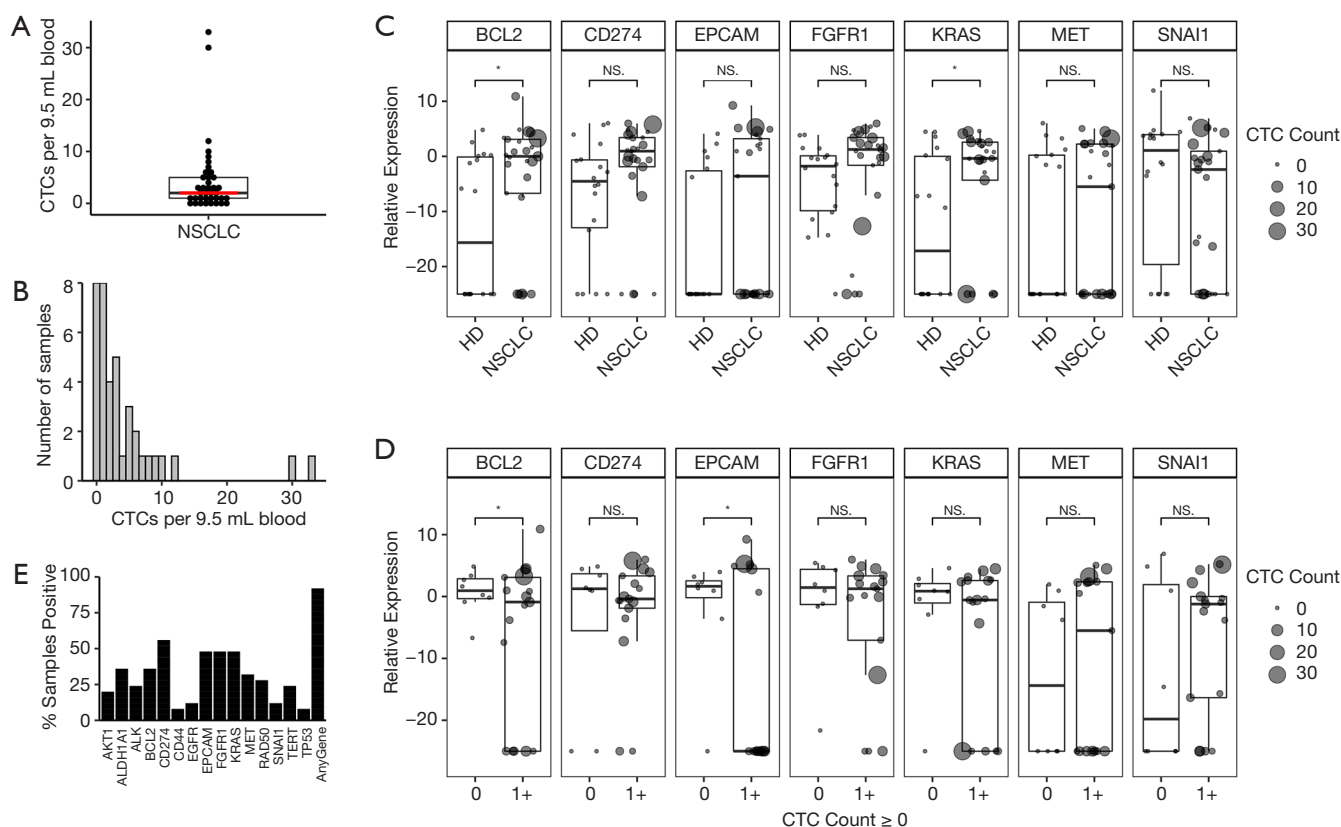


Figure 2 CTC counts and gene expression in NSCLC patients. (A,B) Distribution of CTC counts in NSCLC patients, red bar = median. (C) Relative gene expression in samples from healthy donors (HD) and NSCLC patients. (D) Relative gene expression in samples from patients with and without positive CTC counts. (E) Percentage of patients expressing each gene at higher levels than healthy donors. *, P<0.05. CTC, circulating tumor cell; NSCLC, non-small cell lung cancer.

were nonetheless negative by the measure of EPCAM expression.

CTC count associated with survival

The 38 patients with NSCLC were followed for a median of 43.1 months. Median PFS was 16.4 months and median (Figure 3A,B,C) overall survival was 38.7 months (Figure 3D,E,F). In these baseline blood draws, there was no significant difference in CTC counts between patients whose tumors progressed during the study (Figure 3A) and those who died of their disease (Figure 3D). In receiver operating characteristic curve analysis (ROC) of CTC enumeration, the areas under the curve for PFS and OS were 0.52 and 0.53, respectively. However, all 7 patients with at least 7 CTCs experienced progression (Figure 3B) and eventually died (Figure 3E). When we applied a cut-off of ≥7 CTCs per sample, the baseline CTC count was able

to predict OS with 100% specificity and positive predictive value, but with a sensitivity of 0.350 and negative predictive value of 0.581. For PFS, the sensitivity of this cut-off was 0.269, and the negative predictive value was 0.387. Patients with less than 7 CTCs had a significantly longer PFS compared with patients with 7 or more CTCs (median 24.7 vs. 3.8 months, P=0.0006, HR =4.24, 95% CI, 1.73–10.40, Figure 3C) and longer OS (median time not reached vs. 18.2 months, P<0.0001, HR =8.17, 95% CI, 2.87–23.26, Figure 3F). CTC count remained a significant prognostic factor for both PFS and OS in multivariate analysis that included standard staging (Figure S5).

Gene expression predicts survival

The ability of the positively expressed CTC-related genes to predict PFS and OS in 25 NSCLC patients is reported in Table 2. Positive expression of BCL2, positive expression

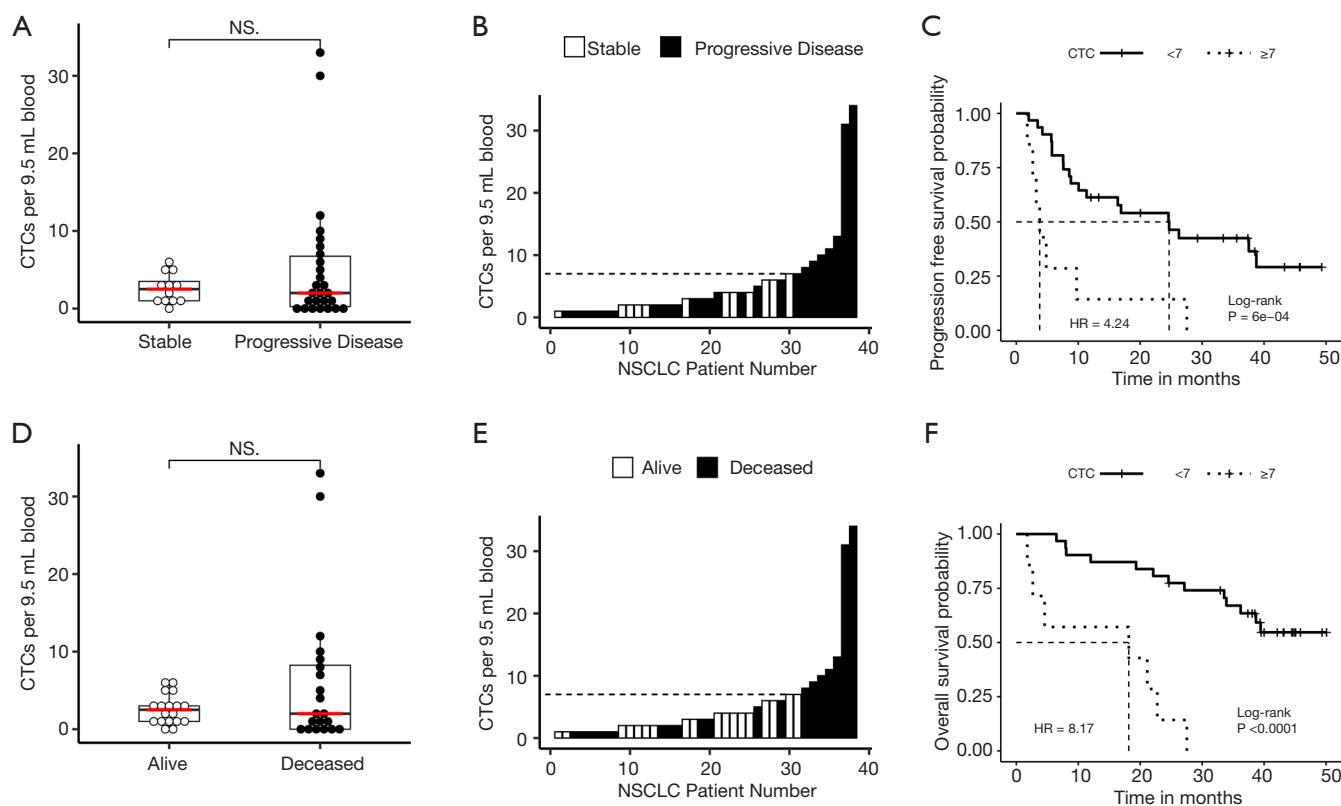


Figure 3 CTC counts were associated with survival. (A,B,C) Progression-free survival. (D,E,F) Overall survival. (A,D) CTC counts in patients whose disease progressed or who died during the study period. (B,E) Displayed as CTC count +1 so that a count of 0 is visible. (C,F) Association of CTC count with progression-free survival (C) and overall survival (F). Red bar = median. CTC, circulating tumor cell.

of MET, and the total number of positive genes were each significantly associated with an increased risk of progression in the univariate Cox proportional hazards model. Similarly, positive expression of BLC2 and positive expression of SNAI1 were significantly associated with an increased risk of death. BCL2 remained a significant prognostic factor for both PFS and OS in multivariate analysis that included standard clinical variables in the initial model and required CTC count in the final model (Table 2), as well as in an unselected model that did not include CTC count (Figure S6). Additionally, FGFR1, MET, SNAI1, and the number of positive genes remained significant predictors in the unselected Cox proportional hazard models for PFS or OS (Tables S1 and S2).

Discussion

In this study, the Hitachi Chemical MCA system was evaluated for enrichment of CTCs from peripheral

blood of patients with NSCLC. This report is the first empirical demonstration that blood samples enriched for CTCs by the MCA system are amenable to molecular characterization, specifically of gene expression by PCR. Furthermore, the gene expression profiles of these enriched CTCs are clinically relevant.

One of the challenges of enriching CTCs from peripheral blood is the rarity of CTCs among blood cellular elements. It has been suggested that for every CTC, there are 10^8 blood cellular elements including leukocytes and erythrocytes, as well as platelets (13). Depletion of leukocytes that co-elute with cancer cells in CTC enrichment systems is therefore crucial in improving the efficiency of these systems for molecular analysis (14). Although leukocyte contamination of CTCs may not affect enumeration, it still may play a significant role in downstream molecular analysis. Here, we have shown that leukocyte depletion with magnetic beads coated with anti-CD45 antibody before CTC enrichment can increase CTC

Table 2 Univariable and multivariable Cox proportional hazards models

Factors	Progression-free survival						Overall survival					
	Univariate			Multivariate			Univariate			Multivariate		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Age	0.98	0.93–1.03	0.478	0.95	0.89–1.02	0.159	1.03	0.97–1.09	0.365			
Sex												
Female	1.00						1.00					
Male	1.58	0.71–3.48	0.261				4.43	1.47–13.3	0.008**	3.91	0.94–16.32	0.061
Stage												
IIIa	1.00											
IIIb	2.41	0.89–6.6	0.141	2.42	0.89–6.58	0.085	1.77	0.73–4.28	0.204	2.27	0.79–6.54	0.130
Histology												
Adenocarcinoma	1.00						1.00					
NSCLC	0.46	0.1–2.09	0.318				0.85	0.18–3.95	0.838			
Squamous cell	0.74	0.33–1.68	0.475				1.25	0.50–3.16	0.636			
Grade												
1	1.00						1.00					
2	1.57	0.17–14.37	0.690				0.23	0.01–3.68	0.298			
3	1.33	0.18–10.06	0.780				0.82	0.11–6.25	0.851			
ECOG	1.59	0.43–5.92	0.488				1.56	0.36–6.84	0.553			
CTC count	1.10	1.03–1.17	0.004**	1.07	0.98–1.17	0.122	1.08	1.03–1.14	0.004**	1.15	0.97–1.37	0.099
AKT1	1.25	0.41–3.78	0.696				1.30	0.37–4.58	0.684			
ALDH1A1	1.12	0.44–2.85	0.811				1.34	0.48–3.70	0.574			
ALK	1.25	0.45–3.5	0.673				0.60	0.17–2.12	0.429			
BCL2	3.11	1.18–8.22	0.022*	3.9	1.36–11.22	0.011*	2.02	0.74–5.46	0.168	3.66	1.12–12	0.032*
CD274	1.88	0.74–4.82	0.187				2.05	0.74–5.74	0.169			
CD44	2.24	0.49–10.13	0.297				2.64	0.08–4.87	0.667			
EGFR	0.79	0.18–3.45	0.749				0.72	0.16–3.19	0.668			
EPCAM	0.95	0.39–2.31	0.902				0.90	0.36–2.66	0.975			
FGFR1	2.25	0.88–5.76	0.092				1.33	0.49–3.59	0.572			
KRAS	1.33	0.55–3.23	0.524				2.24	0.82–6.14	0.118			
MET	2.78	1.03–7.52	0.044*				1.13	0.39–3.27	0.817			
RAD50	1.50	0.58–4.1	0.380				1.00	0.32–3.13	0.998			
SNAI1	2.26	0.63–8.13	0.212				4.14	1.09–14.73	0.037*			
TERT	1.83	0.67–4.98	0.240				2.06	0.71–5.95	0.184			
TP53	2.11	0.47–9.57	0.332				0.79	0.10–6.04	0.822			
No. of positive genes	1.18	1.00–1.38	0.048*				1.16	0.97–1.39	0.115			

ECOG, Eastern Cooperative Oncology Group status. *, P<0.05; **, P<0.01.

purity with minimal effects on CTC recovery.

Removal of plasma prior to loading blood unto the MCA also did not affect the capture efficiency in contrived samples or, by extension, of the CTCs in patient samples. Since plasma is a critical component of liquid biopsy that may provide valuable clinical information, the extraction of plasma before processing blood through the MCA system would be a judicious use of patient samples and provide an excellent resource for additional interrogations.

Our basic development data showing high rates of CTC recovery demonstrated the MCA to be a stable and reliable system for conducting both translational and clinical research with patient samples processed either fresh or after overnight storage. This observation that the CTC enumeration is minimally affected by overnight storage of blood could be beneficial in clinical settings where samples may not be processed immediately.

Using the conditions established in our validation studies, we processed samples from NSCLC patients for CTC enumeration and molecular gene expression. For better statistical power, no CTC count threshold was used in survival modeling with Cox proportional hazards; instead, the HR for each unit increase in CTC was calculated. The resulting HR was small (per CTC) but highly significant.

CTC count thresholds for cancer diagnosis using the CELLSEARCH system have been well-validated for metastatic breast cancer (2) and prostate cancer (4) (for each, 5 CTCs/7.5 mL of blood) and colorectal cancer (3 CTCs/7.5 mL of blood) (3). Although several CTC cut-offs have been proposed for NSCLC, including 1 CTC (15), 2 CTCs (16,17), and 5 CTCs (17-19), none have gained the clinical fortitude of the cut-offs for the aforementioned neoplasms. Using the MCA system, Ichimura and colleagues (20) recently showed in a subset of 12 non-surgical lung cancer patients that a follow-up count of 3 or more CTCs in 3 mL of blood predicted poor survival. This result is similar to the poor OS and PFS predicted by 7 CTCs in 9.5 mL of blood that we observed here; however, Ichimura *et al.* observed no correlation between CTC count and survival in the larger cohort of 38 patients with stage I, II, III and IV lung cancer (20). Here, we found that a cut-off of 7 CTCs per 9.5 mL of blood had 100% prognostic specificity in this training set. We also observed that CTC count was independent of clinical stage and histology in NSCLC, along the lines of some previous reports (15,20). As the small sample size precludes drawing any generalizable conclusions, further investigation is warranted.

The agnostic nature of the CTC enrichment process using the MCA system can add tremendous value to the final product and, consequently, the feasibility of clinical applications. Surface protein expression of the epithelial cell adhesion molecule EPCAM is traditionally used to capture CTCs as well as to differentiate between CTCs and contaminating leukocytes (1). Here, we found only 48% of NSCLC patients have elevated *EPCAM* mRNA. Furthermore, using image analysis, we found a large portion of the samples containing CTCs, identified by CK+CD45- status, to have low levels of *EPCAM* mRNA, within the range of expression by healthy donors. As MCA captures CTC independent of EPCAM, it is not unexpected that the captured cells may or may not express EPCAM. Although EPCAM can both promote and inhibit metastasis, EMT processes can downregulate EPCAM as tumor cells disaggregate and disseminate (21), and inflammation may play a role in downregulating EPCAM expression (22). Therefore, relying on EPCAM for enrichment could potentially underestimate the number of CTC in patients (6,23,24). However, since the prognostic value of the EPCAM negative CTCs may be limited (25), better-powered studies are warranted.

Expression of *BCL2* and *KRAS* transcripts was elevated in MCA-enriched NSCLC blood samples compared with those of healthy donors. In NSCLC samples with at least 1 CTC, *BCL2* and *EPCAM* transcripts were expressed at higher levels. *BCL2* is a critical inhibitor of apoptosis and is overexpressed in NSCLC (26). Furthermore, in contrast to metareviews of primary tumors (27-29), we found that high *BCL2* expression in CTCs of NSCLC patients was correlated with poor survival and was an independent prognostic factor. This result is similar to those of other studies of *BCL2*+ CTCs in lung cancer patients (30) but contrasts studies in metastatic breast cancer where high *BCL2* correlated with better survival (31).

We were also able to detect *PD-L1* (*CD274*) transcripts in enriched CTCs. As studies have shown that patients may show a response to anti-PD-L1 therapy even when the protein is not detected in tissue samples (32), an additional measure that can be evaluated serially may be beneficial. The patients in this study were enrolled before immunotherapy was established as a standard of care, but it is encouraging that the MCA system may be able to track PD-L1 through easily accessible liquid biopsy for patients undergoing immune checkpoint inhibitor therapy.

To our knowledge, this is the first report that MCA-enriched CTCs are amenable to molecular characterization.

Here, we demonstrate that these samples are not only conducive to gene expression analysis but also yield data that correlate well with clinical outcomes.

Analysis of CTC-specific biomarkers has the potential to stratify patients into prognostic groups. This approach may also open avenues for finding effective targets for new chemotherapeutic approaches. Importantly, biomarker testing in CTCs offers the key advantage of conducting these tests using peripheral blood, thereby avoiding the need for repeated invasive procedures to obtain primary tissue. The challenges that have precluded the widespread use of CTCs in *in vitro* diagnostic applications are thus being gradually addressed with recent advances in technology such as the Hitachi Chemical MCA system.

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Footnote

Provenance and Peer Review: This article was commissioned by the editorial office, *Translational Lung Cancer Research* for the series "New era of treatment for unresectable locally advanced non-small cell lung cancer". The article has undergone external peer review.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in compliance with the Declaration of Helsinki (as revised in 2013) and its subsequent amendments and was approved by the Institutional Review Board of the University of Texas MD Anderson Cancer Center (Lab09-0307) and informed consent was taken from all individual participants.

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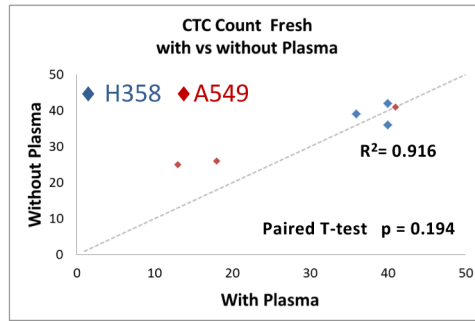


Figure S1 Plasma removal prior to MCA filtration did not interfere with CTC recovery. Paired samples with and without plasma from 3 healthy blood donors spiked with 50 H358 cells or 50 A549 cells in each condition were enumerated after enrichment. Removing plasma had no discernable effect on CTC capture efficiency ($P=0.194$). MCA, Micro Cavity Array; CTC, circulating tumor cell.

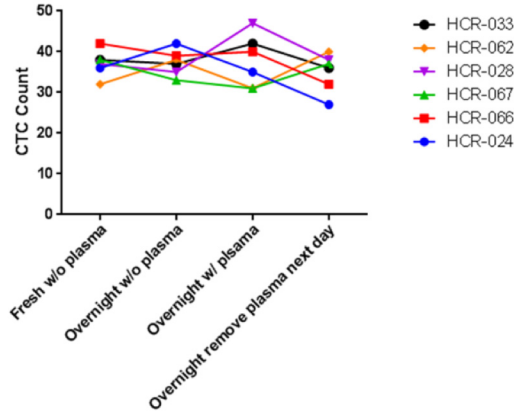


Figure S2 Evaluation of sample stability on CTC counts. Storage of blood overnight with or without plasma did not make a significant difference in the recovery of CTCs. CTC counts in 4 different conditions in 6 normal donors spiked with 50 H358 cells were compared: (I) fresh blood with plasma removed; (II) blood stored overnight with plasma; (III) blood stored overnight without plasma; (IV) blood stored overnight with plasma and removed prior to processing. Different sample handling conditions generated on-par cell recovery. HCR denotes different health blood donors. CTC, circulating tumor cell.

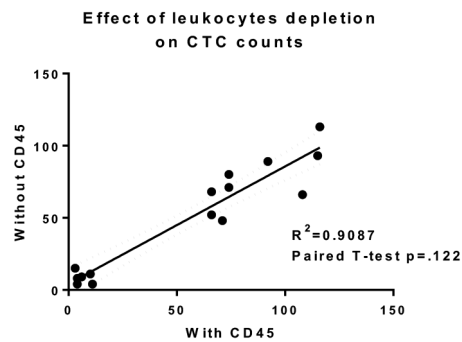


Figure S3 Leukocytes removal before MCA filtration did not affect CTC capture. Leukocyte depletion did not affect the capture of CTCs irrespective of the number of tumor cells spiked into each of 5 HDB samples, 100 H358 cells or 10 H358 cells (P=0.122). MCA, Micro Cavity Array; CTC, circulating tumor cell.

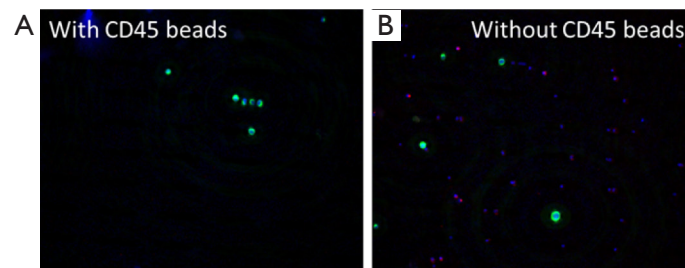
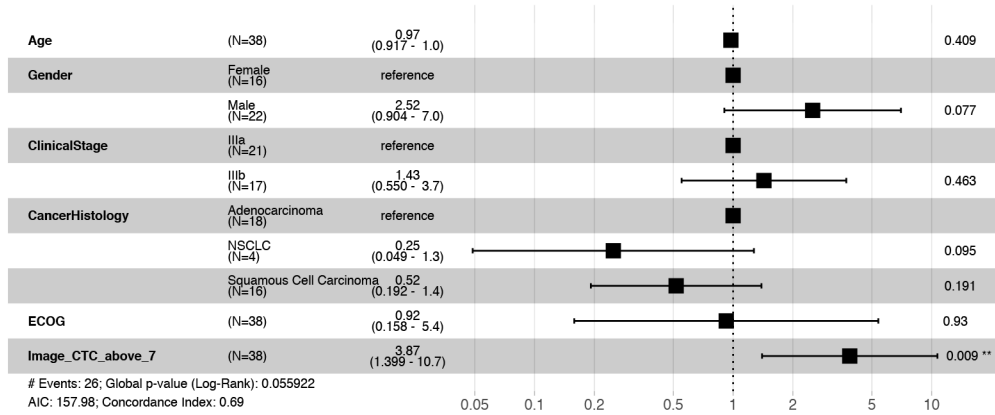
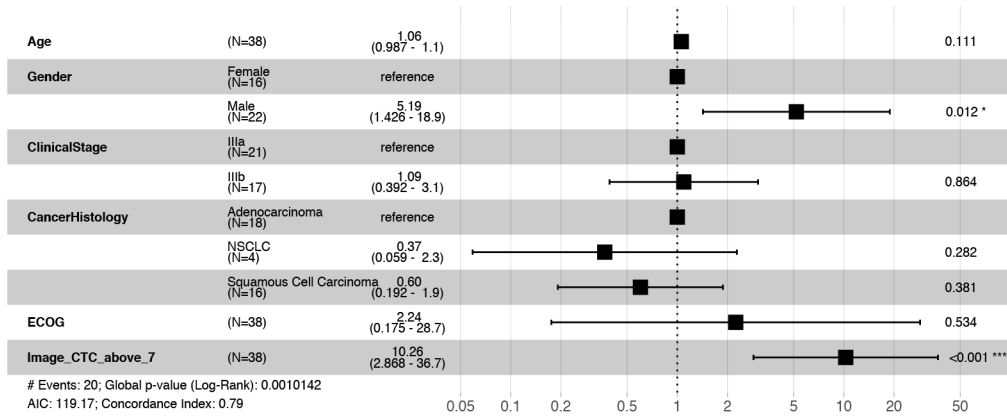


Figure S4 Image of CD45 depletion. (A) Shows an MCA cartridge imaged by immunofluorescence where the leukocytes were depleted using CD45 Microbeads prior to enrichment. (B) Shows high levels of co-eluting leukocytes in an MCA cartridge without pre-treatment with CD45 microbeads. MCA, Micro Cavity Array.

PFS Multivariate Hazard Ratios



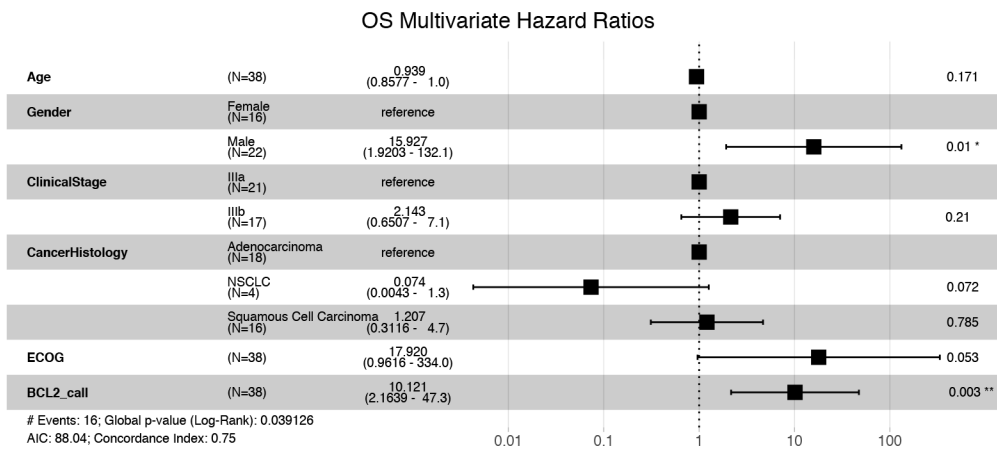
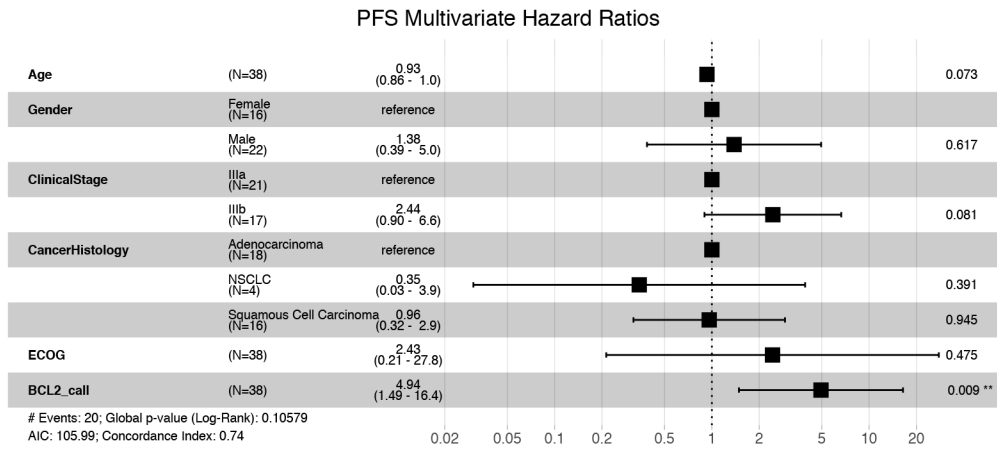
OS Multivariate Hazard Ratios



AIC: Akaike information criterion

Figure S5 Multivariate analysis of CTC counts. *, P<0.05; **, P<0.01; ***, P<0.001. AIC, Akaike information criterion; CTC, circulating tumor cell.

BCL2 Call Only



AIC: Akaike information criterion; BCL2 call: expression higher than HDB

Figure S6 BCL2 call only. *, P<0.05; **, P<0.01. AIC, Akaike information criterion; CTC, circulating tumor cell; BCL2 call, expression higher than HDB.

Table S1 Multivariate analyses

Measure	PFS P value	OS P value
Age, sex, clinical stage, cancer histology, ECOG	–	–
CTC count	0.048*	0.047*
CTC count ≥ 7	0.016*	0.0005***
BCL2	0.424	0.222
FGFR1	0.086	0.235
MET	0.383	0.516
SNAI1	0.027*	0.013*
BCL2_call	0.012*	0.005**
FGFR1_call	0.026*	0.111
MET_call	0.057	0.083
SNAI1_call	0.226	0.282
BCL2_CD45	0.194	0.062
FGFR1_CD45	0.879	0.782
MET_CD45	0.076	0.046*
SNAI1_CD45	0.445	0.511
No. of positive genes	0.031*	0.013*

Each measure above was included in a Cox proportional hazards model with the stated measure and age, sex, clinical stage, cancer histology, and Eastern Cooperative Oncology Group status (ECOG). The effects of each of these covariates differed slightly in each model and are not reported here for simplicity. Tumor grade was not included because of the small sample size. CTC count and gene expression are shown both as continuous variables (no suffix), dichotomized based on HDB expression as described in methods (denoted as call), or dichotomized gene expression after correcting for co-eluting leukocytes by normalizing to the expression of CD45 (denoted as CD45). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table S2 Multivariate analyses with CTC count

Measure	PFS P value	OS P value
Age, gender, clinical stage, cancer histology, ECOG, CTC count	–	–
BCL2	0.297	0.100
FGFR1	0.037*	0.089
MET	0.403	0.548
SNAI1	0.04*	0.004**
BCL2_call	0.013*	0.006**
FGFR1_call	0.052	0.249
MET_call	0.139	0.365
SNAI1_call	0.217	0.230
BCL2_CD45	0.153	0.087
FGFR1_CD45	0.670	0.799
MET_CD45	0.223	0.299
SNAI1_CD45	0.320	0.379
No. of positive genes	0.055	0.046*

Each measure above was included in a Cox proportional hazards model with the stated measure and age, sex, clinical stage, cancer histology, ECOG, and CTC count. The effects of each of these covariates differed slightly in each model and are not reported here for simplicity. Gene expression levels are shown both as continuous variables (no suffix), dichotomized based on HDB expression as described in methods (denoted as call), or dichotomized gene expression after correcting for co-eluting leukocytes by normalizing to the expression of CD45 (denoted as CD45). *, $P < 0.05$; **, $P < 0.01$.