Analysis of Circulating Tumor Cells in Patients with Non-small Cell Lung Cancer Using Epithelial Marker-Dependent and -Independent Approaches

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Introduction: Epithelial circulating tumor cells (CTCs) are detectable in patients with non-small cell lung cancer (NSCLC). However, epithelial to mesenchymal transition, a widely reported prerequisite for metastasis, may lead to an underestimation of CTC number. We compared directly an epithelial marker-dependent (CellSearch) and a marker-independent (isolation by size of epithelial tumor cells [ISET]) technology platform for the ability to identify CTCs. Molecular characteristics of CTCs were also explored.

Methods: Paired peripheral blood samples were collected from 40 chemonäive, stages IIIA to IV NSCLC patients. CTCs were enumerated by Epithelial Cell Adhesion Molecule-based immunomagnetic capture (CellSearch, Veridex) and by filtration (ISET, RareCell Diagnostics). CTCs isolated by filtration were assessed by immunohistochemistry for epithelial marker expression (cytokeratins, Epithelial Cell Adhesion Molecule, epidermal growth factor receptor) and for proliferation status (Ki67).

Results: CTCs were detected using ISET in 32 of 40 (80%) patients compared with 9 of 40 (23%) patients using CellSearch. A subpopulation of CTCs isolated by ISET did not express epithelial markers. Circulating tumor microemboli (CTM, clusters of ≥3 CTCs) were observed in 43% patients using ISET but were undetectable by CellSearch. Up to 62% of single CTCs were positive for the proliferation marker Ki67, whereas cells within CTM were nonproliferative.

Conclusions: Both technology platforms detected NSCLC CTCs. ISET detected higher numbers of CTCs including epithelial marker negative tumor cells. ISET also isolated CTM and permitted molecular characterization. Combined with our previous CellSearch data confirming CTC number as an independent prognostic biomarker for NSCLC, we propose that this complementary dual technology approach to CTC analysis allows more complete exploration of CTCs in patients with NSCLC.

Key Words: Circulating tumor cells, Non-small cell lung cancer, CellSearch, ISET.

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The detection and characterization of circulating tumor cells (CTCs) in cancer patients offer as yet untapped potential to further understand the biology of human cancer metastasis and to identify novel treatment strategies. Recent advances in technology have paved the way to reproducible CTC detection and enumeration and begun to reveal their potential as a real-time, minimally invasive, "virtual biopsy" (reviewed in Hou et al.[1]). The robust, semiautomated CellSearch platform (Veridex, LLC, Raritan, NJ) has been used to demonstrate prognostic significance of CTC numbers in patients with metastatic breast, prostate, and colorectal cancers[2–7] (reviewed by Krebs et al.[8]) and subsequently has been approved by the US Food and Drug Administration as a prognostic biomarker and as an aid to monitoring treatment response in these disease types. We recently reported that CellSearch detection of ≥5 CTCs (per 7.5 ml of blood) in patients with advanced non-small cell lung cancer (NSCLC) is a poor prognostic factor and that a change in CTC number after a single cycle of standard-of-care chemotherapy predicts survival outcome.[9] However, two-thirds of patients with stage IV NSCLC had no detectable CTCs, and CTCs were detectable in less than 5% stage III patients using this technology platform.

Detection of CTCs using CellSearch is dependent on tumor cell expression of Epithelial Cell Adhesion Molecule

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(EpCam), an epithelial cell marker. However, the paradigm of epithelial to mesenchymal transition (EMT) as a predominant mechanism for tumor cell invasion and metastasis raises the possibility that not all cells in the circulation will express epithelial markers.[10–14] Thus, we hypothesized that the low prevalence of CTCs detected with the CellSearch technology in patients with advanced NSCLC, although strongly prognostic, may be due to the loss of EpCam expression. Therefore, we explored an antigen/EpCAM-independent approach for the ability to identify CTCs and for the potential to perform molecular characterization.

ISET(RareCell Diagnostics, Paris, France) is a filter-based, size exclusion technology capable of isolating CTCs independently of their expression of any particular marker. Using this technique, CTCs have been observed previously in patients with hepatocellular carcinoma, breast carcinoma, melanoma, and more recently in patients with early-stage, resectable NSCLC.[15–19] In a previous pilot study using ISET, we noted that CTCs were detectable in patients with advanced-stage NSCLC, with heterogeneity in both epithelial (E-cadherin and cytokeratin [CK]) and mesenchymal (N-cadherin and vimentin) marker expression.[20] In addition, circulating tumor microemboli (CTM; defined as clusters of cells with three or more nuclei) were identified, whereas CTM were not detected in our previous evaluation of tumor cells in 101 patients with NSCLC using CellSearch.[9]

Our goal was to compare the CellSearch (epithelial dependent) and ISET (antigen/EpCAM independent) platforms directly for the ability to isolate CTCs in a cohort of NSCLC patients. Secondary objectives were to explore the prevalence of CTM in NSCLC patients and to examine molecular characteristics of cells isolated by filtration, specifically in relation to their epithelial marker expression and proliferative status.

PATIENTS AND METHODS

Patient Eligibility and Clinical Characteristics

This was a prospective study conducted at the Christie Hospital, Manchester, United Kingdom. Patients with chemonäive, histologically proven NSCLC were eligible. Inclusion criteria included radiologically confirmed stage IIIA or IIIB/IV disease; World Health Organization performance status 0–2; age ≥18 years; and standard-of-care chemotherapy was to be administered. Patients with a history of prior malignancy, within the previous 5 years, were excluded. All patients provided written informed consent according to ethics board approved study protocols. Data were collected for age, ethnicity, histological subtype, stage of disease, smoking status, sites of metastases, and treatment received.

Blood Sampling

Peripheral blood (a maximum of 20 ml) samples were collected up to 1 hour before patients commencing their first cycle of chemotherapy. Blood (10 ml) was collected in a CellSave preservative tube (Veridex LLC, NJ), stored at room temperature, and processed according to standard Veridex protocols within 96 hours of collection. A further 8 to 10 ml was collected in an ethylenediaminetetraacetic acid tube (Becton Dickinson, NJ), stored at 4°C, and processed by ISET within 4 hours of collection. Samples were processed in accordance with the UK Clinical Trials regulations for compliance to Good Clinical Practice for laboratories.[21]

CTC Detection by CellSearch

Samples were analyzed as previously described.[22,23] A CTC was defined according to the criteria of round to oval morphology, cell size more than 4 μm, 4′,6-diamidino-2-phenylindole (DAPI) positive nucleus, CK positive staining, and absence of CD45 expression. CTC number is reported per 7.5 ml of blood. The sensitivity, accuracy, linearity, and reproducibility of the CellSearch system have been previously described.[22,24] Samples were considered positive for CTCs if ≥2 per 7.5 ml of blood were detected as one CTC has previously been reported as a normal finding.[22]

CTC Detection by ISET

Blood samples were divided into 1 ml aliquots and diluted 1:10 with red cell lysis buffer, containing 0.8% formaldehyde (RareCell Diagnostics), as per manufacturer's instructions. Each aliquot was placed into an individual well of a 10-well ISET filter module (consisting of a polycarbonate track-etched-type membrane punctured by 8-μm cylindrical pores, supported beneath a 10-well plastic reservoir).[15–18] Filtration was achieved by attaching the module to the ISET instrument and by applying regulated, gentle suction. Unfiltered cells were deposited on a 0.6-cm diameter, circular "spot" on the membrane beneath each well. After filtration, each spot was washed once with phosphate-buffered saline and then the membrane disassembled from the module and allowed to air dry at room temperature. Filters were subsequently stored at −20°C. The sensitivity of the ISET system has been previously reported as 1 cell per ml,[15,17,18] and similar results were found in our study (see Supplemental Table 1, http://links.lww.com/JTO/A198). Previous studies have reported that CTCs are not detectable in blood samples from healthy donors using the ISET technique.[18,19]

Identification of CTCs Isolated on ISET Filters

Tumor cells were identified according to absence of the leukocyte common antigen, CD45, by immunohistochemistry (IHC) and by their large, hyperchromatic, irregular-shaped nuclei. Individual spots (each representing 1 ml of filtered blood) were excised from the filter and placed in pH 6 antigen retrieval buffer (S1699, Dako) in a 99°C waterbath for 40 minutes. Spots were washed in tris-buffered saline, placed in 0.2% triton for 10 minutes to permeabilize cell membranes, and exposed for 30 minutes to 3% hydrogen peroxide–methanol solution to block endogenous peroxidases. Spots were subsequently washed in water and incubated overnight at 4°C with monoclonal mouse antihuman CD45 primary antibody (clone T29/33, Dako) diluted 1:30 in antibody diluent (S0809, Dako). CD45 staining was achieved with standard Envision Kits and the Liquid DAB+ Substrate Chromagen System according to manufacturer's instructions (K5007, Dako) and counterstained with Gill's hematoxylin. Spots were mounted on glass slides and scanned at 400× magnification using an Olympus BX52 microscope linked

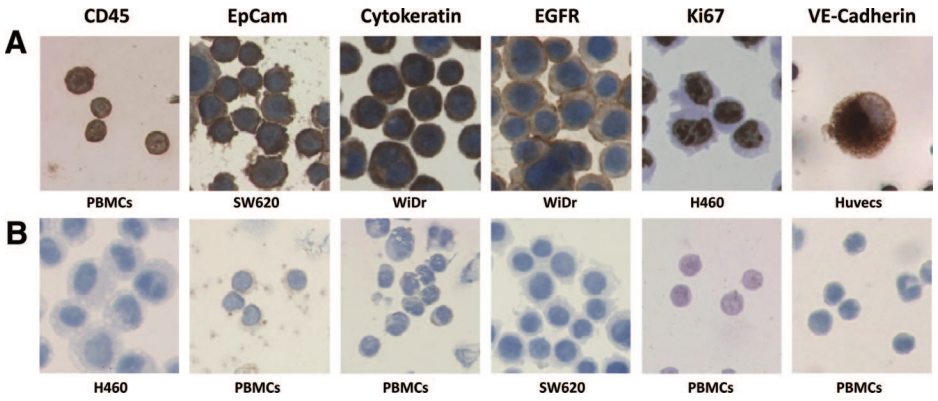

FIGURE 1. Biological controls for immunohistochemistry of circulating tumor cells (CTCs) isolated by ISET. (*A*) Positive-staining controls and (*B*) negative-staining controls for each of the markers used in this study to characterize CTCs/CTM. Control staining was performed on cell lines or peripheral blood mononuclear cells (PBMCs), cytospun onto glass slides. All images were acquired at ×400 magnification using a Bioview imaging system. ISET, isolation by size of epithelial tumor cells; CTM, circulating tumor microemboli.

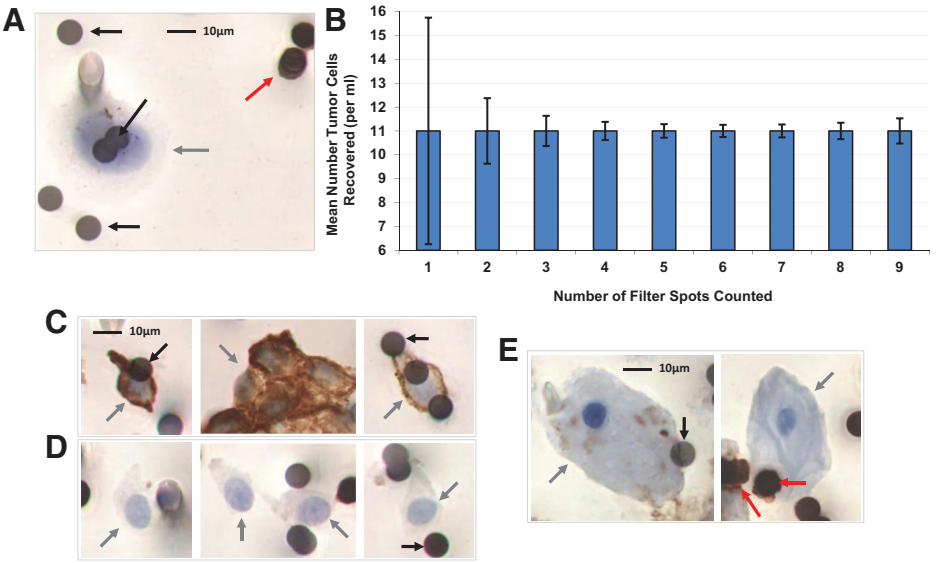

FIGURE 2. Exclusion of nontumor cell contaminants on ISET filters and circulating tumor cell (CTC) enumeration strategy. *A*, CD45 immunostaining of H460 tumor cells spiked into healthy donor blood and processed through the ISET filtration system to exclude white blood cells. Filter pores appear dark and circular or cylindrical (black arrows), white blood cells stain brown due to DAB-substrate reaction (red arrow), and tumor cells appear blue due to the hematoxylin counterstain (blue arrow). *B*, Bar chart demonstrating the mean and SE for CTC number depending on the number of spots analyzed. Four spots or more exhibited the most representative mean CTC value. *C*, VE-cadherin (CD144) staining on clinical samples processed by ISET to exclude mature circulating endothelial cells (CECs). CECs exhibited small, round, or oval, pale nuclei with low nuclear-to-cytoplasmic ratio and were positive for CD144 (blue arrows). Nuclei appear blue due to hematoxylin counterstain. A cluster of CECs is shown in the central panel. Filter pores appear dark and circular (black arrows). *D*, CD45 staining on clinical samples processed by ISET. Cells with CEC morphology are seen with hematoxylin-stained nuclei (blue arrows). These cells were considered to be mature CECs on the basis on their morphological similarity with CD144-positive cells (*C*) and absence of CD45 expression. *E*, Typical CD45 negative squamous skin cells exhibiting small, round, pyknotic nuclei with abundant cytoplasm (blue arrows). Filter pores appear dark and circular (exemplified by black arrow). ISET, isolation by size of epithelial tumor cells.

with image analysis software (Allegro Plus, Bioview, Rehovot, Israel). Staining for CD45 was optimized using cytospins of peripheral blood mononuclear cells as the positive control and human NSCLC H460 cells (American Type Culture Collection) as the negative control (Figure 1). H460 cells were spiked into healthy donor blood, and staining was validated on filtered spots (Figure 2*A*).

Images were reviewed manually and cells assigned as tumor if they had (i) negative CD45 expression, (ii) presence of hyperchromatic nuclei, (iii) irregularly shaped nuclei, (iv) nuclear size ≥ 12 μm, (v) nuclear to cytoplasmic ratio more than 50%, and/or (vi) presence of clusters of CD45 negative cells containing three or more nuclei with morphology as described in points (ii) to (v), defined, in this study, as CTM. Images were reviewed by a Consultant Histopathologist (Dr. Daisuke Nonaka). For each clinical sample, an average CTC number was determined from four spots and extrapolated to 7.5 ml for direct comparison with CellSearch (see "Statistical Considerations" section for determination of optimum number of spots to stain for reliable enumeration).

TABLE 1. Number of Membrane Spots (Generated after ISET Blood Filtration) Interrogated with a Range of Antibodies to Explore Molecular Characteristics of Isolated Tumor Cells

Marker	Number of Patients Evaluated (1 Spot Each)	Antibody and Concentration Used	Cells Used as Positive Control for IHC[a]	Cells Used as Negative Control for IHC[a]
EpCam	9	Anti-human EpCam; clone VU-1D9, Thermo Scientific; 1:100	SW620	PBMCs
Cytokeratin (4, 5, 6, 8, 10, 13, 18)	9	Anti-human cytokeratin; clone C-11, Thermo Scientific; 1:100	WiDr	PBMCs
EGFR	4	Anti-human epidermal growth factor receptor clone E30, Dako; 1:25	WiDr	SW620
VE-cadherin	4	Anti-human endothelial cell marker CD144; Clone 16B1, eBioscience; 1:50	Huvecs	PBMCs
Ki67	14	Anti-human Ki-67; clone MIB-1, Dako; 1:100	H460	PBMCs
IgG control	4	IgG1κ control; clone G01, Dako; 1:25	—	H1299

Positive- and negative-staining controls are listed.

[a] SW620 and WiDr are colorectal cancer cell lines; H460 and H1299 are non-small cell lung cancer cell lines, and Huvecs are an endothelial cell line.

ISET, isolation by size of epithelial tumor cells; IHC, immunohistochemistry; PBMCs, peripheral blood mononuclear cells; EGFR, epidermal growth factor receptor; EpCam, Epithelial Cell Adhesion Molecule.

Additional Characterization of CTCs on ISET Filters

To further characterize the captured cells, a series of filter spots from patient samples were stained for three epithelial markers (EpCam, CKs, and epidermal growth factor receptor [EGFR]). In addition, the proliferative status of CTCs and CTM was examined using Ki67. An endothelial marker (VE-cadherin) was used to exclude the presence of circulating endothelial cells (CECs). After enumeration, which used four filter spots per patient, there were up to six spots remaining per patient for CTC molecular characterization (some patients had less depending on the total amount of blood acquired). To evaluate the full range of markers, a single filter spot per patient was used for each marker. To maximize the probability of finding CTCs on a single spot, only patients with 15 or more CTCs per 7.5 ml of blood by ISET, equating to an average of 2 CTCs per spot, were chosen for this aspect of the study. With a limited number of spots exhibiting this criterion, the numbers of patients evaluated for each marker are detailed in Table 1.

Slides from archival tumor biopsies, procured at diagnosis, were obtained from four patients with the highest numbers of CTCs/CTM for comparison of morphology. Hematoxylin and eosin or Papanicalou staining was performed by pathologists at the referring hospitals (as part of the diagnostic process). Slides were scanned at 400× magnification using an Olympus BX52 microscope linked with image analysis software (Allegro Plus, Bioview, Rehovot, Israel) for direct comparison with ISET-isolated CTCs/CTM.

Cell Culture

Cytospins were prepared from cultured cell lines or peripheral blood mononuclear cells for use as positive or negative staining controls for IHC (see Table 1 and Figure 1). Details of cell culture methodology are included in Supplementary data (http://links.lww.com/JTO/A198).

Statistical Considerations

Concordance between CTC numbers detected by CellSearch and ISET were analyzed by Bland-Altman plots. Pitman's variance ratio test was used to assess agreement.

Enumeration of CTCs/CTM by ISET is a nonautomated, labor-intensive, and lengthy process. To determine the minimum number of spots to stain with CD45 for a robust and representative CTC count per milliliter, 10 ml of healthy donor blood was collected in ethylenediaminetetraacetic acid tubes and spiked with 200 NCI-H1299 human lung cancer cells. Samples were processed according to ISET methodology and recovered tumor cells counted on each of the 10 spots, each containing cells trapped from 1 ml of blood. CTC counts were then calculated from all possible combinations of spots (i.e., any 1, any 2, any 3, etc.) and mean CTC number ($\pm$SE) with 95% confidence intervals calculated for each (Figure 2*B*). The total number of cells recovered was 110 (mean = 11). Enumerating CTCs from only 1, 2, or 3 spots exhibited a wide error in mean CTC number estimation. Analyzing four spots or more provided an accurate estimation of the true mean (mean $\pm$ SE = 11 $\pm$ 0.19, 95% confidence interval: 10.6–11.4). Replicate experiments confirmed four spots to provide the most reliable estimation, and therefore each patient had four spots stained with CD45 for CTC enumeration, and CTC numbers were extrapolated to 7.5 ml for comparison with CellSearch.

RESULTS

Patient Demographics

Forty-five patients with NSCLC were recruited to the study between April 2008 and May 2010. Five patients were not evaluable for CTC analysis because of insufficient blood volume for processing by both CellSearch and ISET. Thus, 40 patients with paired blood samples were evaluable for the study. Patient characteristics are reported in Table 2.

TABLE 2. Patient Demographics

Characteristic	Patients ($N = 40$)
Age at baseline, yr	
Median	68
Range	46–77
Sex, *n* (%)	
Female	18 (45)
Male	22 (55)
Race, *n* (%)	
White British	39 (97.5)
South Asian	1 (2.5)
Stage at diagnosis, *n* (%)	
IIIA	5 (13)
IIIB	12 (30)
IV	23 (57)
Histological subtype, *n* (%)	
Squamous cell carcinoma (SCC)	15 (37)
Adenocarcinoma	10 (25)
Poorly differentiated	2 (5)
Bronchioloalveolar	2 (5)
NSCLC (not otherwise specified)	11 (28)
Baseline WHO PS, *n* (%)	
0	3 (8)
1	29 (72)
2	8 (20)
Smoking status, *n* (%)	
Current smoker	12 (30)
Ex-smoker	16 (40)
Never-smoker	2 (5)
Not documented	10 (25)

NSCLC, non-small cell lung cancer; WHO, World Health Organization; PS, performance status.

CTC Enumeration—CellSearch versus ISET

CTCs were detected using ISET in 32 (80%) patients out of the 40 evaluated compared with only 9 (23%) patients using CellSearch. Seven patients had CTCs detected by both ISET and CellSearch; 2 patients had CTCs detected by CellSearch only, and 25 patients had CTCs detected by ISET only. Considering both techniques together, 34 (85%) patients had detectable CTCs. Numbers of CTCs isolated, according to disease stage, are detailed in Table 3. The numbers of CTCs detected by ISET (mean, 71; range, 0–1045) were higher than those detected by CellSearch (mean, 4; range, 0–78). There was no statistical concordance between the numbers of CTCs detected by the two techniques using Bland–Altman analysis (Pitman's test of difference in variance, $n = 40$, $p = <0.001$, $r = 0.988$).

CTC Morphology by CellSearch and ISET

Examples of images of cells isolated by CellSearch and ISET are shown in Figures 3*A*, *B*. The size range of cells detected by CellSearch was 4 to 18 μm and by ISET was 12 to 30 μm. Cellular morphology was seen with greater clarity using ISET (in conjunction with hematoxylin staining and IHC) compared with CellSearch.

Circulating Tumor Microemboli

CTM, which we define here as contiguous clusters of cells containing three or more nuclei, were observed by ISET in 15 (38%) of 40 patients including 10 of 23 patients with stage IV disease, 5 of 12 patients with stage IIIB disease, and 0 of 5 patients with stage IIIA disease (Table 3). There was no association between histological subtype and the presence of CTM (Fisher's exact test $p = 0.44$). The number of intact nuclei within a single CTM ranged from 3 to 45 and the size of CTM ranged from 20 to 130 μm in diameter. Examples of typical CTM morphology, confirmed as malignant phenotype by pathology review, are shown in Figure 3*C*. Their appearance showed morphological similarity to clusters of cells within the corresponding primary tumor biopsy/cytology specimens (Figure 3*D*). In contrast, no CTM were detected in any of the 40 patients in parallel blood samples analyzed by CellSearch.

CECs and Contaminant Skin Cells

Previous studies, using CellSearch technology, have shown a normal finding of 1 to 20 CECs per milliliter of blood in healthy individuals and up to 387 CECs per milliliter of blood in patients with lung cancer.[25] Therefore, it is critical that CECs (reported size >10 μm.[26]) are not falsely designated as CTCs on ISET filters. This issue was explored by staining filters for the endothelial marker VE-cadherin (CD144) that is not expressed on tumor or on white blood cells. CECs were identified by positive VE-cadherin staining and exhibited morphology clearly distinct from CTCs/CTM (Figure 2*C*). CECs displayed regular-shaped, round, or oval nuclei, low nuclear-to-cytoplasmic ratio, and paler hematoxylin-stained nuclei compared with CTCs/CTM which exhibited irregular-shaped, large, hyperchromatic nuclei with high nuclear-to-cytoplasmic ratio. On the basis of these morphological characteristics, mature CECs could be identified and discounted when using CD45 negative selection alone (Figure 2*D*). However, a caveat is that progenitor CECs (a minority population) can express CD45 weakly and may not be excluded by negative selection using CD45. Contaminant skin cells were infrequently detected using ISET and were readily identified as polygonal-shaped cells with abundant cytoplasm and small round pyknotic nuclei with absence of CD45 expression (Figure 2*E*).

EpCam Staining in CTCs Isolated by ISET

To explore the hypothesis that a proportion of CTCs in patients with NSCLC may be EpCam negative, a series of nine patient spots were subjected to EpCam staining by IHC (positive and negative controls presented in Figure 1). All CTCs/CTM detected from the nine patients tested were negative for EpCam expression (Figure 4*A*). Nonspecific nonmembranous staining was seen in a minority of CTM, but this staining was considered to be negative (representative image in Figure 4*B*). Of the nine patients, six (67%) had no CTCs detected by CellSearch, whereas ISET detected between 12 and 188 CTCs/7.5 ml of blood. The other three patients had between 3 and 18 CTCs/7.5 ml of blood detected by CellSearch but higher numbers of cells isolated by ISET (17–84 CTCs/7.5 ml of blood).

TABLE 3. Comparison of the Number of CTCs and CTM Identified by CellSearch and ISET Expressed per 7.5 ml Blood

Patient ID	Stage	Histology	CTC No. by CellSearch per 7.5 ml Blood	CTC No. by ISET per 7.5 ml Blood	CTM No. by ISET per 7.5 ml Blood
93	IIIA	SCC	0	0	0
94	IIIA	SCC	1	0	0
57	IIIA	NOS	1	4	0
73	IIIA	SCC	0	6	0
100	IIIA	SCC	0	68	0
Mean ± SE			0.4 ± 0.2	15 ± 13.1	0 ± 0
104	IIIB	BCA	1	0	0
105	IIIB	SCC	0	0	0
89	IIIB	Adeno	3	2	0
112	IIIB	Poor diff	0	2	0
115	IIIB	SCC	0	4	0
113	IIIB	Adeno	0	8	0
90	IIIB	SCC	0	9	2
86	IIIB	NOS	0	60	4
114	IIIB	SCC	0	71	13
110	IIIB	NOS	0	71	0
91	IIIB	Adeno	1	81	6
80	IIIB	SCC	0	188	13
Mean ± SE			0.4 ± 0.3	41 ± 16.3	3 ± 1.4
18	IV	NOS	3	0	0
45	IV	SCC	1	0	0
84	IV	Adeno	78	0	0
92	IV	SCC	0	0	0
118	IV	Adeno	0	4	0
17	IV	Adeno	0	6	0
106	IV	Poor diff	0	6	0
126	IV	NOS	0	8	0
107	IV	Adeno	18	17	0
82	IV	SCC	3	24	4
68	IV	Adeno	0	34	0
13	IV	NOS	1	38	4
85	IV	NOS	30	38	0
25	IV	SCC	1	47	6
121	IV	SCC	0	49	13
83	IV	BCA	0	79	17
127	IV	NOS	3	84	0
109	IV	NOS	2	116	2
123	IV	Adeno	6	131	0
122	IV	Adeno	1	137	21
117	IV	NOS	0	193	2
116	IV	SCC	0	201	26
101	IV	NOS	0	1045	39
Mean ± SE			6 ± 3.6	98 ± 44.9	6 ± 2.2

Adeno, Adenocarcinoma; BCA, bronchioalveolar; CTCs, circulating tumor cells; CTM, circulating tumor microemboli; NOS, not otherwise specified; Poor diff, poorly differentiated; SCC, squamous cell carcinoma.

Molecular Characterization of CTCs/CTM by ISET

To further characterize CTCs/CTM identified by ISET, cells were stained by IHC for the epithelial markers CKs and EGFR (positive and negative controls shown in Figure 1). Of the nine patients tested for CK staining, eight had identifiable CTCs/CTM on the single spot tested. All CTM, where present, were positive for CK, whereas the proportion of positive single cells was more heterogeneous, ranging from 0 to 90% between patients (representative images in Figure 4*C*). Of the four patients tested for EGFR staining, all had identifiable CTCs/CTM on the single spot tested. All CTM were again positive for EGFR, whereas the proportion of positive single cells was heterogeneous, ranging from 0 to 85% between patients (representative images in Figure 4*D*).

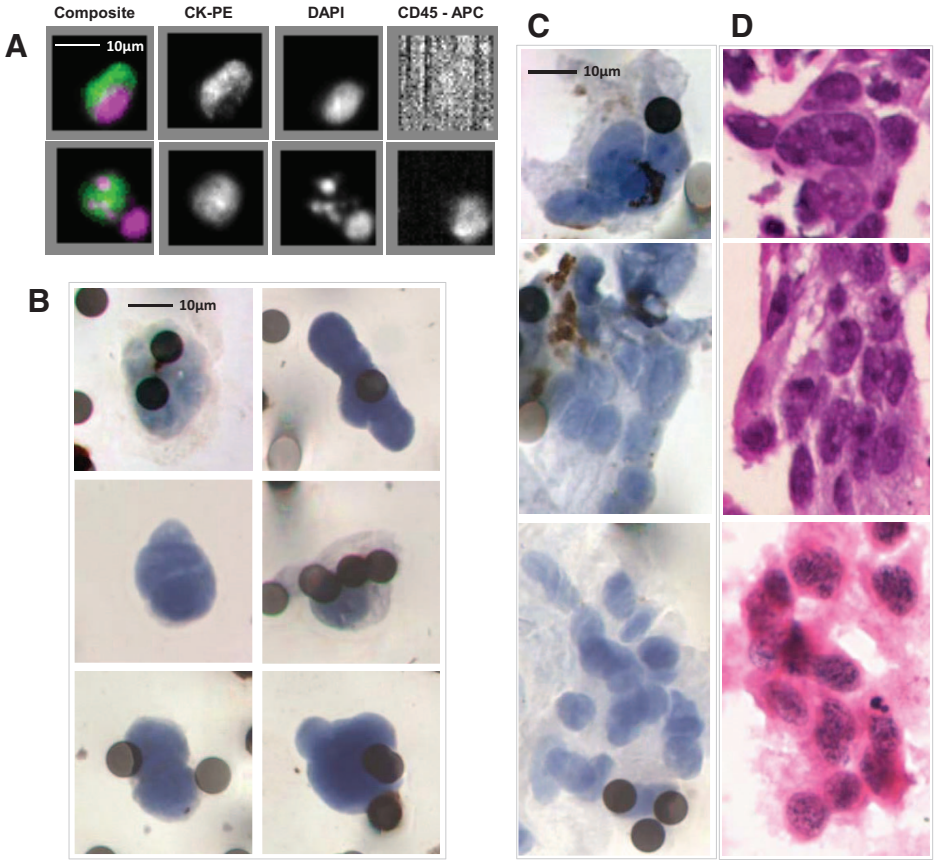

FIGURE 3. Circulating tumor cells (CTCs) and circulating tumor microemboli (CTM) from patients with non-small cell lung cancer (NSCLC) using CellSearch and ISET in comparison to primary tumor biopsies. *A*, Representative images of cells isolated by CellSearch. CTCs are defined as positive for cytokeratin and DAPI, CD45 negative, and cell size >4 μm. *B*, Representative images of cells isolated by ISET. CTCs exhibited large, hyperchromatic, irregular-shaped nuclei (identified by hematoxylin counterstain), cell size ≥12 μm, high nuclear-to-cytoplasmic ratio, and were CD45 negative. *C*, Representative images of CTM from NSCLC patients. CTM were CD45 negative, containing groups of irregularly shaped/pleomophic nuclei and enlarged nucleoli (stained by hematoxylin counterstain). *D*, Matched primary tumor biopsies/cytology specimens (top and middle panels stained with hematoxylin and eosin; bottom panel stained with papanicolau) show striking morphological similarity between groups of cells within tumor and CTM in a paired blood sample (adjacent images in panel C show CTM from matched patient blood samples). APC, allophycocyanin; CK, cytokeratin; PE, phycoerythrin; ISET, isolation by size of epithelial tumor cells.

CTC and CTM Proliferative Status

The proliferative status of CTCs and cells within CTM was explored using Ki67 in 14 patients (11, stage IV; 2, stage IIIB; and 1, stage IIIA disease). CTCs and/or CTM were identified in all cases. The proportion of CTCs staining positive for Ki67 ranged from 0 to 62% between patients with no trend according to the disease stage. In contrast, all CTM were negative for Ki67 staining (Figure 4*E*).

DISCUSSION

The introduction of the CellSearch platform has enabled robust and reliable CTC enumeration in several epithelial tumor types,[2–7,22] including NSCLC,[9] but the technique is dependent on the presence of EpCAM expression on CTCs. With EMT increasingly being reported as an important mechanism for cancer cell invasion and metastasis (with the loss of epithelial markers), this study explored the filtration-based, antigen-independent ISET system and found that higher numbers of CTCs were detected in advanced NSCLC patients compared with CellSearch. Eighty percent of patients were positive for CTCs by ISET compared with 23% by CellSearch, in support of our hypothesis. A minority of patients were positive for CTCs by CellSearch but were negative by ISET, thus changes in protein expression cannot solely account for the differences in cell capture between the two systems. Individual CTCs isolated by ISET (18–30 μm) tended to be larger than those isolated by CellSearch (4–18 μm), and these physical characteristics may also contribute to the differences in numbers of CTCs isolated by each system.

One of the most intriguing findings of this study was the isolation and prevalence of CTM by ISET. Preclinical in vivo studies have demonstrated that CTM may be an important mechanism of metastasis with survival advantages over single cells and greater propensity to seed distant metastates.[27–30] Although reported in preclinical models,[30–32] studies investigating CTM in humans have been less forthcoming, mainly due to the

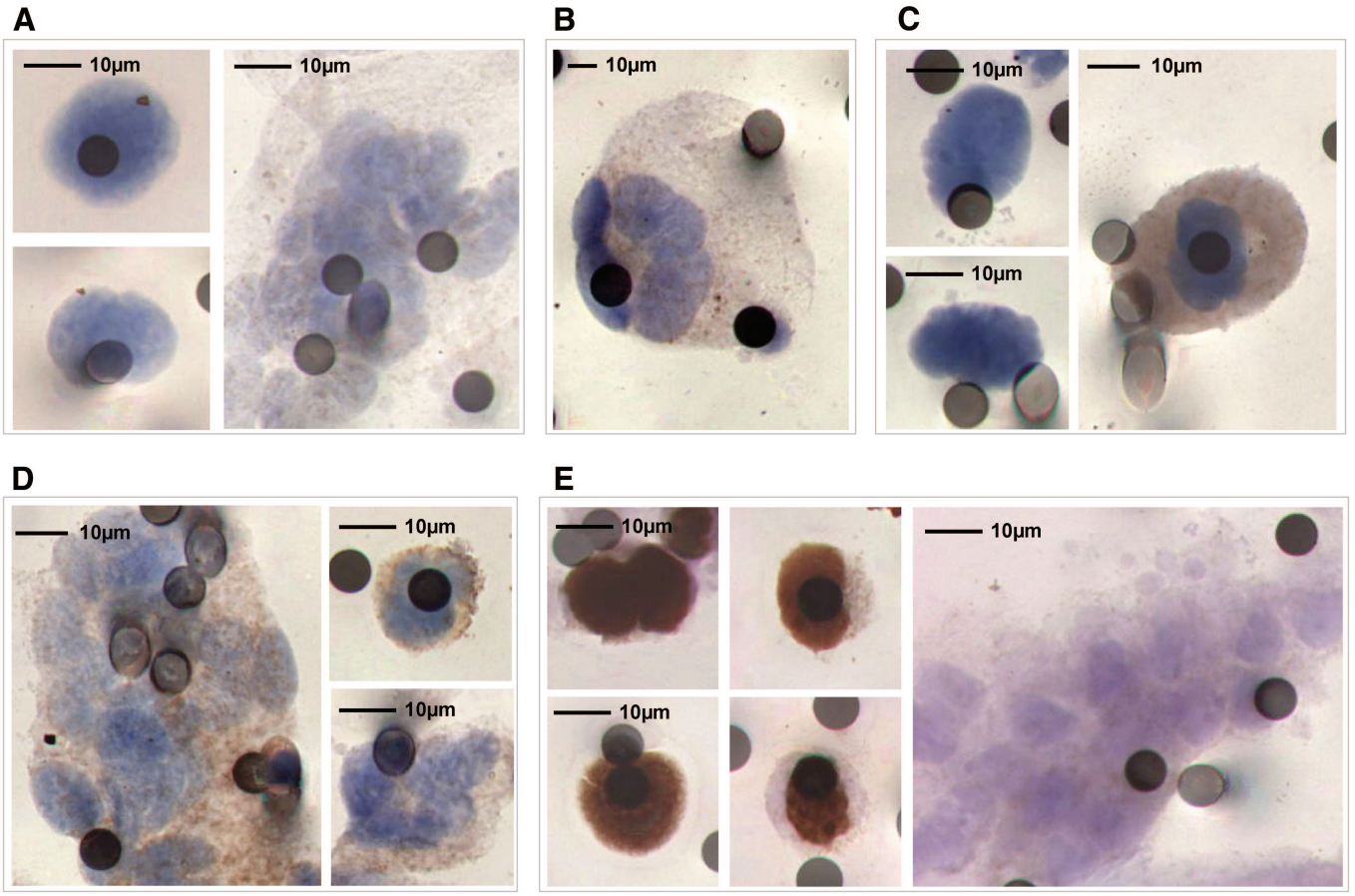

FIGURE 4. Molecular characterization of circulating tumor cells (CTCs)/circulating tumor microemboli (CTM) isolated by ISET. Filtered membrane spots were interrogated with a range of antibodies by immunohistochemistry. *A*, EpCam staining in two CTCs and CTM which were all negative for this marker. *B*, Equivocal EpCam staining within a CTM. *C*, Cytokeratin staining was heterogeneous with the images of negative CTCs shown on the left and a positive CTC on the right. *D*, Variably positive epidermal growth factor receptor (EGFR) staining in two CTM (left and bottom right images) and a CTC (top right). *E*, Ki67 (proliferative status) was positive with nuclear staining in the majority of CTCs (four images shown on the left) and was negative in all CTM (representative image shown on the right). All images were taken at ×400 magnification and representative images are shown. ISET, isolation by size of epithelial tumor cells.

difficulty in isolating clusters of cells from the blood. However, anecdotal observations of CTM in clinical samples have been reported in several cancer types using filtration techniques,[15–19] and here CTM were identified in 43% of patients with stage IIIB/IV NSCLC by ISET. Curiously, CTM were never isolated by matched samples processed by CellSearch, and this was also the case in our previous evaluation of 101 patients with advanced NSCLC by CellSearch.[9] A possible explanation may relate to magnetic beads (employed by CellSearch) having insufficient capacity to enrich CTM of large physical size (upto 130 μm) and weight.

It is important to consider, however, the possibility that CTM may be a methodological artifact of filtration. The evidence to suggest that this is not the case is that using ISET, CTM are not seen universally in patients with NSCLC; that cell groups are not forced together when single cells are spiked into human blood unless intentionally spiked as groups of cells; and that in SCLC, CTM are seen in a subset of patients using both ISET and CellSearch, perhaps because the groups of cells are smaller and lighter or because they express more EpCam or for both reasons.[20,33] Furthermore, CTM in this study showed clear morphological similarities with primary tumor biopsy/cytology specimens (Figures 3*C*, *D*). A number of other studies have detected CTM using nonfiltration approaches in patients with renal, prostate, and colorectal cancers.[34–36] Stott et al.[37] demonstrated the presence of CTM in patients with advanced NSCLC or prostate cancer using the novel herringbone-chip which is a microfluidic mixing device with EpCam-coated chevrons. The design of this device facilitates the mixing of blood as it passes through the chip allowing contact and attachment of cells with the antibody-coated surface. Cells are then directly visualized by light microscopy, and CTM were identified in a small number of patients. This lends support to the existence of CTM in patients with NSCLC as a real entity and not a methodological artifact.

Here we demonstrate that the ISET system is versatile for the molecular characterization of CTCs and CTM by IHC. This is an invaluable tool as CTC research moves beyond CTC enumeration. For example, to identify predictive and/or

pharmacodynamic biomarkers to facilitate drug development, drug target levels and downstream effects of target inhibition of a novel agent may be analyzed through serial sampling of CTCs rather than undertaking serial biopsies, often challenging to obtain in lung cancer patients. CTC characterization may also provide novel insight into the biology and mechanisms of metastasis such as EMT. In this study, as proof of principle that characterization is achievable, IHC was performed on ISET filters on an exploratory basis and cells interrogated for epithelial markers and proliferative status.

Analysis of EpCam expression on ISET-isolated CTCs/CTM showed that all cells were negative for this marker, consistent with lack of CTC detection by CellSearch in the majority (six of nine) of these patients. In three of nine patients, CTCs were detected unexpectedly by CellSearch but in much lower numbers than those isolated by ISET. The reasons for this may relate to the concept of partial EMT[10] (where cells express both epithelial and mesenchymal characteristics) and differential sensitivity of the assays or may be due to the choice of EpCam antibody used. Analysis of the epithelial markers EGFR and CK showed that single cells were heterogeneous for these markers, again supporting the concept that at least a proportion of circulating cells lack epithelial characteristics. In addition, a recent study by Hofman et al.[38] compared CellSearch and ISET for the detection of CTCs in patients with early-stage, resectable NSCLC. Similar findings were reported in that higher numbers of CTCs were isolated by ISET compared with CellSearch, and a proportion of cells isolated by ISET lacked epithelial characteristics. Cells within CTM tended to be positive for EGFR and CK expression, in our study, and that they seem to maintain their cell–cell interactions within CTM may suggest that they undergo EMT to a lesser degree than single CTCs. These hypotheses warrant further interrogation and provide an impetus to examine further CTC and CTM biology.

As an initial step, we compared the proliferative activity (by Ki67 staining) of cells within CTM and single CTCs and revealed that cells within CTM were nonproliferative, in contrast to a majority subpopulation of solitary CTCs. This suggests that at the time of sampling, CTM do not represent groups of cells actively dividing during transit in the blood, rather the lack of proliferation is more consistent with the hypothesis that cell clusters break off from the primary tumor, invade the stroma and local vasculature en mass, and appear in the blood as the product of collective migration.[29] In contrast, up to 62% of single CTCs stained positively for Ki67. In a previous study of breast cancer patients, single CTCs were negative for Ki67.[39] The difference in the proliferative status between breast cancer CTCs and lung cancer CTCs is consistent with disseminated breast cancer cells existing for many years in the bone marrow with a more protracted clinical course.[39–41] The natural history of lung cancer is more rapid and aggressive than the majority of breast cancers, and the finding of Ki67-positive CTCs may thus mirror the differential kinetics of disease progression. The biological and clinical significance of the observed heterogeneity of CTCs/CTM within and between NSCLC patients remains unclear, but the opportunity now exists to explore this in further detail.

TABLE 4. Advantages and Disadvantages of the CellSearch and ISET Techniques for the Isolation of CTCs from Patients with Non-small Cell Lung Cancer

CellSearch—EpCam-Based	ISET—Filtration-Based
Pros	
FDA approved for prognostic use (and as an aid to monitoring treatment) in patients with breast, prostate, and colorectal cancer	Nonantigen dependent—able to detect wider range and higher number of cells than CellSearch
Robust, reliable, reproducible, semiautomated processing	Able to isolate CTM Better image quality of cells by IHC
Stains with CK/CD45/DAPI and option to stain with one further antibody, i.e., multiparameter staining	Cheaper than CellSearch (but still expensive)
Nonsize dependent—capable of detecting smaller CTCs	Cells amenable to interrogation by IHC, IF, FISH, and potentially DNA/RNA extraction for genetic analyses—potential to explore CTC biology
Standardized kits	Filters can be stored at −20°C for later analysis
Cons	
EpCam dependent—may miss cells if they have undergone EMT process (i.e., lack epithelial markers)	Exploratory—needs further validation before routine clinical use
Expensive	Manual processing limits robustness and reproducibility (but potential to semi-automate)
Limited ability to further characterize cells unless present in high numbers	Size dependent—may miss cells less than 8 μm in size
Numbers of CTCs low in NSCLC and no CTM detectable	This study used single-staining IHC but multiparameter staining readily achievable by IHC or IF

FDA, Food and Drug Administration; CK, cytokeratin; FISH, fluorescent in situ hybridization; IF, immunofluorescence; IHC, immunohistochemistry; CTC, circulating tumor cell; NSCLC, non-small cell lung cancer; CTM, circulating tumor microemboli; EMT, epithelial to mesenchymal transition; EpCam, Epithelial Cell Adhesion Molecule.

There are relative advantages and disadvantages to both CTC isolation techniques used in this study, but when used together they have a complementary role. ISET isolated higher numbers of CTCs and CTM than CellSearch, was versatile for molecular characterization of cells, and has the useful attribute that it allows storage of filters at −20°C for analysis of a completed patient cohort of samples at a later time. However, ISET currently lacks the acknowledged sufficient level of robust and reliable validation from regulatory agencies as afforded to CellSearch[22] with respect to accuracy and reproducibility for CTC enumeration, and this currently restricts ISET use for formally accredited CTC enumeration and prognostication. For this reason, ISET numbers were not correlated with clinical characteristics or clinical outcomes in this study. Our previous study showed the strong prognostic utility of CellSearch detected CTC number and the association of CTCs with stage IV disease and

presence of liver or bone metastases compared with other sites of metastatic disease. Advantages and disadvantages of the CellSearch and ISET techniques are detailed in Table 4.

This study demonstrates the complementary use of two different technologies to explore and characterize CTCs/CTM in patients with NSCLC. Our findings highlight a rationale for using combinations of approaches to delineate how CTCs/CTM can be evaluated optimally to further understanding of metastasis biology with the goal of improved therapeutics and clinical management of patients with NSCLC.

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REFERENCES

1. Hou J-M, Krebs M, Ward T, et al. Circulating tumor cells, enumeration and beyond. *Cancers* 2010;2:1236–1250.
2. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 2004;351:781–791.
3. Cristofanilli M, Hayes DF, Budd GT, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *J Clin Oncol* 2005;23:1420–1430.
4. Hayes DF, Cristofanilli M, Budd GT, et al. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. *Clin Cancer Res* 2006;12:4218–4224.
5. Cohen SJ, Punt CJ, Iannotti N, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J Clin Oncol* 2008;26:3213–3221.
6. Cohen SJ, Punt CJ, Iannotti N, et al. Prognostic significance of circulating tumor cells in patients with metastatic colorectal cancer. *Ann Oncol* 2009;20:1223–1229.
7. de Bono JS, Scher HI, Montgomery RB, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin Cancer Res* 2008;14:6302–6309.
8. Krebs MG, Hou J-M, Ward T, et al. Circulating tumour cells: their utility in cancer management and predicting outcomes. *Ther Adv Med Oncol* 2010;2:351–365.
9. Krebs MG, Sloane R, Priest L, et al. Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. *J Clin Oncol* 2011;29:1556–1563.
10. Christiansen JJ, Rajasekaran AK. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer Res* 2006;66:8319–8326.
11. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002;2:442–454.
12. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* 2006;7:131–142.
13. Raimondi C, Gradilone A, Naso G, et al. Epithelial-mesenchymal transition and stemness features in circulating tumor cells from breast cancer patients. *Breast Cancer Res Treat* 2011;130:449–455.
14. Mikolajczyk SD, Millar LS, Tsinberg P, et al. Detection of EpCAM-negative and cytokeratin-negative circulating tumor cells in peripheral blood. *J Oncol* 2011;2011:252361.
15. Vona G, Sabile A, Louha M, et al. Isolation by size of epithelial tumor cells: a new method for the immunomorphological and molecular characterization of circulating tumor cells. *Am J Pathol* 2000;156:57–63.
16. Vona G, Estepa L, Beroud C, et al. Impact of cytomorphological detection of circulating tumor cells in patients with liver cancer. *Hepatology* 2004;39:792–797.
17. Pinzani P, Salvadori B, Simi L, et al. Isolation by size of epithelial tumor cells in peripheral blood of patients with breast cancer: correlation with real-time reverse transcriptase-polymerase chain reaction results and feasibility of molecular analysis by laser microdissection. *Hum Pathol* 2006;37:711–718.
18. De Giorgi V, Pinzani P, Salvianti F, et al. Application of a filtration- and isolation-by-size technique for the detection of circulating tumor cells in cutaneous melanoma. *J Invest Dermatol* 2010;130:2440–2447.
19. Hofman V, Bonnetaud C, Ilie MI, et al. Preoperative circulating tumor cell detection using the isolation by size of epithelial tumor cell method for patients with lung cancer is a new prognostic biomarker. *Clin Cancer Res* 2011;17:827–835.
20. Hou JM, Krebs M, Ward T, et al. Circulating tumor cells as a window on metastasis biology in lung cancer. *Am J Pathol* 2011;178:989–996.
21. The Medicines for Human Use (Clinical Trials) Regulations (UK Gov). 2004. Available at: http://www.legislation.gov.uk/uksi/2004/1031/contents/made.
22. Allard WJ, Matera J, Miller MC, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res* 2004;10:6897–6904.
23. Miller MC, Doyle GV, Terstappen LW. Significance of circulating tumor cells detected by the CellSearch system in patients with metastatic breast colorectal and prostate cancer. *J Oncol* 2010;2010:617421.
24. Riethdorf S, Fritsche H, Muller V, et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. *Clin Cancer Res* 2007;13:920–928.
25. Rowand JL, Martin G, Doyle GV, et al. Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. *Cytometry A* 2007;71:105–113.
26. Woywodt A, Goldberg C, Scheer J, et al. An improved assay for enumeration of circulating endothelial cells. *Ann Hematol* 2004;83:491–494.
27. Fidler IJ. The relationship of embolic homogeneity, number, size and viability to the incidence of experimental metastasis. *Eur J Cancer* 1973;9:223–227.
28. Liotta LA, Saidel MG, Kleinerman J. The significance of hematogenous tumor cell clumps in the metastatic process. *Cancer Res* 1976;36:889–894.
29. Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol* 2009;10:445–457.
30. Ilina O, Friedl P. Mechanisms of collective cell migration at a glance. *J Cell Sci* 2009;122:3203–3208.
31. Liotta LA, Kleinerman J, Saidel GM. Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. *Cancer Res* 1974;34:997–1004.
32. Alpaugh ML, Tomlinson JS, Kasraeian S, et al. Cooperative role of E-cadherin and sialyl-Lewis X/A-deficient MUC1 in the passive dissemination of tumor emboli in inflammatory breast carcinoma. *Oncogene* 2002;21:3631–3643.
33. Hou JM, Greystoke A, Lancashire L, et al. Evaluation of circulating tumor cells and serological cell death biomarkers in small cell lung cancer patients undergoing chemotherapy. *Am J Pathol* 2009;175:808–816.
34. Kats-Ugurlu G, Roodink I, de Weijert M, et al. Circulating tumour tissue fragments in patients with pulmonary metastasis of clear cell renal cell carcinoma. *J Pathol* 2009;219:287–293.
35. Brandt B, Junker R, Griwatz C, et al. Isolation of prostate-derived single cells and cell clusters from human peripheral blood. *Cancer Res* 1996;56:4556–4561.
36. Molnar B, Ladanyi A, Tanko L, et al. Circulating tumor cell clusters in the peripheral blood of colorectal cancer patients. *Clin Cancer Res* 2001;7:4080–4085.
37. Stott SL, Hsu CH, Tsukrov DI, et al. Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. *Proc Natl Acad Sci USA* 2010;107:18392–18397.
38. Hofman V, Ilie MI, Long E, et al. Detection of circulating tumor cells as a prognostic factor in patients undergoing radical surgery for non-small-cell lung carcinoma: comparison of the efficacy of the CellSearch assay and the isolation by size of epithelial tumor cell method. *Int J Cancer* 2011;129:1651–1660.
39. Muller V, Stahmann N, Riethdorf S, et al. Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. *Clin Cancer Res* 2005;11:3678–3685.
40. Hedley BD, Chambers AF. Tumor dormancy and metastasis. *Adv Cancer Res* 2009;102:67–101.
41. Meng S, Tripathy D, Frenkel EP, et al. Circulating tumor cells in patients with breast cancer dormancy. *Clin Cancer Res* 2004;10:8152–8162.

