

Epithelial–Mesenchymal Transitioned Circulating Tumor Cells Capture for Detecting Tumor Progression

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Abstract

Purpose: This study aimed to detect cell-surface vimentin (CSV) on the surface of epithelial–mesenchymal transitioned (EMT) circulating tumor cells (CTC) from blood of patients with epithelial cancers.

Experimental Design: In this study, 101 patients undergoing postsurgery adjuvant chemotherapy for metastatic colon cancer were recruited. EMT CTCs were detected from blood of patients using the 84-1 monoclonal antibody against CSV as a marker. EMT CTCs isolated were characterized further using EMT-specific markers, fluorescent *in situ* hybridization, and single-cell mutation analysis.

Results: Using the 84-1 antibody, we detected CSV exclusively on EMT CTCs from a variety of tumor types but not in the surrounding normal cells in the blood. The antibody exhibited very high spec-

ificity and sensitivity toward different epithelial cancer cells. With this antibody, we detected and enumerated EMT CTCs from patients. From our observations, we defined a cutoff of <5 or ≥ 5 EMT CTCs as the optimal threshold with respect to therapeutic response using ROC curves. Using this defined threshold, the presence of ≥ 5 EMT CTCs was associated with progressive disease, whereas patients with <5 EMT CTCs showed therapeutic response.

Conclusion: Taken together, the number of EMT CTCs detected correlated with the therapeutic outcome of the disease. These results establish CSV as a universal marker for EMT CTCs from a wide variety of tumor types and thus provide the foundation for emerging CTC detection technologies and for studying the molecular regulation of these EMT CTCs. *Clin Cancer Res*; 21(4): 899–906. ©2014 AACR.

Introduction

Metastasis is the main cause for cancer-related deaths worldwide and circulating tumor cells (CTC) are considered to be the roots of metastases (1). These cells are emerging as a novel target for early detection of metastasis and for monitoring the therapeutic efficacy of anticancer drugs (2). Current CTC technology relies on the capture of these cells with antibodies against the epithelial phenotype-specific markers EpCAM and cytokeratins (2). A major drawback with these markers is their inability to detect CTCs that no longer express EpCAM after undergoing epithelial–mesenchymal transition (EMT; i.e., EMT CTCs), a cellular process in which epithelial cells acquire a mesenchymal

phenotype and thus become more aggressive and invasive (3). These EMT CTCs are considered the key cell subtype that causes metastasis (4). Although EMT CTCs have been gaining attention, the absence of a cell-surface mesenchyme-specific marker hampers research in the field of CTC detection.

EMT in cancer cells has been associated with an increasingly invasive, chemoresistant, and metastatic phenotype in a wide variety of cancer types. The EMT process is associated mainly with overexpression of vimentin (5), and single-cell profiling of CTCs isolated from patients with cancer has indicated overexpression of vimentin transcript compared with established cancer cell lines (6), indicating a mesenchymal phenotype in these CTCs. However, intracellular expression of vimentin in normal mesenchymal cells, including most white blood cells, limits the use of this protein as a CTC marker. We and others have previously reported the detection of vimentin on the surface of cancer cells (5, 7–9). Unlike intracellular vimentin, the expression of cell-surface vimentin (CSV) is mainly associated with cancer cells only. We therefore hypothesized that CSV can serve as a marker for EMT CTCs.

Siewerts and colleagues previously showed that the CellSearch detection method does not identify cells that have undergone EMT (3). Although a few researchers have reported detecting transitioned CTCs with a panel of markers (4, 10) or individual markers (11, 12), the uncertainty regarding their ability to detect these cells from a wide variety of solid tumors using the existing technologies or markers calls for the discovery of novel single and specific markers for EMT CTCs. Moreover, those few reported EMT CTC markers have not been used to test the correlation between EMT CTCs and disease progression. Here, we report the discovery

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Translational Relevance

In the present study, epithelial–mesenchymal transitioned (EMT) circulating tumor cells (CTC) were detected and isolated from patients with colon cancer using cell-surface vimentin (CSV) as a newly developed mesenchymal CTC marker. Patients with progressive disease showed an increasing number of EMT CTC in comparison with patients who are responding/have stable disease. This is of potential clinical importance, as it provides evidence that EMT CTC are critical in detecting patients with tumor progression and are missed using the conventional EpCAM-based CTC isolation technologies. These results help provide more convincing evidence for using EMT CTC detection in future clinical practice.

of cancer cell CSV as a marker of EMT CTCs with a monoclonal antibody we developed that shows high specificity and sensitivity toward different cancer types, thus making it a universal marker for EMT CTCs. Using our antibody, we were able to correlate counts of EMT CTCs with disease status by using blood samples from patients with colorectal cancer and other independent clinical diagnostic methods.

Materials and Methods

Cell culture

All cell lines used in this study were obtained from the American Type Culture Collection and were grown according to the supplier's recommendations. All cell lines were cultured within three passages from the time of purchase. Cell lines with no particular culture recommendations were grown in DMEM-F12 medium (Sigma-Aldrich) with 10% fetal bovine serum, 1% L-glutamine, and 0.1% penicillin/streptomycin (Gibco; Invitrogen). NCM-356, normal human colonic cell line was used as control. Primary cultures obtained from human colon (HPC), liver (HLM, primary colon cancer metastasized to liver), and lung (CPM, primary colon cancer metastasized to lung) cancers were obtained after cell dissociation from the primary tumors and cultured in DMEM/F-12 medium with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, 100 µg/mL Primocin (InvivoGen), and 0.1% penicillin/streptomycin. Medium was changed once every 4 days. All cells were maintained at 37°C in an incubator with a 5% CO₂.

Geltrex thin layer method

HPC-1 cells were grown on Geltrex reduced growth factor basement membrane matrix (Invitrogen). This matrix is a soluble form of basement membrane purified from Engelbreth-Holm-Swarm tumor, which gels at 37°C to form a reconstituted basement membrane that provides the matrix for the culture of cells. Major components of Geltrex include various growth factors and laminin, collagen IV, and entactin. According to the manufacturer's recommendation, Geltrex was thawed on ice and 100 µL was used to coat the Lab-Tek chamber slides (Thermo) 1 hour before the cells were plated. Later, 1,000 HPC-1 cells in 100 µL of cold serum-free DMEM/F-12 medium with 2% Geltrex were plated on the chamber slides with a thin gel coating. The cells were grown at 37°C in a humidified atmosphere of 5% CO₂ in air and were observed through a bright-field microscope for the

formation of spheres. Sphere-containing chamber slides were then processed for immunofluorescence staining.

Study cohort

Patients of any age with metastatic colorectal cancer refractory to 5-fluorouracil who were undergoing palliative chemotherapy at The University of Texas MD Anderson Cancer Center were eligible for this study. Patients were at different stages of treatment with different therapeutic regimens as listed in the Supplementary Table S1. Routine diagnostic workup included diagnostic imaging, chest X-rays, bone scan, blood sampling, and clinical examination. Study age matched blood samples from healthy blood donors were obtained from the Gulf Coast Blood Center in Houston, TX. Nine patients had died during the course of this study. For preliminary analysis only, blood samples from patients with breast, bladder, and liver cancer were collected.

Blood collection and processing

Human blood samples for CTC analysis were obtained after informed consent had been obtained from the patient or blood donor, per the Institutional Review Board protocol at the MD Anderson Cancer Center. CTC detection was conducted as and when possible; no attempt made to reach a defined statistical power. At any given blood draw, a maximum of 7.5 mL of blood was obtained using CPT Vacutainer tubes (BD Biosciences). Single nucleated cells were isolated within 48 hours of blood collection, per the manufacturer's recommendation. Cells were then washed in phosphate-buffered saline (PBS) and used for further analysis. Neither patients nor clinicians were informed of the results from the CTC analysis.

84-1⁺ Cell selection

Method of cell isolation, confirmation for positive selection and validation steps were described in (9). Briefly, first CD45⁺ cells were depleted using an EasySep human CD45 depletion kit (StemCell Technologies) according to the manufacturer's recommendation. To minimize nonspecific binding, antibody against human Fc receptor (Miltenyi Biotec) was added to the cocktail. Second, the CD45⁻ cell fraction was subjected to 84-1⁺ selection. Cells were labeled with the 84-1 anti-vimentin antibody, and later mouse IgG-binding microbeads (Miltenyi Biotec) were added to the mixture. 84-1⁺ Cells were then extracted using the magnetic column according to the manufacturer's recommendation (Miltenyi Biotec). The cells thus obtained were 84-1⁺ and CD45⁻ and ready for further analysis.

Antibodies

Antibodies against the specific markers EpCAM (D1B3) Rabbit mAb #2626, SLUG (C19G7) Rabbit mAb #9585, E-cadherin (24E10) Rabbit mAb #3195, β-catenin (D10A8) XP Rabbit mAb #8480, and c-myc (D84C12) XP Rabbit mAb #5605 were obtained from Cell Signaling Technology. Antibodies against the specific markers FOXC2 (AF5044), TWIST-1 (AF6230), and SNAIL (AF3639) were obtained from R&D Systems.

Flow cytometry

A total of 5×10^5 cells were detached with a nonenzymatic dissociation buffer, washed, and stained for 20 minutes on ice in the dark. For CSV analysis, cells were stained with the 84-1 monoclonal antibody we developed (1:100); mouse primary

antibody (Invitrogen) was used as an isotype control. Later, cells were rinsed twice in PBS and labeled for secondary antibody using Alexa Fluor-405, -488, or -555 secondary antibody (Invitrogen). Cells were then washed twice in PBS and immediately used for data acquisition using an Attune flow cytometer (Applied Biosystems). Fifty thousand cells were counted for the analysis. The data were analyzed using the FlowJo software (TreeStar).

Spiking assay

To demonstrate the precision and reproducibility of CTC capture by the 84-1 antibody, cultured cancer cells were spiked into blood collected from healthy donors. For the sensitivity assay, approximately 2, 5, 10, or 25 Calcein AM-labeled (EMD Bioscience) HLM-3 cells were spiked into a sample containing 1×10^6 peripheral blood mononuclear cells. To demonstrate specificity, approximately 5 Calcein AM-labeled HLM-3 cells were spiked into samples containing 1×10^6 , 2×10^6 , or 2.5×10^6 peripheral blood mononuclear cells. All cells used for spiking experiments were subjected to 84-1⁺ selection a day before the spiking analysis to increase the fraction of 84-1⁺ cells. For cell counting, cells were harvested in culture medium and then serially diluted to achieve the required counts, which were then confirmed

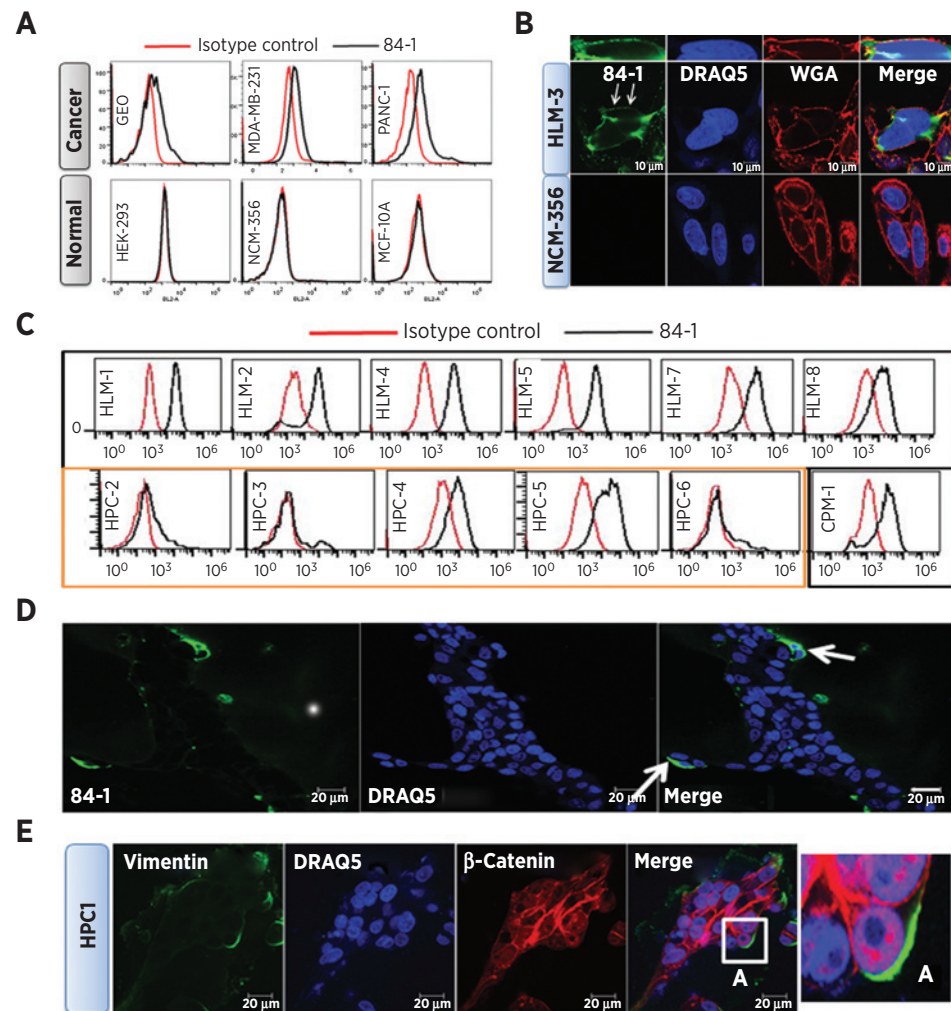
in a series of 5- μ L spots under a microscope. If lower or higher numbers of cells were observed, we calculated the necessary counts of cells required to be spiked. Spiking experiments were performed in triplicate to ensure the sensitivity and specificity of the method. For negative controls, CSV⁻ HEK293T and NCM-356 cells were spiked into blood and analyzed for 84-1⁺ selection. Also, as an additional control mouse IgG was used for the isolation procedure and probed with anti-mouse secondary antibody.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism software, where $P < 0.05$ was considered significant. Differences in baseline characteristics between treatment responders and nonresponders were analyzed using the Fisher exact and *t* tests. Diagnostic performance of CTC count was assessed by constructing a receiver operating characteristic (ROC) curve, and was further evaluated by calculating the area under each ROC curve (AUC-ROC; ref. 13). An AUC-ROC of value 1 denotes that the test method is able to discriminate perfectly, whereas an AUC-ROC of value 0.5 would denote a worst discrimination of the test. *P* value was calculated for the difference between each AUC-ROC.

Figure 1.

CSV expression is limited to cancer cells. A, immunologic assessment of CSV expression in cancer (top) and normal (bottom) cell lines using flow cytometry. CSV was detected by the 84-1 antibody in only the cancer cell lines. Isotype controls were used as negative controls. B, cell-surface staining analysis for CSV in liver cancer cell line HLM-3 and normal colon cell line NCM-356 using confocal microscopy. Cells were stained for CSV (with the 84-1 antibody; green), nuclei (with DRAQ5; blue), and the cell-surface marker WGA (red). Arrows, cell surface vimentin colocalized with WGA. Scale bar, 10 μ m. C, immunologic assessment of CSV in human primary colon cancer (HPC) cells, human colon cells metastasized to the liver (HLM), and human colon cells metastasized to the lung (CPM-1). Metastatic cancers are outlined in black; primary tumors are outlined in orange. D, CSV detection in HPC-1-derived spheres on thin Geltrex-coated wells using confocal microscopy. CSV (green) was detectable in a few cells at the periphery of the sphere (arrows). DRAQ5 was used to demarcate the cells. Scale bar, 20 μ m. E, vimentin and β -catenin expression in HPC-1-derived spheres on thin Geltrex-coated wells using confocal microscopy. Total vimentin (green) was detectable in a few cells at the periphery of the sphere, which correlated with the increased nuclear accumulation of β -catenin (red). DRAQ5 was used to demarcate the cells. Scale bar, 20 μ m. Inset A in "Merge" image is enlarged at right.



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Microscopy image capture and analysis is included in Supplementary Methods.

Results

CSV expression is restricted to cancer cells

Because commercial antibodies against CSV are not available, we generated a CSV-specific monoclonal antibody, called 84-1, using a differential expression screening strategy described in ref. (9). Briefly, to screen for CSV-specific antibodies produced against full-length vimentin, we used the cell line LM7 (human metastatic osteosarcoma cells) to represent CSV-positive cells and the cell lines NCM-356 (human colon epithelial cells) and hFOB (human fetal osteoblasts) to represent CSV-negative cells. The monoclonal antibody had very high affinity for cancer cell CSV and did not bind to normal epithelial or mesenchymal cells. Screening for established cancer [GEO (colonic), MDA-MB-231 (breast), and PANC-1 (pancreatic)] and normal (HEK-293, NCM-356, and MCF-10A) cell lines indicated the presence of vimentin only on the surface of cancer cells (Fig. 1A). It is evident from these results that only a fraction of cells was positive for CSV. This fraction may be strongly associated with metastasis. Furthermore, our 84-1 antibody was specific to cancer cells and did not exhibit binding affinity toward any of the subsets of white blood cells. We have also analyzed several different established cancer cell lines (breast, liver, colon, brain, bladder, and pancreas) for CSV expression using flow cytometry and our results indicate that majority of these cancer cell lines are positive for CSV (Table 1). These results thus prove CSV to be an excellent universal marker for any given tumor type.

To confirm the surface expression of vimentin, we performed immunocytochemical analysis of human liver metastatic cells (HLM-3) and normal human colon cells (NCM-356). The results indicated that vimentin was present on the surface of the cancer cells (Fig. 1B) but not on the surface of the normal cells. In addition, vimentin colocalized with the cell-surface marker wheat germ agglutinin (WGA). Permeabilizing these cells revealed the

presence of cytoplasmic vimentin in only the cancer cells and not in the normal cells (Supplementary Fig. S1A). These results confirmed that the surface expression of vimentin was restricted to cancer cells.

To analyze whether CSV-expressing cancer cells are metastatic, we used flow cytometry to analyze CSV expression in primary cancer cells isolated from human colon (HPC), human colon cells metastasized to the liver (HLM), and human colon cells metastasized to the lung (CPM). The results indicated overexpression of CSV in metastatic tumors compared with primary tumors (Fig. 1C), suggesting that CSV expression is mainly associated with metastasis and could serve as a potential biomarker for metastasis. This result also suggested that a subpopulation of primary colon cancer cells that express CSV have an invasive or metastatic phenotype and are shed into the blood circulation for spreading to distant organs. This possibility was supported by the observed presence of CSV⁺ cells at the periphery of the sphere in a three-dimensional HPC-derived sphere model (Fig. 1D). Cells at the periphery were more aggressive and invasive, which is supported by the increasing nuclear accumulation of β -catenin that denotes the invasive phenotype of these cells (ref. 14; Fig. 1E). From these observations, it was evident that CSV could serve as a specific marker for detecting metastatic cancer cells.

84-1 Antibody detects spiked cancer cells with high sensitivity and specificity

On the basis of our detection of CSV in a range of metastatic tumors, we postulated that CSV could serve as a biomarker to detect metastatic CTCs from epithelial cancers. We verified the detection of 84-1⁺ HLM-3 cells, which were labeled with Calcein-AM tracking dye, and spiked different numbers of cells into 7.5 mL of normal human blood. After CD45 depletion and 84-1⁺ selection, the cells recovered were subjected to immunofluorescence staining. From fluorescence microscopy micrographs, it was evident that even a single labeled cell could be isolated from whole blood by using the 84-1 antibody (Fig. 2A). Because the sensitivity (limit of detection) and specificity (no background/unwanted cells) of detection are important parameters for using an antibody for CTC enumeration, we evaluated these parameters in our spiking assay using 84-1⁺ HLM-3 cells. Linear regression of the number of detected tumor cells versus the number of tumor cells spiked yielded a correlation coefficient (R^2) of 0.971 ($P < 0.001$; Fig. 2B) with approximately 100% specificity. Normal cells spiked in the blood were undetectable by the 84-1 antibody and tumor cells were undetectable using mouse IgG used as a control. Taken together, these results indicated a very high specificity and sensitivity of the antibody to detect spiked cells at various concentrations.

84-1 Antibody detects EMT CTCs that express EMT-specific markers

Because our spiking assays showed very high specificity for spiked cancer cells, we used the above-described method to detect CTCs from patients with colorectal cancer as well as several methods to confirm the cancerous phenotype of these isolated CTCs. β -Catenin and c-myc proteins are overexpressed in colorectal cancers (15), and in our study we detected and isolated 84-1⁺ CD45⁻ CTCs from blood of patients with colorectal cancer and confirmed the overexpression of these proteins in these cells (Fig. 2C). The localization of these proteins in the nucleus indicated that these were active transitioned cells (16). For further

Table 1. Cell-surface vimentin expression analysis in different cancer cell lines

Cell line	CSV	Cell line	CSV
Breast		Colon	
MCF-7 (H)	+	DLD-1 (H)	++
SKBR3 (H)	+	GEO (H)	++
MDA-MB-231 (H)	+	OS-187 (H)	++
MDA-MB-453 (H)	+	SW620 (H)	+
MDA-MB-458 (H)	++	SW480 (H)	+
4T1 (M)	+	HCT-116 (H)	+
Liver		Bladder	
AMC14 (M)	++	HT-29 (H)	++
Brain		Pancreas	
SKNAS (H)	++	Caco-2 (H)	+
SKNBE2 (H)	+++	CT-26 (M)	+
NGP (H)	+	Bladder	
SH-SY5Y (H)	++	RT4V6 (H)	+
LAN5 (H)	++	T24 (H)	++
KCN (H)	+	Pancreas	
DBT (M)	+	PANC-1 (H)	++
U251 (H)	+	MiaPACA-2 (H)	+

NOTE: CSV was scored using flow cytometric analysis by measuring mean fluorescence intensity of CSV.

Abbreviations: H, human; M, mouse.

"+, ++, +++": <2-, <4-, >4-fold presence compared with isotype control.

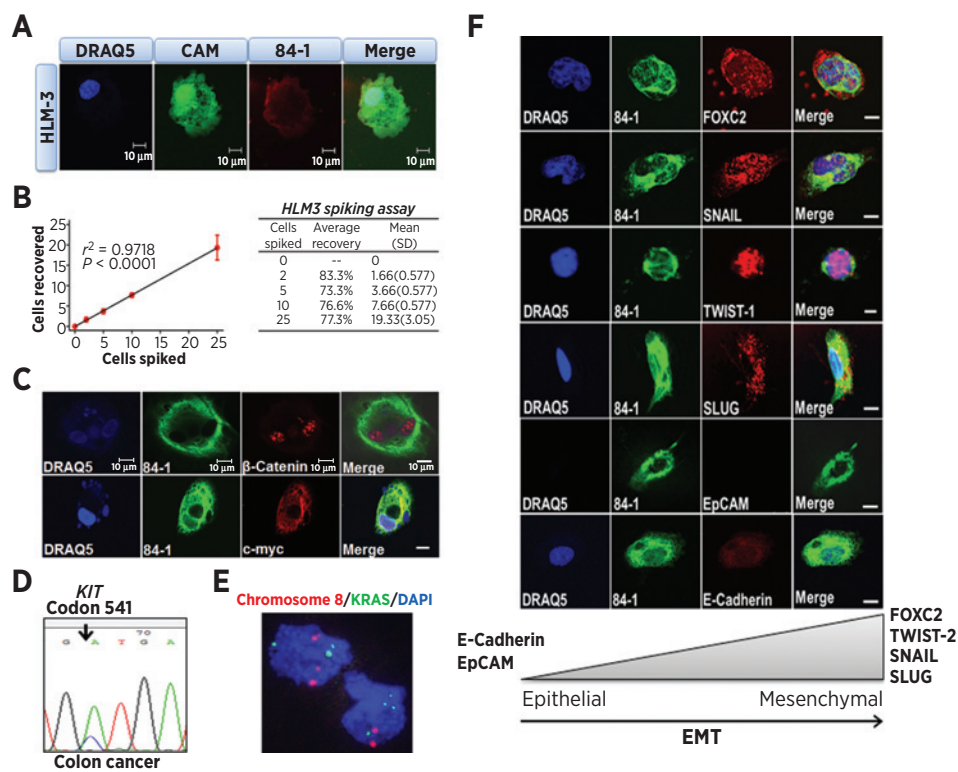


Figure 2. Spiking assay and detection of CTCs from human colorectal cancer patients. A, detection of HLM-3 cells labeled with tracker dye Calcein-AM (CAM; green) that were spiked into 7.5 mL of blood using fluorescence microscopy. Cells were also stained for nuclei (with DRAQ5; blue) and vimentin (with the 84-1 antibody; red). B, regression analysis of capture efficiency for up to 25 HLM-3 cells spiked in human blood. C, analysis of 84-1⁺ CD45⁻ CTCs from human colon cancer samples that were isolated and stained for nuclei (with DRAQ5; blue), total vimentin (with 84-1; green), β -catenin (red), and c-myc (red). The results indicated complete nuclear localization of β -catenin and c-myc. Scale bar, 10 μ m. D, mutational analysis of *KIT* in colon cancer-derived CTC. E, dual-probe FISH with a centromere probe for chromosome 8 (red) and region-specific probe for *KRAS* (green). Three chromosome 8 signals and three *KRAS* signals were detected in the cell at top, whereas two chromosome 8 signals and four *KRAS* signals were detected in the cell at bottom. Nuclei were counterstained with DAPI (blue). F, analysis of colon cancer-derived 84-1⁺ CD45⁻ CTCs from patient samples for specific molecular EMT markers. CTCs were stained for vimentin (with 84-1 antibody) and for FOXC2, SNAIL, TWIST-1, SLUG, EpCAM, and E-cadherin with the respective antibodies. Scale bar, 5 μ m.

validation of 84-1⁺ CD45⁻ CTCs isolated from all cancers, we subjected these cells to cancer-specific mutation analysis. A representative colon cancer sample was positive for mutations in *KIT*, *KRAS*, and *APC*; however, CTCs isolated from this patient harbored a mutation in *KIT* only (Fig. 2D). Mutational analysis from other patients is listed in Supplementary Table S1. Heterogeneity among CTCs from the same patient have been reported (17), and from our observations it was interesting to note that CTCs harbored only specific mutations.

As a second step for validation, we used fluorescent in situ hybridization (FISH) to detect amplification of the *KRAS* gene and chromosome 8 signals in EMT CTCs from a representative sample (Fig. 2E). The FISH data indicated a heterogeneous amplification of targets, again highlighting the heterogeneous nature of these CTCs. Taken together, our results indicated that the cells isolated from the blood of patients with cancer were of cancerous origin and were heterogeneous.

Because we were able to detect CSV⁺ CTCs in cancers of epithelial origin and establish vimentin as a marker for EMT, we hypothesized that 84-1⁺ CD45⁻ CTCs had undergone EMT. An analysis of vimentin-positive CTCs with known EMT-specific markers (18) revealed increased expression of FOXC2, SNAIL, TWIST-1, and SLUG and markedly decreased expression of epi-

thelium-specific markers E-cadherin and EpCAM (Fig. 2F), thus confirming the mesenchymal nature of 84-1⁺ CD45⁻ CTCs. Expression of EMT-inducing transcription factors, including the same four proteins, has been associated with tumor invasion and metastasis (18), and TWIST-1 (19) and SLUG (20) overexpression has been shown to be independent prognostic parameters for poor survival in patients with colorectal carcinoma. Thus, "metastatic-like CTCs" might be detected using CSV as a marker.

Moreover, we detected EMT CTCs from other solid tumors, including those of the breast, bladder, and liver (Fig. 3A), which suggests that CSV has a universal capacity for detecting EMT CTCs. With the use of CSV as a marker, further characterization of transitioned CTCs and better understanding of their contribution to the metastatic spread of a wide variety of cancer types are now possible.

84-1⁺ CTCs isolated from patients with colorectal cancer correlate with disease status

Because we were able to confirm that the CTCs detected with our anti-CSV antibody were cancerous and had transitioned to the mesenchymal phenotype, we next tested human blood samples from 12 healthy blood donors and from 101 patients with metastatic colorectal cancer for 84-1⁺ CD45⁻ CTCs. In this

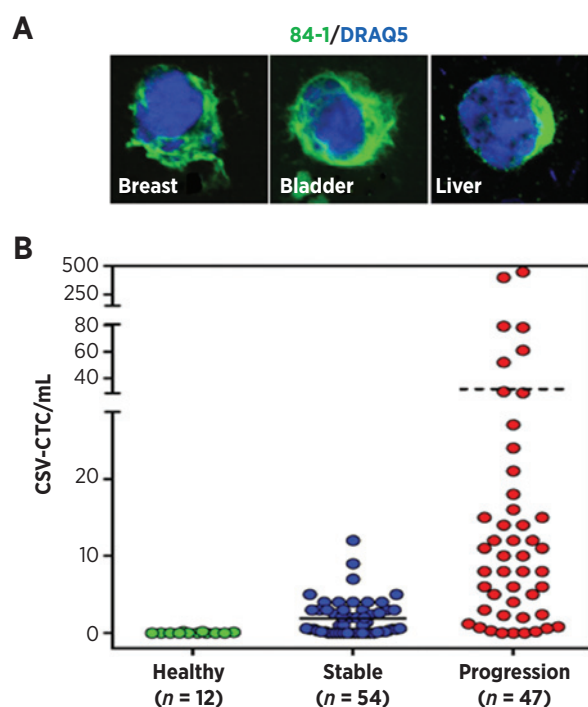


Figure 3. A, EMT CTCs isolated from breast, bladder, and liver cancers. CTCs were stained for vimentin (green) and nuclei (DRAQ5). B, enumeration of CSV⁺ CTCs from blood specimens from healthy volunteers and from patients with metastatic colorectal cancer. Patients were categorized on the basis of whether the disease was stable or progressive. Horizontal bar in the graph represents the mean value of the CTC.

study, fluorescent images were reviewed independently by researchers blinded to treatment and outcome. No adverse events or complications had been reported during the blood collections. Blood samples from the healthy donors were analyzed under the same conditions. None of the healthy donors had detectable CTCs, indicating a high specificity of the 84-1 antibody. CTCs were detectable in 85 of the 101 (84%) metastatic colorectal cancer patient samples. The number of CTCs isolated from these patients ranged from 0 to 450 per mL (Fig. 3B). We divided the patient population into two groups based on RECIST guidelines: (i) stable or responding disease, (ii) progressive disease. Of these 101 patients, 54 (53%) exhibited radiographic response or stable disease, and 47 (46%) had progressive disease (Table 2). We observed a significant difference in response to therapy by CTC count: patients with ≥ 5 per

mL were more likely to resist treatment and exhibit progressive disease, whereas patients with < 5 per mL were more likely to be sensitive to treatment and exhibit stable disease ($P = 0.0195$). This threshold cutoff range of 5 CTCs/mL was calculated using ROC curves (Supplementary Fig. S2 and Supplementary Table S2). Fourteen patients (14%) with a count of < 5 per mL showed progressive disease, whereas 5 patients (5%) with a count of ≥ 5 per mL had stable disease. This discrepancy might be attributable to the clinical stage of the disease and the treatment type used for each patient. From the analysis, it was observed that CTC are better prognostic markers for evaluating therapeutic responses.

Sensitivity, specificity, and positive predictive value

From our study data, we determined that enumerating EMT CTCs is highly specific (91%) in identifying the progressive cell population and has a positive predictive value of 87% for the therapeutic outcome of the disease.

Discussion

Many approaches have been implemented to detect CTCs from blood samples, including flow cytometry, size-based separation, and optical imaging-based technology (21). Although these techniques are relatively new, antibody-based separation of CTCs has several advantages that are already making those technologies obsolete. One of the most successful antibody-dependent technologies is the CellSearch (22) method by Veridex, which is the only FDA-approved CTC detection platform available for detecting CTCs from patients. CellSearch technology is dependent on anti-EpCAM antibody for isolating CTCs and on staining with cytokeratins from the blood of patients with epithelial cancers. Although this platform has gained much attention, in recent years, it has been shown that by using EpCAM, cytokeratins, or both as a CTC target, CellSearch and other technologies have overlooked CTCs that have undergone EMT and have lost the ability to express EpCAM (3) and other epithelial markers. EMT CTCs have been shown to correlate with disease progression (4) or relapse and their presence could be a key determinant of metastasis and poor prognosis.

The main goal of our laboratory was to identify EMT CTCs from the blood of patients by using a single, specific marker instead of a multitude of markers and probes. As a first step toward this goal, we analyzed the proteins that are transported to the cell surface of cancer cells during EMT. We previously demonstrated that vimentin is an EMT marker and is localized on the surface of cancer cells (5), so it served as an interesting target for us. However, due to the lack of a specific antibody that can bind to vimentin on the cell surface (perhaps due to a structural change in the protein as it is

Table 2. Patient characteristics

Characteristic	Patients, n (%)	CTC count/mL		
		0	<5	>5
Age				
≤ 50 years	34 (34%)	3	16	15
> 50 years	67 (66%)	13	31	23
Sex				
Male	59 (58%)	13	29	17
Female	42 (42%)	3	18	21
Disease status				
Stable	54 (53%)	13	36	5
Progressive	47 (47%)	3	11	33
Overall	101 (100%)	16	47	38

transported to the surface); we were unable to detect this antigen on the cell surface of cancer cells. Therefore, we created the specific monoclonal antibody 84-1 to fill this gap in technology. This antibody showed very high specificity toward cancer cells and had very high sensitivity as assessed from spiking assays (see Fig. 2B). These characteristics thus fulfilled the basic requirements for establishing a CTC marker: sensitivity, specificity, and reproducibility. Moreover, this technique of isolating CTCs provides an opportunity to study the morphologic and other characteristics of these CTCs and to further use a wide variety of markers, including protein markers and FISH probes (Fig. 2E and F).

EMT involves a functional transition of epithelial cells into mobile mesenchymal cells (23). The common mesenchymal cell markers used to identify EMT cells include FOXC2 (24), TWIST1 (19), SNAIL (25), SLUG (20), and vimentin (5). Although negative staining results for epithelial markers suggests EMT, researchers mainly rely on mesenchymal EMT markers. In this study, we showed that EMT CTCs are in fact associated with multiple EMT markers. However, it remains unclear whether the expression of multiple EMT markers in different CTCs is heterogeneous. Also, colocalization of epithelial and EMT-specific markers may define an intermediate phenotype of EMT cells (4), i.e., cells that are in transition. In our study, we observed that most EMT CTCs had lost the ability to express EpCAM and just a few of the EMT CTCs still retained the epithelial phenotype. Not much is known about the function or role of intermediate EMT cells.

Cytoplasmic vimentin is overexpressed during EMT and is associated with metastasis, invasion, and proliferation (5). It is still unclear what the role of vimentin transported to the cell surface is. In this study, we observed a positive correlation between CSV⁺ EMT CTCs and disease progression, which suggests a role for CSV in promoting disease progression. Because vimentin is an intermediate filament protein and is known to form vimentin adhesion networks (5), CSV could be involved in routing CTCs to the metastatic site and assisting in the invasion process to reseed the metastatic niche, thus promoting tumor progression. Also, CSV⁺ EMT CTCs might represent a unique subset of CTCs that are chemoresistant and thus do not respond to chemotherapeutic regimens. In addition, CSV could be associated with cancer stem-like cells, which have the propensity to form stable colonies. These possibilities need to be tested to confirm the role of CSV in cancer.

CTCs have been used in several studies to monitor response to chemotherapy (26). However, an exclusive correlation between EMT CTC count and disease progression has never been reported. To our knowledge, we are the first to report a correlation between therapeutic outcome in metastatic patients with colorectal cancer and EMT CTC count. Because our cohort consisted of only patients undergoing chemotherapy for metastatic colorectal cancer, the results suggested that the CTCs detected in the bloodstream were released mainly from the metastatic sites. Also, from our study, it is evident that these CTCs were heterogeneous, so these cells should be assessed in regard to their propensity to successfully form metastases or reseed the primary tumor. Capturing and culturing these CTCs and determining their propensities would allow characterization their phenotypes using *in vitro* and *in vivo* modeling, studies of which are currently in progress.

Our study had a few limitations. For example, we retrospectively analyzed information on patients with colorectal cancer

treated with different chemotherapeutic protocols. Because this was a pilot study to assess the therapeutic outcome based on EMT CTC counts, we recruited postsurgery patients who were undergoing chemotherapy. It would be interesting to follow up with a large group of patients at different stages of treatment (before surgery, preneoadjuvant chemotherapy, postneoadjuvant chemotherapy, and after surgery). Also, long-term follow-up would be essential to understanding how EMT CTCs could be used to predict relapse. Technical limitations of the study included the optimization of assays, positive selection of cancer cells a day before spiking experiments, timing of blood collection, and preservation of the integrity of the CTCs in blood. In addition, because several types of host cells in the blood are mesenchymal and express vimentin, the CTCs isolated must be tested for EMT-specific markers that are absent in host cells. The protein Plastin3 (11) has been used to identify EMT CTCs from blood samples of patients with colon cancer, but it has not been tested with other types of solid tumors. We presented here, for the first time, evidence that EMT CTCs can be detected in multiple solid tumor types by using a single, specific marker.

In conclusion, we demonstrated that CSV is a marker for EMT CTCs and that by using the 84-1 anti-vimentin antibody we developed, these CTCs can be isolated from the blood of cancer patients with metastatic colorectal cancer. A key protein that is overexpressed during EMT, vimentin has been shown to be overexpressed in other types of cancers that undergo EMT, thereby making it a universal EMT CTC marker. Also, by using this antibody, we can not only isolate or detect EMT CTCs but also characterize the nature of these cells by using other specific markers. Therefore, isolating CSV⁺ CTCs will help us understand the metastatic precursor cell subpopulation and will help in developing novel diagnostic, treatment, and prognostic options based on therapeutic monitoring.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Satelli, S. Kopetz, L.M. Ellis, S. Li
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Satelli, Z. Brownlee, S. Kopetz, Q.H. Meng, S. Li
Writing, review, and/or revision of the manuscript: A. Satelli, Z. Brownlee, M.J. Overman, S. Kopetz, S. Li
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Satelli
Study supervision: A. Satelli, Q.H. Meng

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