



# Young Investigator Challenge: Application of Cytologic Techniques to Circulating Tumor Cell Specimens: Detecting Activation of the Oncogenic Transcription Factor STAT3

Alarice C. Lowe, MD<sup>1,2</sup>; Jean-Christophe Pignon, PhD<sup>1</sup>; Ingrid Carvo<sup>1</sup>; Michael G. Drage, MD, PhD<sup>1,2</sup>; Natalie M. Constantine, CT(ASCP)<sup>1</sup>; Nichole Jones<sup>3</sup>; Yasmin Kroll<sup>3</sup>; David A. Frank, MD, PhD<sup>2,3</sup>; Sabina Signoretti, MD<sup>1,2</sup>; and Edmund S. Cibas, MD<sup>1,2</sup>

**BACKGROUND:** The circulating tumor cell (CTC) field is rapidly advancing with the advent of continuously improving technologies for enriching these rare neoplastic cells from blood. CTC enumeration provides prognostic information, and CTC characterization has the potential to provide more useful information for the clinical decision-making process in this era of personalized medicine and targeted therapeutics. Proof-of-principle studies have shown that CTC samples can be characterized with a variety of techniques in the research laboratory environment. The goal of the current study was to validate routine cytologic techniques and immunohistochemical markers in CTC samples in a clinical cytology laboratory, using inducible phosphorylated signal transducer and activator of transcription 3 (pSTAT3) as a clinically important example and Ki-67 as a positive control. **METHODS:** Whole blood from noncancer patients was spiked with breast cancer cell lines with constitutive or inducible pSTAT3 expression and underwent CTC processing in the CellSearch system. The resulting CTC samples were subjected to various cytologic/immunocytochemical techniques and were compared with non-CTC-processed cultured cell controls. **RESULTS:** CTC-processed samples showed a morphology comparable to that of controls in cytospin, ThinPrep, and cell block preparations. Immunocytochemistry for Ki-67 and pSTAT3 provided biological information from CTC samples, showing uniform Ki-67 staining across all samples, pSTAT3 positivity in the constitutive and induced cells, and an absence of pSTAT3 expression in the noninduced cells, as expected. **CONCLUSIONS:** CTC samples can be processed in the cytology laboratory with routine methods. CTC morphologic and immunophenotypic analysis can be easily integrated into the existing clinical workflow, moving the field closer to a true peripheral blood liquid biopsy for cancer patients. *Cancer (Cancer Cytopathol)* 2015;123:696-705. © 2015 American Cancer Society.

**KEY WORDS:** circulating tumor cell (CTC); cytological techniques; cytology; immunocytochemistry; signal transducer and activator of transcription 3 (STAT3).

## INTRODUCTION

Circulating tumor cells (CTCs) are the neoplastic cells found in the peripheral blood of patients with early- or late-stage malignancies, and they arise from the primary tumor or metastatic sites. Although they were first described more than a century ago,<sup>1</sup> their low frequency limited their identification because they constitute approximately 1 in a million to 1 in a billion of the nucleated cells in the blood. Recent technological advances have led to the development of a variety of methodologies for capturing these rare cells. Accordingly, early studies

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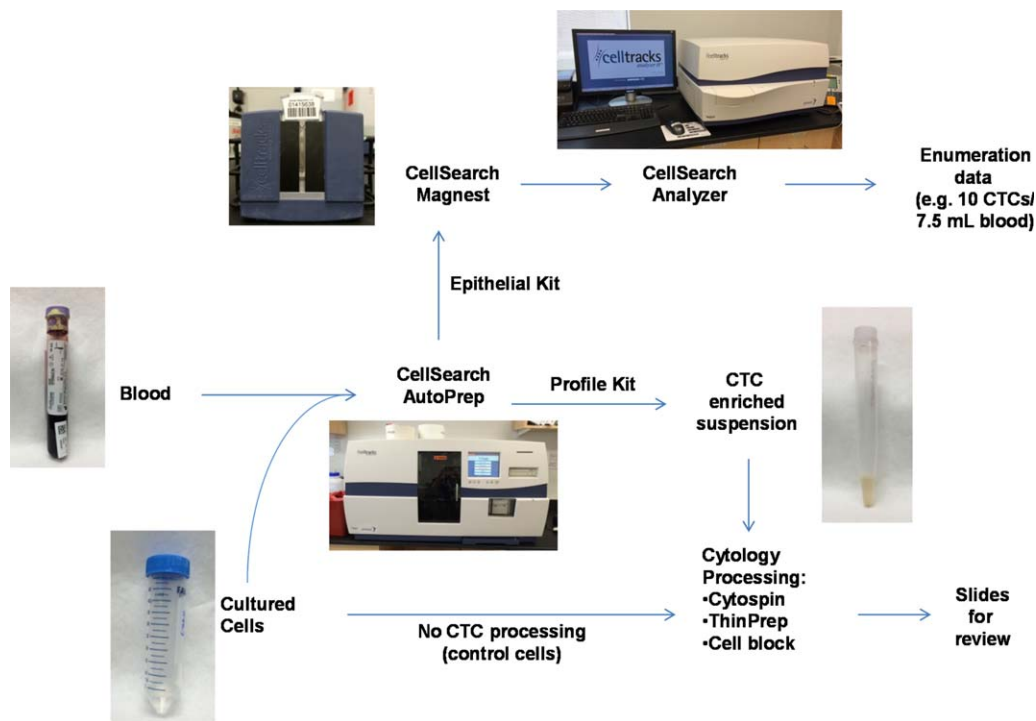
**Corresponding author:** Alarice C. Lowe, MD, Department of Pathology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115; Fax: (617) 739-6192; aclowe@partners.org

<sup>1</sup>Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts; <sup>2</sup>Harvard Medical School, Boston, Massachusetts; <sup>3</sup>Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts

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**Figure 1.** Overview of CTC processing via the CellSearch system. Whole blood can be processed with the CellSearch AutoPrep system via the Epithelial Kit pathway or the Profile Kit pathway. The Epithelial Kit pathway requires additional processing, which includes the morphologic analysis of images presented on the computer monitor, and this results in a CTC count. The Profile Kit pathway results in a CTC-enriched suspension for downstream analysis. In our study, downstream analysis performed directly on cultured cells in suspension served as a control, and it was compared with cultured cells spiked into whole blood and processed with the Profile Kit. CTC indicates circulating tumor cell.

in the field found a significant association between high CTC counts and worse progression-free and overall survival at the time of the initial diagnosis and over the course of treatment.<sup>2-4</sup> Some prospective studies evaluating the effect of treatment options based on CTC counts have recently been concluded,<sup>5</sup> and others are ongoing.<sup>6</sup>

Currently, the focus of the CTC field is shifting from the prognostic significance of CTC enumeration to the predictive value of CTC characterization. Studies have demonstrated that many techniques can be applied to CTC samples, including cytology,<sup>7</sup> immunocytochemistry,<sup>8</sup> immunofluorescence,<sup>9,10</sup> in situ hybridization,<sup>11</sup> molecular analysis,<sup>12,13</sup> and cell culture.<sup>14</sup> These characterization studies have occurred within the research laboratory setting and have not become contemporary practice in the clinical setting. Nevertheless, current clinical trials are evaluating treatment decisions based on the CTC immunophenotype.<sup>6,15</sup>

The Epithelial Kit of the CellSearch system (Janssen Diagnostics, Raritan, NJ) is the only CTC technology that has obtained Food and Drug Administration approval for

monitoring patients with metastatic breast, colorectal, or prostate carcinoma. This immunomagnetic capture system uses iron particles conjugated to anti-epithelial cell adhesion molecule (anti-EpCAM) antibodies (called ferrofluid) to bind the EpCAM-expressing CTCs. The ferrofluid-CTC complex is then sequestered by magnets. After red blood cell lysis, the CTC-enriched suspension is stained with immunofluorescence for cytokeratin (CK), CD45, and 4,6-diamidino-2-phenylindole (DAPI), and subjected to a semi-automated morphologic analysis, which confirms each CTC individually. With this CTC methodology, CTCs are defined as cells larger than  $16 \mu\text{m}^2$  that show positive cytoplasmic staining for CK, no cytoplasmic staining for CD45, the presence of a nucleus, and no degenerative morphologic changes. The end result is the total number of CTCs identified (Epithelial Kit pathway; Fig. 1). An alternative kit called the Profile Kit is available via the CellSearch system in which no CTC count is obtained; instead, the process is truncated at the sequestration step, and the result is a buffer-based suspension of CTCs (Profile Kit pathway; Fig. 1). This suspension is depleted of red

blood cells and can be used as a substrate for further analysis. These CTC samples are liquid specimens containing cells of interest at a low frequency and are similar to other specimen types encountered in routine cytology practice, such as cerebrospinal fluids and effusions with a low tumor burden.

Our objective was to determine whether morphological and immunocytochemical analysis could be performed in a clinical cytology laboratory setting on whole blood spiked with cultured cells that had been processed through the CellSearch CTC system with the Profile Kit. We sought to evaluate these CTC specimens with a variety of cytologic techniques and examine the effects of CTC processing on the morphology and immunocytochemistry. We focused on the detection of phosphorylation of the oncogenic transcription factor signal transducer and activator of transcription 3 (STAT3). Although the activating tyrosine phosphorylation of STAT3 is tightly controlled in normal cells, STAT3 is inappropriately and constitutively activated in a majority of breast cancers<sup>16</sup> and in many other human malignancies.<sup>17</sup> Genes regulated by STAT3 control many key cellular processes, including proliferation, survival, self-renewal, and invasion. Constitutive activation of STAT3 directly drives the pathogenesis of breast cancer,<sup>18</sup> and inhibiting its function has been an ongoing goal in drug discovery.<sup>19</sup> Thus, the ability to follow and assess the activation state of STAT3 in CTCs during the course of targeted therapy would be a major advance in personalized cancer therapy. We also chose to evaluate Ki-67, functioning both as a nuclear antigen–positive control and as a clinically useful indicator of cellular proliferation with the potential to be an important pharmacodynamic biomarker for clinical trials of targeted therapies.<sup>20</sup>

## MATERIALS AND METHODS

### *Whole Blood*

With the approval of the institutional review board, peripheral blood samples were obtained in CellSave tubes (Janssen Diagnostics) from donors with no known history of malignancy ( $n = 52$ ). Each donor provided 1 or 2 tubes. Each tube contained approximately 8 mL of blood.

### *CellSearch Processing*

The CellSave tubes ( $n = 92$ ) were spiked with known numbers of cultured breast cancer cells (0, 10, 25, 100, 250, 500, 1000, 2000, 2500, 4000, 10,000, 20,000,

25,000, 50,000,  $10^5$ ,  $2.5 \times 10^5$ , or  $10^6$ ; at least 1 of each). The spiked tubes were processed at room temperature (RT) on the CellSearch system with the CellSearch Profile Kit, using 7.5 mL of whole blood for each sample analysis. Processing resulted in a 900- $\mu$ L buffer-based suspension enriched for the spiked tumor cells and depleted of red blood cells, designated the CTC-processed specimen.

### *Controls*

For a subset of the experiments ( $n = 42$ ), suspensions of cultured breast cancer cells only (ie, no blood products) in 1X phosphate-buffered saline (PBS) were used as controls. They were matched for the original spiked cell number (0, 10, 100, 500, 1000, 2000, 4000, 10,000, 20,000, 50,000,  $10^5$ ,  $2.5 \times 10^5$ , or  $10^6$ ; at least 1 of each) and volume (the final 900- $\mu$ L postprocessing volume of the CTC-processed specimens). Controls were processed immediately after they had been taken off ice or after a RT incubation of 2 to 4 hours (chosen to match the CTC processing time). Additional controls containing only whole blood were initially evaluated but were not pursued because of marked obscuring by red blood cells.

### *Breast Cancer Cell Lines*

Both human epidermal growth factor receptor 2–overexpressing SKBR3 and triple-negative MDA-MB-468 breast cancer cell lines were authenticated in January 2013 by Genetica DNA Laboratories (Burlington, NC). Cells were kept in culture for no more than 6 months before a new authenticated vial was thawed. The SKBR3 cell line lacks basal phosphorylated STAT3 (pSTAT3), which can be induced by treatment with the cytokine interleukin-6 (IL-6); the MDA-MB-468 cell line displays constitutive pSTAT3 expression.

### *Cytologic Techniques*

Both the CTC-processed and control specimens (Fig. 1) were processed by the cytologic techniques described next.

### *Cytospin*

Cytospin slides were made from 140- to 440- $\mu$ L aliquots of CTC-processed and control specimens with the Stat-Spin cytocentrifuge (Beckman Coulter, Brea, Calif) for 6 minutes at 8500 rpm. The slides were air-dried, immediately alcohol-fixed with a spray cytologic fixative (Fisher Scientific, Pittsburgh, Pa), or air-dried and subsequently

fixed in 10% neutral buffered formalin for 20 minutes and rinsed twice in PBS.

### ThinPrep

CTC-processed and control samples (900  $\mu$ L) were transferred into ThinPrep PreservCyt containers (Hologic, Bedford, Mass) and processed with the ThinPrep 2000 system (Hologic) with the fine-needle aspiration program. The resulting ThinPrep slides were Papanicolaou-stained.

### Cell block

CTC-processed and control specimens (900  $\mu$ L) were centrifuged at 1800 rpm for 10 minutes, the supernatant was removed, and plasma and thrombin were mixed with the cell pellet. The resulting clot was fixed in 10% neutral buffered formalin, processed on the Leica Peloris according to the automated 4-hour xylene processing protocol, paraffin-embedded, cut into 5- $\mu$ m sections, and stained with hematoxylin and eosin (H&E). Modified Gomori iron staining was performed with the Ventana BenchMark Special Stains automated slide stainer (Ventana Medical Systems, Tucson, Ariz).

### Romanowsky and Papanicolaou staining

Cytospins that had only been air-dried were stained with Hemacolor stain (EMD Millipore, Billerica, Mass). Alcohol-fixed cytospins and ThinPrep slides were stained with the Papanicolaou stain with the Varistain Gemini automated stainer (Thermo Scientific, Waltham, Mass).

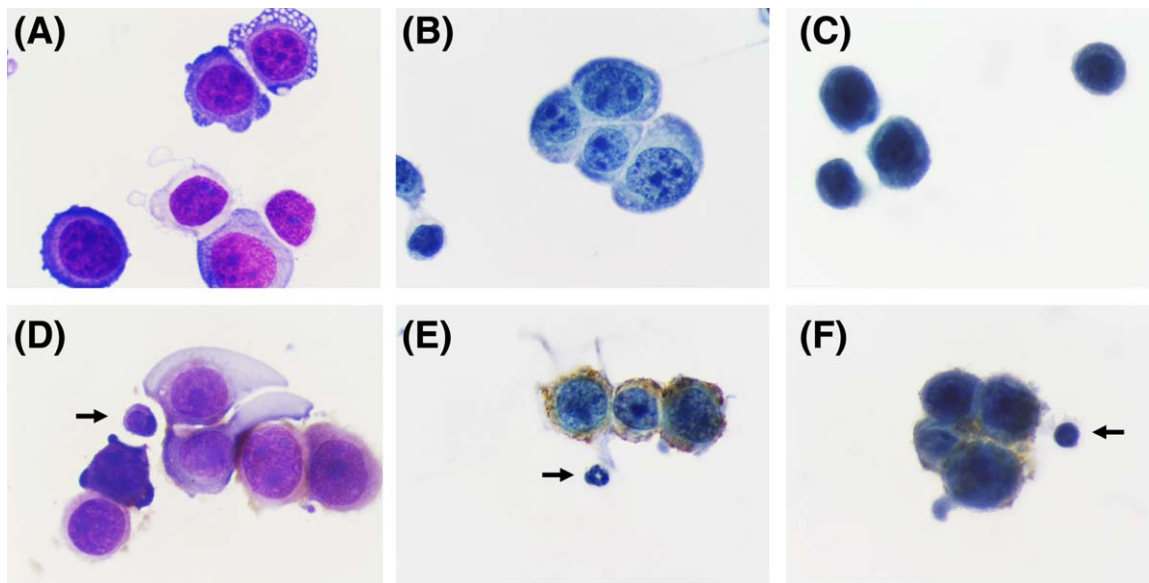
### Immunocytochemistry/immunohistochemistry

Multiplex immunocytochemistry was performed on cytospin slides from CTC-processed and control samples by sequential immunostaining with brown, blue, and red chromogens. Antigen retrieval was performed via the boiling of the slides in an ethylenediaminetetraacetic acid buffer (pH 8) with a high-pressure cooker at 125 °C for 30 seconds. After cooling down at RT, slides were bathed in a washing buffer (0.05 M trishydroxymethylaminomethane buffer [pH 7.4], 0.1% Tween-20) for 5 minutes and then incubated with a peroxidase and alkaline phosphatase (AP) block (Dual Endogenous Enzyme Block; Dako, Carpinteria, Calif) for an additional 5 minutes. The peroxidase and AP block was removed via the washing of the cells for 5 minutes with the washing buffer. The protein block (Serum-Free Block; Dako) was then applied for 5 minutes. The excess protein block was removed, and slides were incubated for 1 hour at RT with the first primary anti-

body, a polyclonal rabbit anti-pSTAT3 (Tyr705) antibody (1/50; Cell Signaling, Danvers, Mass) or a monoclonal rabbit anti-Ki-67 antibody (1/100; clone SP6; Vector Labs, Burlingame, Calif), diluted in an antibody diluent (Antibody Diluent with Background Reducing Components; Dako). Slides were washed for 5 minutes with the washing buffer and then incubated with a secondary goat, anti-rabbit, horseradish peroxidase (HRP)-conjugated antibody (EnVision system; Dako) for 30 minutes at RT. HRP was then visualized with 3,3-diaminobenzidine chromogen (Liquid DAB+; Dako) for 3 minutes. The reaction was stopped by the bathing of the slides with distilled water followed by bathing with the washing buffer for 5 minutes. Residual HRP activity was then blocked by incubation of the slides for 10 minutes with the peroxidase and AP block. After a 5-minute wash in the washing buffer, the slides were incubated for 1 hour at RT with the second primary antibody, a rat anti-CK8 antibody (1/50; TROMA-1; Developmental Studies Hybridoma Bank, Iowa City, Iowa), diluted in the antibody diluent. The slides were then washed for 5 minutes in a washing buffer and incubated with a secondary goat, anti-rat, HRP-conjugated antibody (ImmPRESS HRP anti-rat immunoglobulin G polymer; Vector Labs) for 30 minutes at RT. HRP was then visualized with the HIGHDEF blue immunohistochemistry chromogen (Enzo Life Sciences, Farmingdale, NY) for 3 minutes. The reaction was stopped by the bathing of the slides with distilled water followed by bathing in the washing buffer for 5 minutes. The slides were then incubated for 1 hour at RT with the third primary antibody, a mouse monoclonal anti-CD45 antibody (1/50; clone F10-89-4; Millipore, Billerica, Mass), diluted in the antibody diluent. The slides were washed for 5 minutes and then incubated for 30 minutes at RT with a secondary goat, anti-mouse IgG2a, AP-conjugated antibody (1/100; SouthernBiotech, Birmingham, Ala) diluted in the antibody diluent. AP was then visualized with the Vulcan Fast Red Chromogen Kit (Biocare Medical, Concord, Calif) for 10 minutes. The reaction was stopped by the bathing of the slides in distilled water. Finally, the slides were coverslipped with the Faramount Aqueous Mounting Medium (Dako). Parallel immunohistochemistry was also performed on cell block material.

### Recovery

For a subset of the specimens, the number of morphologically identifiable tumor cells on the prepared slides was



**Figure 2.** Morphology of (A-C) control MDA468 cells and (D-F) MDA468 cells that were spiked into whole blood and processed via the CellSearch system: (A,D) air-dried, Romanowsky-stained cytopspins; (B,E) alcohol-fixed, Papanicolaou-stained cytopspins; and (C,F) Papanicolaou-stained ThinPrep preparations (original magnification  $\times 1000$ ). The CTC-processed cells showed preserved nuclear and cytoplasmic morphology in comparison with the matched controls, associated leukocytes (arrows), and a faint to conspicuous brown halo surrounding many cells. (C,F) Control and CTC-processed ThinPrep preparations showed decreased nuclear detail. CTC indicates circulating tumor cell.

counted by 1 pathologist (A.C.L.) for both CTC-processed and control samples. In rare instances, a second pathologist (E.S.C.) reviewed cases containing cells that were morphologically suspicious for tumor. For cytopsin slides, all slides made from a single sample were counted. For ThinPrep specimens, all slides made from the Preserv-Cyt vial before the sample was exhausted were counted. For cell block specimens, a single slide was counted.

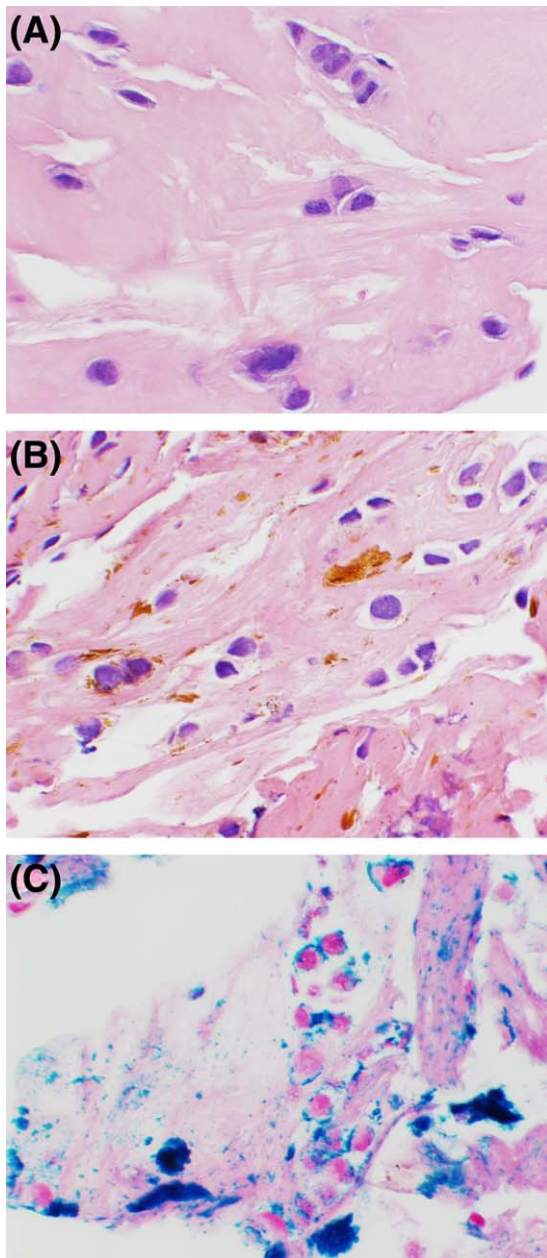
## RESULTS

### Morphology

Cytopsin and ThinPrep slides made from the CTC-processed samples, consisting of whole blood spiked with breast cancer cell line cells and processed in the CellSearch system with the Profile Kit, showed single cells and loosely cohesive aggregates of tumor cells that were morphologically similar to the matched controls made directly from the cell line suspensions, regardless of concentration. Increased aggregation was seen in the CTC-processed specimens. Romanowsky- and Papanicolaou-stained slides showed similar results (Fig. 2). ThinPrep slides showed decreased nuclear detail and fewer cell aggregates in comparison with cytopsin slides for both CTC-processed and control specimens. CTC-processed and control slides

showed tumor cells of similar sizes and nuclear-to-cytoplasmic ratios. Nuclear membrane irregularities, nucleoli, chromatin texture, cytoplasmic vacuolation, and cytoplasmic blebs, akin to those seen in cerebrospinal fluid specimens, were preserved after CTC processing. The CTC-processed samples showed red blood cells, white blood cells, and a brown, finely granular material in isolation and clinging to the surface of tumor cells (Fig. 2D-F); none of these features were present in the controls (Fig. 2A-C). This brown material was seen with all fixatives and preparation types. It was rarely seen attached to the white blood cells but was present on most tumor cells ( $>50\%$ ). When associated with cells, the brown material imparted a halo or clinging diathesis appearance on both ThinPrep and cytopsin preparations. Some tumor cells ( $<5\%$ ) and white blood cells in the CTC-processed samples showed degenerative changes, including a loss of cytoplasm, a loss of nuclear chromatin detail, and cell swelling (not shown). Similar changes were also seen in a smaller proportion ( $<1\%$ ) of the tumor cells of the control specimens. There did not appear to be an association between the presence of the brown material and cell degeneration. The tumor cell concentration did not affect the cell morphology.

The cell morphologies of cell block preparations made from the CTC-processed samples and the control



**Figure 3.** Cell blocks made from (A) control and (B) CTC-processed samples showed similar morphologies (H&E, original magnification  $\times 400$ ). CTC processing produced an abundance of amorphous, brown material associated with the cancer cells and in isolation. (C) Iron stain highlighted the brown material in the CTC-processed specimen (original magnification  $\times 400$ ) and was negative in the control (not shown). CTC indicates circulating tumor cell.

samples were also similar (Fig. 3A,B). The previously noted brown, amorphous material was present in the CellSearch samples only (Fig. 3B), both in association with the tumor cells and in isolation. Iron stains strongly highlighted the brown material in the CTC-sorted samples

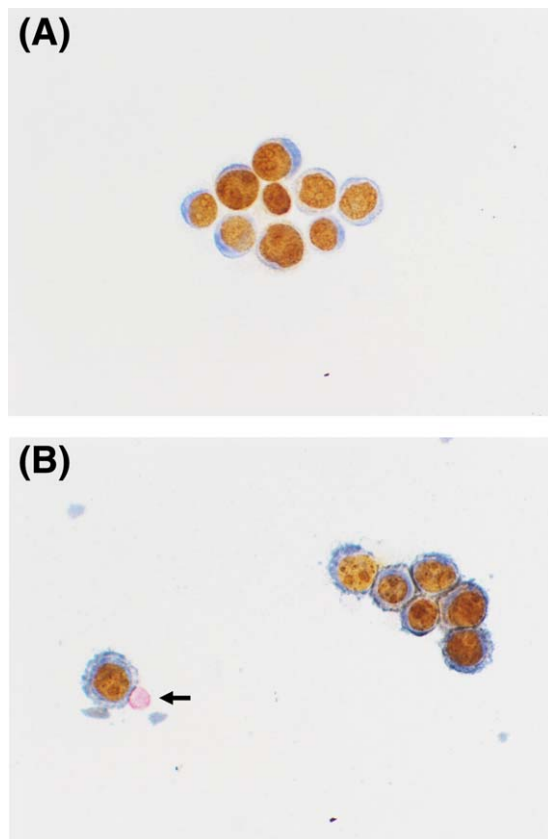
(Fig. 3C) and were negative in the control samples (not shown).

### **Assessment of STAT3 Signaling Pathway Activation**

To detect STAT3 signaling pathway activation in CTCs, immunocytochemical triple staining was performed on cytospin preparations with pSTAT3, CK8, and CD45 antibodies; the latter 2 antibodies were used to distinguish CTCs (CK8-positive, CD45-negative) from leukocytes (CK8-negative, CD45-positive) and mimicked the CK and CD45 staining obtained with the Food and Drug Administration–cleared CellSearch Epithelial Kit enumeration specimens. Immunocytochemical triple staining for Ki-67, CK8, and CD45 was also performed, with Ki-67 functioning as a nuclear antigen positive control.

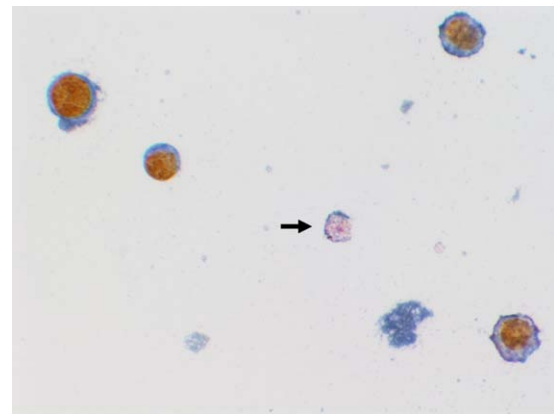
Triple staining for Ki-67 (brown chromogen), CK8 (blue chromogen), and CD45 (red chromogen) showed tumor cells to be positive for nuclear Ki-67 and cytoplasmic CK8 and negative for CD45 in both the CTC-processed and control samples (Fig. 4). In both sample types, the cytoplasmic CK8 staining varied from weak to strong but was interpretable for most cells ( $>90\%$ ). Ki-67 staining was strongly positive in most cells ( $>95\%$ ). The CTC-processed samples showed CD45-positive leukocytes, which were negative for Ki-67 and CK8 (arrow in Fig. 4B). No CD45-positive cells were seen in the controls. The brown, amorphous material seen on the other CTC-processed preparations was also present on the immunocytochemistry-stained slides and appeared brown/blue. Some of the white blood cells and rare tumor cells had marked degenerative changes in the CTC-processed samples. Rare degenerating indeterminate cells were present and appeared to show positivity for both CK8 and CD45 (Fig. 5). There was no difference in the Ki-67 staining qualities between the SKBR3 cells that had and had not been stimulated with IL-6.

Immunocytochemical triple staining for pSTAT3 (brown), CK8 (blue), and CD45 (red) was performed on cytospins that had been made immediately from non-CTC-processed SKBR3 cells with or without pSTAT3 stimulation by IL-6. The unstimulated control cells showed cytoplasmic positivity for CK8, were negative for CD45, and showed no nuclear pSTAT3 staining (Fig. 6A). In contrast, the stimulated control cells showed moderate to strong nuclear pSTAT3 staining in most cells (Fig. 6B). In comparison with the control cells, the CTC-



**Figure 4.** Immunocytochemical staining for Ki-67 (brown), CK8 (blue), and CD45 (red) on cytospin slides made from (A) control and (B) CTC-processed MDA468 cells showed similar staining patterns in the cancer cells (original magnification  $\times 400$ ). The CTC-processed sample showed a leukocyte (arrow) adjacent to a tumor cell and blue/brown, amorphous material in isolation and in association with tumor cells. CK indicates cytokeratin; CTC, circulating tumor cell.

processed, IL-6-stimulated SKBR3 cells showed decreased pSTAT3 staining with respect to both the staining intensity and the fraction of positive cells (Fig. 6C), and this reflected the expected time-dependent decrease in pSTAT3 during the 3-hour CTC processing.<sup>21</sup> A similar decrement in pSTAT3 staining was seen in non-CTC-processed, IL-6-stimulated cells that were kept at RT for the duration of CTC processing (Fig. 6D). A milder decrease in pSTAT3 staining was seen in MDA-MB-468 cells with constitutive pSTAT3 expression that had been CTC-processed or maintained at RT for the duration of CTC processing in comparison with the control cells; this possibly reflected activation of phosphatases (not shown). Similar staining patterns were seen in control and CTC-processed specimens at all tumor cell concentrations. Immunohistochemistry was also performed on cell block material made from



**Figure 5.** Immunocytochemical staining of circulating tumor cell-processed MDA468 cells placed on cytospin slides for Ki-67 (brown), CK8 (blue), and CD45 (red) showed a rare indeterminate cell (arrow), which stained weakly for both blue and red chromogens (original magnification  $\times 400$ ).

CTC-processed and control stimulated SKBR3 cells, unstimulated SKBR3 cells, and MDA-MB-468 cells and showed similar results to those seen with the cytospin preparations (not shown).

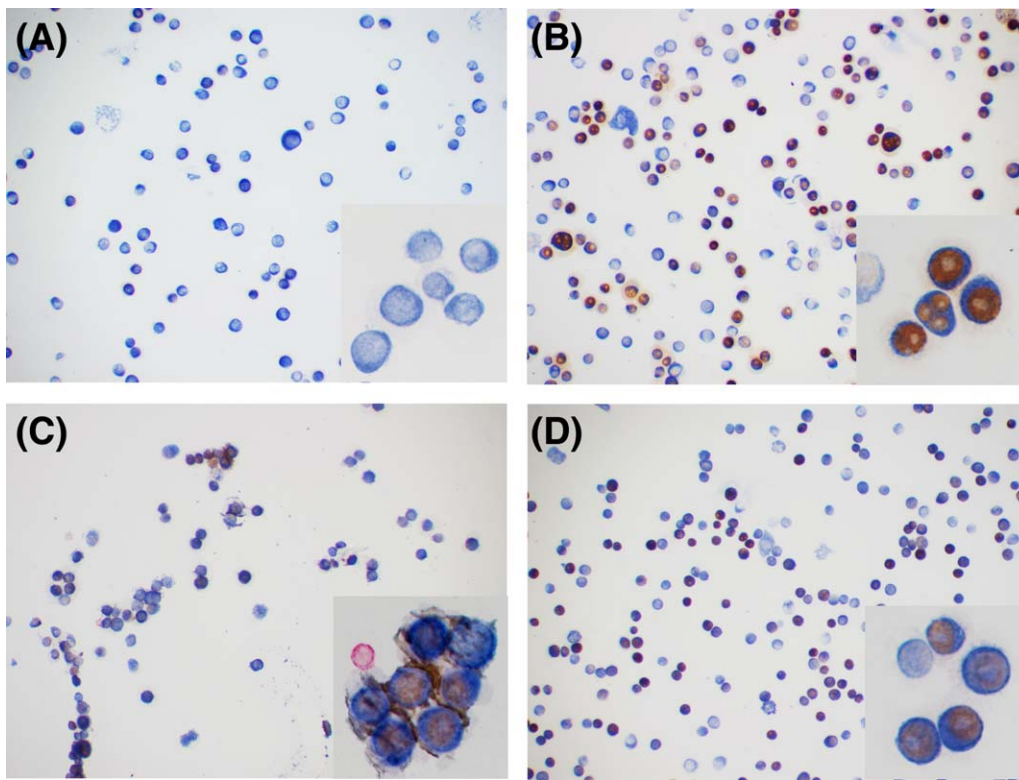
### Recovery

The number of morphologically identifiable cancer cells recovered from the various cytologic preparations is reported in Table 1. Although parallel controls were not performed for all specimens, the lower limit of recovery was similar between CTC-processed and control specimens. All CTC-processed samples spiked with at least 1000 cells recovered cells on the evaluated ThinPrep, cytospin, and cell block slides. No morphologic or immunophenotypic differences were seen in tested specimens, even at the lowest concentrations evaluated.

### DISCUSSION

With the current focus on targetable biomarkers, we are increasingly striving to gain more information from smaller samples obtained by less invasive methods. CTCs are a promising source of material for the surveillance of patients with known malignant disease. Although CTC enumeration provides prognostic information for patients at the time of diagnosis and during treatment,<sup>2</sup> the iterative characterization of CTCs to monitor the evolution of molecular drivers and the development of tumor resistance is one of the many exciting frontiers of medicine.

Various studies have successfully evaluated CTCs via a variety of morphologic and molecular techniques.



**Figure 6.** (A) Immunocytochemical staining for pSTAT3 (brown), CK8 (blue), and CD45 (red) on unstimulated SKBR3 control cells showed no nuclear pSTAT3. (B) IL-6-stimulated SKBR3 control cells showed moderate to strong pSTAT3 staining in most cells. (C) CTC-processed SKBR3 cells that had been stimulated with IL-6 showed weaker pSTAT3 staining and fewer positive cells than those seen in panel B. (D) Stimulated SKBR3 controls cells incubated at room temperature for the duration of the CTC processing showed a similar decrement in pSTAT3 (original magnification  $\times 100$ , inset magnification  $\times 400$ ). CK8 indicates cytokeratin 8; CTC, circulating tumor cell; IL-6, interleukin-6; pSTAT3, phosphorylated signal transducer and activator of transcription 3.

**TABLE 1.** Number of Morphologically Identifiable Tumor Cells Recovered from Control and CTC-Processed Specimens Spiked with Various Numbers of Cultured Cells

No. of Cells Spiked	No. of Cells Recovered by Preparation Method					
	ThinPrep		Cytospin		Cell Block	
	Control	CTC	Control	CTC	Control	CTC
0	0	0	0	0	0	0
10	2	0, 0, 1	ND	ND	0	0
25	ND	ND	ND	3	ND	ND
100	11	0, 1, 5	0, 6	8, 11, 18	2 <sup>a</sup>	0
250	ND	ND	ND	17	ND	ND
500	ND	0	28	1, 1, 1, 2, 3, 5, 85	ND	1 <sup>a</sup>
1000	250	59, 78	20, 36, 64, 91	47, 59, 79, 105, 112, 205	13, 2 <sup>a</sup>	4
2000	ND	ND	574	235	ND	ND
2500	ND	ND	ND	86	ND	ND
4000	ND	ND	>1000	360	ND	ND
10,000	>1000	152	>1000	>1000	10, 20	17, 23
20,000	ND	ND	ND	ND	36	38, 53
25,000	ND	ND	ND	508	ND	ND
50,000	ND	ND	ND	ND	29, 204	591

Abbreviations: CTC, circulating tumor cell; ND, no data

Each value represents a single sample. Multiple samples performed with the same spiked cell number and preparation are separated by commas.

<sup>a</sup>Morphology suspicious for tumor.



Romanowsky<sup>22</sup> and Papanicolaou staining<sup>8</sup> has been performed directly on the devices used to isolate CTCs. Immunocytochemical<sup>8,13</sup> and immunofluorescent staining<sup>9,10</sup> has also been described in the context of CTC identification (eg, cells positive for prostate specific antigen or thyroid transcription factor 1 were classified as CTCs). RNA in situ hybridization has been performed to evaluate CTCs for epithelial or mesenchymal phenotypes, with serial analysis exhibiting phenotypic shifts.<sup>11</sup> Nucleic acid sequencing has identified tumor-specific RNA transcripts such as TMRSS2-ERG fusion<sup>13</sup> and compared entire exomes of multiple CTCs with those of the primary and metastatic tumors.<sup>12</sup> These studies have all been performed in the research setting, and, in some instances, the results were the defining feature that classified the examined cells as CTCs.

Despite the continuing advances in molecular technology, the current clinical landscape of genomic testing relies on evaluating a limited number of targetable mutations, rearrangements, or copy number variations in the context of specific tumor types. Therefore, immunocytochemical/immunohistochemical methods continue to be widely used for diagnostic purposes, often as a surrogate for or a screen before more costly molecular methods. Determining a logical method of implementation to evaluate CTCs for targetable biomarkers, ideally within the existing infrastructure, is the necessary first step to their widespread adoption as a clinical test.

The cytology laboratory is where liquid specimens containing single cells and tissue fragments too small for processing via conventional histologic means are placed on slides through a variety of methods for morphologic and immunocytochemical analysis. In addition to routinely performing cytomorphologic analysis, cytopathologists triage cytologic material for clinically relevant ancillary testing and integrate these morphologic, immunophenotypic, flow cytometry, cytogenetic, and molecular data to provide the patient and the referring clinician with a final diagnosis. We propose that the cytology laboratory is the ideal environment for the clinical application of CTC specimens because of the liquid nature of the sample and the complexity of the cytologic, immunophenotypic, and genomic interpretation required to provide the most clinically actionable information.

In the current study, we applied commonly used cytologic techniques to cultured breast cancer cells that had been spiked into whole blood to simulate the presence of

CTCs in a cancer patient and processed the samples with the CellSearch system. The resulting cytospin, ThinPrep, and cell block slides were morphologically interpretable by practicing cytopathologists and were comparable to the control samples. Although Romanowsky<sup>22</sup> and Papanicolaou staining<sup>8,13</sup> has been studied to evaluate CTCs on the capturing devices, our study is the first to demonstrate that cytospin, ThinPrep, and cell block preparations are feasible cytologic techniques for the morphologic evaluation of CTCs.

In addition, the finely granular brown pigment found in the CTC-processed samples that was identified in all preparations with all stain types has not yet been described in the literature. Its membranous association with the tumor cells (but not with white blood cells) and the positive staining with the Gomori iron stain suggest that it is remnant ferrofluid. This material was abundant and seen on the surface of a significant number of tumor cells. In the current study, we intentionally selected antibodies that would not interfere with the anti-EpCAM ferrofluid used by CellSearch to isolate tumor cells. This morphologic finding further supports the need for compatible antibody selection when one is pursuing immunophenotypic characterization of CTCs isolated by this methodology.

In our study, some but not all CTC-processed specimens that were spiked with as few as 10, 25, or 100 cells recovered rare cells in the ThinPrep, cytospin, and cell-block preparations, respectively. Although this information is useful in suggesting the possibility of the clinical utility of these techniques within the concentration of cells likely to be encountered within patient samples, few samples were tested, and additional studies should be performed to determine the reproducibility of our findings and the lower limit of detection. Recovery was most robust across all techniques in samples spiked with greater than 1000 cells, a number that may be more attainable as CTC technologies improve.

Because of the role that activated STAT3 plays in many cancer types,<sup>16,18</sup> the ability to monitor the activation state of STAT3 in CTCs during the course of targeted therapy is of clinical interest. Ki-67 also has the potential to be an important CTC biomarker.<sup>20</sup> In the current study, we developed methods for multiplex Ki-67 and pSTAT3 immunocytochemical/immunohistochemical staining, and the resulting slides were interpretable and provided biologically relevant information. Establishing similar methods for other targetable post-translational protein biomarkers would provide unique opportunities to

assess signaling pathway activation in a variety of cancer settings for the identification of candidate therapies and for the monitoring of treatment response.

We recognize that our freshly spiked cell line tumor cells are not equivalent to clinical CTC specimens and that additional studies using patient samples are needed to confirm the clinical applicability of our findings. In addition, CTCs may exhibit a range of morphologies, including smaller cells with less cytologic atypia than that seen in our cultured cell lines. This issue speaks to the need for immunophenotypic confirmation and quantitation of these atypical cell types before a specimen is referred for costly and time-consuming ancillary testing. As cytopathologists, we use similar techniques daily as we examine fine-needle aspiration specimens or body cavity fluids for primary tumors or metastatic disease. For this reason, we believe that the cytology laboratory is the logical arena for the integration of CTC sample analysis into clinical care and research investigations, and this study demonstrates its feasibility for the clinical application of CTC evaluation.

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