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Blood-Based Biopsies—Clinical Utility Beyond Circulating Tumor Cells

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Abstract

Circulating tumor cells (CTCs), epithelial–mesenchymal transition (EMT) cells, as well as a number of circulating cancer stromal cells (CStCs) are known to shed into the blood of cancer patients. Individually, and together, these cells provide biological and clinical information about the cancers. Filtration is a method used to isolate all of these cells, while eliminating red and white blood cells from whole peripheral blood. We have previously shown that accurate identification of these cell types is paramount to proper clinical assessment by describing the overlapping phenotypes of CTCs to one such CStC, the cancer-associated macrophage-like cell (CAML). We report that CAMLs possess a number of parallel applications to CTCs but have a broader range of clinical utility, including cancer screening, companion diagnostics, diagnosis, prognosis, monitoring of treatment response, and detection of recurrence. © 2018 The Authors. Cytometry Part A published by Wiley Periodicals, Inc. on behalf of ISAC.

Key terms

Cancer-associated macrophage-like cells; CAMLs; liquid pathology; circulating tumor cells; CTCs; microfiltration; microfilters; CellSieve; liquid biopsy; liquid cell biopsy; blood-based biopsy

THE World Health Organization has estimated that 8.2 million people died of cancer in 2012 (1). It has been shown that early detection of cancer and early detection of cancer recurrence provides for better outcomes in patients with cancer. Noninvasive companion diagnostics, rapid determination of treatment response, and patient prognosis are highly beneficial to patients receiving therapy. Since 2004, circulating tumor cells (CTCs) used as a "liquid biopsy" has been shown to provide important clinical information for late-stage cancer patients undergoing treatment (2–5). However, for patients with early-stage disease, for cancer subtypes that do not have CTCs, and for early detection of recurrence, CTCs provide little clinical utility due to their rarity in these patient populations. To that end, we have developed a number of blood based biopsy tests that provide additional clinical utility beyond CTC enumeration. By collecting and analyzing both CTCs and other circulating cancer stromal cells (CStCs), we expand beyond mere CTC assessment. Clinically, one important CStC subtype is the cancer-associated macrophage-like cell (CAML) (6). CAMLs are more prevalent than CTCs in many solid tumor subtypes, and unlike CTCs, CAMLs are found in all stages of solid tumors (6–10). Combined, our data suggest that CAMLs and CTCs isolated from the same patient blood sample provide more robust clinical utility than CTCs alone.

Materials and Methods

CellSieve™ Microfilter and Filtration System

A number of methods to isolate rare cells from the billions of hematopoietic cells in whole blood sample have been investigated. Of these methods,

microfiltration is one of the effective methods to isolate CTCs and circulating tumor-associated cells from multiple types of cancers (6–17). Filtration by size is a suitable method to consistently capture multiple types of tumor-associated cells in the blood (i.e., CTCs, EMTs, and CStCs). Many filter types, materials, and fabrication methods have been published for CTC capture (11–17). We developed commercially available microfilters with uniform pore size and distribution and filtration system for isolation of all types of circulating cancer-associated cells (6–10). A scanning electron micrograph (SEM) of the CellSieve™ microfilter is shown in Figure 1. The characteristics and benefits of CellSieve microfilters are summarized here.

PROPERTIES	BENEFITS
• Uniform pore size (7 μm diameter) and distribution	The optimal pore size was selected for depleting all red blood cells and 99.99% of white blood cells but maximizing the capture efficiency for CTCs, CAMLs, and cell clusters
• 10 μm thick	Thin films minimize the pressure and stress on the cells. Cell morphology is well maintained
• 180,000 pores in a 9 mm diameter area	The large number of pores enables rapid, gentle filtration, 5 ml/min. The 9 mm diameter filtration area enables rapid imaging
• Low autofluorescent background	Enables high definition images of cell features. Ability to quantify the staining intensity of markers of interest on the cells, such as PD-L1 and PD-L2
• Strong	No additional support structure is needed
• Lies flat on glass slides	Ease in preparing slides, facilitates microscope imaging

The CellSieve low-pressure filtration system, using CellSieve microfilters, provides a straightforward and clean manual operation (8,9,18,19). The filter is held inside a filter holder, which also serves as the assay reaction well, for minimal manipulation of the cells. Whole blood is placed into the input syringe and drawn through the filter into a waste syringe. 7.5 ml of whole blood diluted by 7.5 ml of prefixation buffer is filtered in 3 min. The assay steps (fixation, permeabilization, and staining) are performed inside the holder. After staining, the filter is removed and mounted on a glass slide with a cover slip.

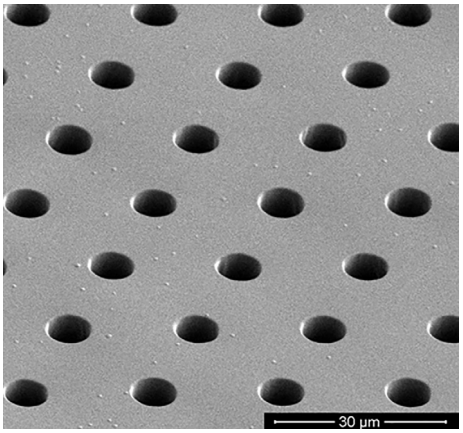

Figure 1. A scanning electron micrograph (SEM) of the CellSieve™ microfilter membrane.

Cell Identification

For epithelial cancers, CTCs have generally been identified as cells in peripheral blood that stain positive for DAPI and cytokeratin (CK) 8, 18, 19, and negative for CD45. Careful analysis has revealed that many other nonepithelial circulating cell types also stain with the same CK/CD45 biomarkers as CTCs. In order to accurately classify these different cell types, we developed more precise definitions of the CK(+) cells found on the filter. Pathologically defined CTCs (PDCTCs) are classic CTCs in which the CK appears in filamentous patterns (Fig. 2A), while in apoptotic CTCs the CK becomes spotted, for example, blebs (Fig. 2B) (8,20). Our data have shown that the morphological features and the CK-staining patterns should always be included in the cell identification process. We have shown that the clinically relevant cells detected by CellSearch® can potentially be detected using this microfiltration approach indicating that cell identification not EpCAM may be the most important criteria for CTC detection (20). However, although the microfiltration approach provides the high efficiency of CTC isolation independent of surface marker's expression, it should be noted that a fraction of small CTCs might not be recovered if the cell size is smaller than the pore size.

Circulating cancer-associated vascular endothelial cells (CAVEs), a subtype of circulating endothelial cells belonging to the CStC category, and epithelial–mesenchymal transition (EMT) cells are often counted as CTCs, because they are CK 8, 18, 19(+), and CD45(−). However, these cell types are not isolated by EpCAM surface marker systems, such as CellSearch. Furthermore, CAVEs are identified to be CD14(−), vimentin(+), and CD31(+) (21). EMTs are often found in clusters and appear similar to CAVEs with weak CK 8, 18, 19 expressions, CD45(−), and vimentin(+), but importantly are CD31(−) (10,22). Interestingly, the markers on CAVEs and EMTs might provide useful information such as PD-L1 for immunotherapy (10).

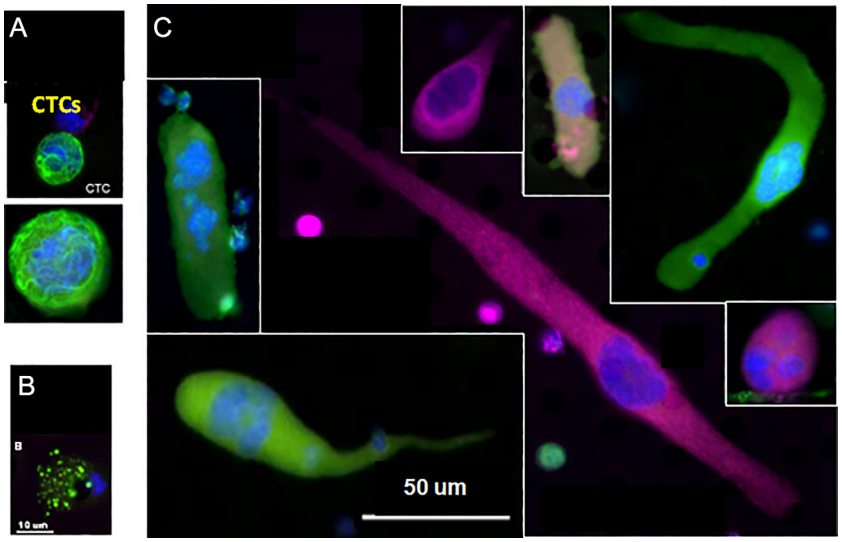

Figure 2. Morphological features and the antibody staining patterns of filter-captured cell populations. (**A**) Pathologically defined CTCs (PDCTCs) are classic CTCs in which the cytokeratin appears in filamentous patterns. (**B**) In the apoptotic CTCs, the cytokeratin becomes spots (aka blebs). (**C**) Cancer-associated macrophage-like cells (CAMLs) from carcinomas show CK 8, 18, 19(+), polyploids, very large in size (25–300 μm), but whose CK has a diffuse staining pattern throughout the cytoplasm.

In patients with carcinomas, an additional CStC cell type that is CK 8, 18, 19(+) is the giant polyploid CAML, which appears very large in size, 25–300 μm, but whose CK has a diffuse staining pattern throughout its cytoplasm (Fig. 2C) (6–10). These polyploid cells have been shown to be either CD45(−) or CD45(+) though mostly express CD11c or CD14, which confirms their origin as myeloid lineage. Thus CAMLs are not CTCs, as determined by morphology, myeloid phenotype and myeloid lineage. Interestingly, CAMLs can be found on other CTC techniques with the same characteristics described earlier, such as by the CellSearch CTC System (23,24).

Clinical Applications

Blood-based biopsies utilizing the CellSieve microfiltration method can simultaneously collect multiple types of circulating cancer-associated cells. These cells together provide a wide variety of research and clinical applications that are not possible utilizing CTCs alone. Some examples of clinical applications enabled by these cells are given below.

a. **Early detection of cancer.** CAMLs have been found to be common in cancer patients but not in healthy controls and rarely seen in people with benign growths. CAMLs have been found in high percentages in all stages of cancer, including stage I disease. The prevalence of CAMLs was shown for the following six cancer types (n = 293): breast (n = 59), prostate (n = 52), pancreatic (n = 59), non-small-cell lung cancer (NSCLC) (n = 59), renal cell carcinoma (RCC) (n = 37), and esophageal (n = 27) cancers. CAMLs were found in Stage I (84%), Stage II (94%), Stage III (95%), and Stage IV (97%).

The objective of an initial breast cancer screening study was to determine whether a blood test can determine presence of breast cancer, and whether such a test can be used in place of traditional biopsy. A biopsy is routinely performed on subjects whose breast mammography is categorized as BIRAD 4 or 5, which is considered to be at high risk for malignancy. A blood based test is a less invasive and lower cost alternative. We tested for the presence of CAMLs as an early detection marker in a double-blind study of BIRAD 4 and 5 subjects. Biopsy results were compared with the presence of one or more CAML ≥30 μm expressing CD14 (7). Biopsy reported the following: noninvasive (n = 5), Stage I (n = 4), Stage II (n = 10), Stage III (n = 1), unknown (n = 2), and benign conditions (n = 19). CAMLs were detected in the following: non-invasive (n = 5), Stage I (n = 2), Stage II (n = 10), Stage III (n = 1), unknown (n = 2), and benign conditions (n = 5). The ROC curve comparing invasive breast cancer (n = 17) to benign conditions (n = 19) showed AUC = 0.78. The CAML screening results were: sensitivity = 88%; specificity = 74%; PPV = 75; NPV = 88%. Of the two "false negatives" by the CAML result both patients were low grade, stage 1 invasive ductal carcinoma, with small nodes 1.4 or 1.8 cm in size. Of the five "false positives" by the CAML assay, two subjects were later determined to have DCIS and invasive cancer at 15 and 27 months after biopsy, respectively, indicating that the CAML blood test may at times detect cancer sooner than the biopsy.

b. **Companion diagnostics.** A blood-based biopsy can be used as a diagnostic test to predict the efficacy of a drug targeted to a patient's specific cancer. We developed a technique to stain isolated cells for 12 identification and subtyping markers (22). Using a fluorescent quenching chemical that has no adverse effects on biological proteins allows the subtyping of cells based on drug applicable targets. We give a specific example for immunotherapy as an

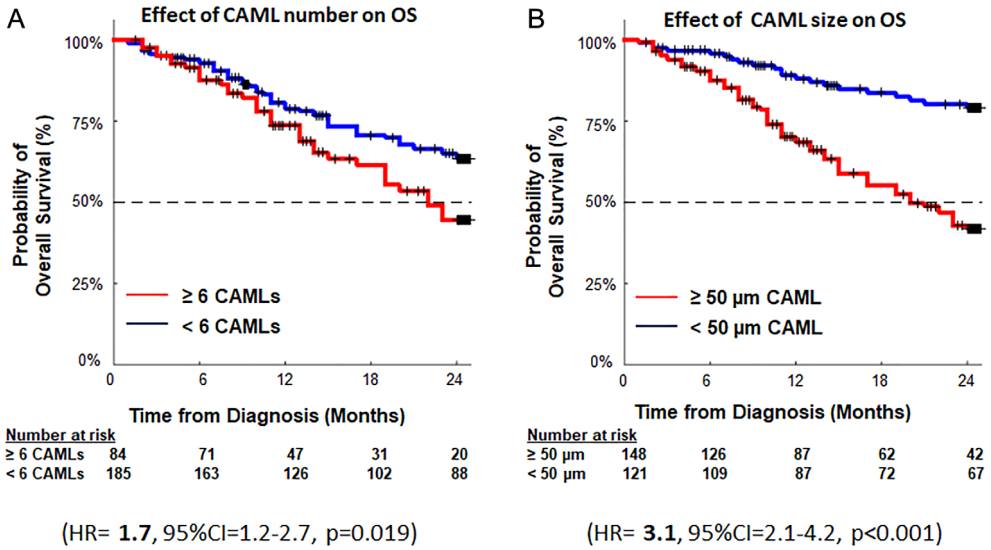

Figure 3. Comparing overall survival (OS) in 269 patients based on CAML enumeration or cell size using 7.5 mL of peripheral blood. (**A**) An optimal patient cohort stratification of ≥6 CAMLs (red) (n = 84) curve had shorter OS than patients with <6 CAMLs (blue) (n = 185). (**B**) Patients with one or more CAML ≥50 µm (red) (n = 148) had shorter OS than patients only have CAMLs <50 µm (blue) (n = 121).

illustration (10). Immunotherapy is achieving significant success for melanoma, NSCLC, kidney cancer, and others. Currently, FDA-approved companion diagnostics for immunotherapies are based on the expression of PD-L1 on tissue biopsy (25,26). However, biopsies cannot always be obtained and the cancer can also mutate from the time of the initial biopsy, possibly necessitating a change in therapy. The PD-L1 expression on CTCs, EMTs, CAVEs, and CAMLs can possibly provide more current tumor biomarker information for better determining PD-L1 expression in real time. The technique is also applicable to a broad range of therapy targets (22).

c. **Monitor treatment response.** Actively monitoring tumors in real time is paramount in determining whether the patient is responding to a treatment, or whether a second-line therapy should be started. Blood based biomarkers (e.g., PSA, CEA, and CA125) can be used to track real-time progression of disease in parallel with imaging. However while numerous blood biomarkers exist that are specific to a cancer type (i.e., PSA to prostate and CEA to colon), they do not appear in all diseased individuals and may not be detected in smaller tumors. Recent data have demonstrated that sequential monitoring by observing changes in the CAMLs indicates disease progression or response to treatment in multiple solid tumors.

d. **Prognosis.** It is well established that CTC number can provide prognosis in late-stage disease of certain cancer types using the CellSearch system (2–5). However, subtyping of CTCs and including CAMLs provides valuable prognostic information.

- The presence of a single CTC undergoing mitosis is a strong predictor of poor prognosis, with a hazard ratio of 11.1, compared to CTC enumeration, with a hazard ration of 5.2 (27). This was based on a clinical analysis of 36 breast cancer patients.
- CAML number and size can provide prognostic information. It is particularly useful, because CAMLs can be found in most blood samples of patients with major solid tumors and in all stages of the disease. A multivariate analysis was performed on n = 269 patients: breast (n = 57), prostate (n = 43), pancreatic (n = 59), NSCLC (n = 54), RCC (n = 35), and esophageal (n = 21) cancers. CAML size was found to be the best independent predictor of survival compared to all other clinical variables, including CTCs. The Kaplan–Meier curves of the overall survival (OS) were analyzed to evaluate the effect of CAML number and CAML size. Figure 3A shows that patients with ≥6 CAMLs (red) curve have shorter OS than patients with <6 CAMLs (blue). Figure 3B shows that patients with one or more CAML larger than ≥50 µm (red) have the OS much shorter than patients only have CAMLs <50 µm (blue). CAML size is also a strong indicator of Progression Free Survival.

e. **Monitoring of minimal residual disease and recurrence.** Cancer patients in remission are usually monitored by CT scan and MRI 2–4 times per year. Imaging can only determine recurrence if the tumor grows by 5 mm or more and changes size perceptibly over time. The presence of CAML and CTCs in peripheral blood can indicate minimal residual disease and provide early detection of recurrence or new cancer.

The CellSieve microfiltration system provides a platform to collect multiple cancer-associated cells, which can enable better research and clinical data assessment during clinical evaluation. We have published multiple additional techniques, but not discussed here, including cryo-preservation (9), bone marrow processing (28), cell transport solution preserving live cells (29), and culturing live cells directly on the filter (30). To date, we

have analyzed 15 different solid tumors (breast, prostate, esophageal, lung, liver, pancreatic, bladder, ovarian, and colorectal cancers, RCC, melanoma, uterine sarcoma, Ewing's sarcoma, neuroblastoma, and head and neck cancers), more than 3,000 patient samples, and in all stages of cancer. CAMLs were found in all the 15 solid tumor types. These patient samples enable the identification of a whole spectrum of clinical applications from cancer screening, companion diagnostics, monitoring treatment response, provide prognosis, and early detection cancer recurrence.

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