Rapid and Simple Isolation of Circulating Tumor Cells for Clinical and Research Applications Using ScreenCell®

Eric R. Schuur, PhD
VMWA LLC, Palo Alto, CA

Introduction

Circulating tumor cells (CTCs) are malignant cells shed by the primary tumor or metastases into the peripheral circulation. CTCs give rise to distant metastases that are usually the ultimate cause of cancer-related death. A significant proportion of patients with early stage cancer in whom no metastases are identifiable will ultimately relapse as a result of hematogenous spread of tumor cells that were undetected at initial diagnosis and treatment.

The importance of CTCs is suggested by the observation that elevated CTC levels are associated with diminished survival[1], a result that was subsequently confirmed in prospective clinical trials. In their landmark study, Cristofanilli and colleagues measured CTCs in 177 patients with previously diagnosed and treated metastatic breast cancer[2]. They determined that a cutoff level of 5 CTC per 7.5 mL of blood could categorize patients into groups at higher or lower risk of relapse. Ensuing studies validated the prognostic utility of CTC information in breast cancer (BC), colorectal cancer (CRC), and prostate cancer (PC)[3].

However, simple enumeration of CTCs is not enough; it has since become apparent that cancer is not a single, homogeneous disease, but rather a constellation of diseases with a variety of pathologic alterations that impact prognosis. To further complicate matters, the recent appreciation of the heterogeneity of genetic alterations and biomarker expression[4], such as HER2 and KRAS, within tumors means that a single biopsy sample is no longer adequate.

CTC analysis has the potential to enable accurate biomarker assessment in the face of tumor heterogeneity. Recent studies suggest that CTC information is representative of the spectrum of molecular and cellular information available in the primary tumor[5]. Furthermore, the accessibility of CTCs through a simple blood sample makes them an attractive source of tumor cells for serially monitoring biomarker status.

CTCs are typically present at vanishingly low concentrations. The rarity of CTCs has posed a challenge to their use to address these issues. While immunomagnetic isolation using antibodies to epithelial cell adhesion molecule (EpCAM) has enabled wider clinical application, the drawbacks to this technology include inability to isolate cells that do not express EpCAM[6] and inability to process cells for downstream biomarker analysis[7].

The next generation of CTC analysis technology must enable molecular analysis of CTCs, in addition to accurate enumeration of CTCs in patient blood samples. The ScreenCell® devices described below address both of these technical issues, enabling rapid, consistent, and inexpensive isolation of CTCs from blood samples for clinical or research use.

The ScreenCell® Device

The ScreenCell® device is a self-contained, disposable device for isolating rare cells from blood samples. Three configurations of the ScreenCell® device are available that address different analysis needs: ScreenCell® Cyto for cell enumeration and cytology, ScreenCell® CC for culture of CTCs, and ScreenCell® MB for molecular analysis. The devices share a fundamental principle for CTC isolation; in addition, each type has specialized features to

support specific applications.

Operational Principles and Applications

The ScreenCell® device is designed to capture CTCs from whole blood samples based on size selection[8]. The device uses microporous membrane filters to capture CTCs under standardized conditions. The standardized conditions have been developed to support downstream analysis of the captured CTCs, such as enumeration, immunohistochemistry, and nucleic acid isolation.

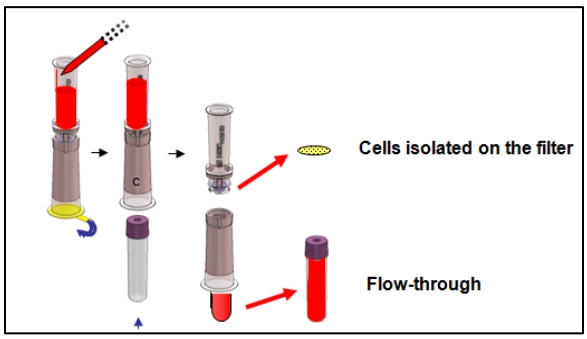

Figure 1. Illustration of the ScreenCell® device operation. Diluted sample is placed in the sample reservoir. A vacuum is applied across the microporous membrane filter drawing fluid through the filter. The filter with captured cells is ejected from the device for further processing.

The ScreenCell® devices are size-selective, taking advantage of the large size of CTCs compared to nucleated blood cells for isolation[9]. The devices consist of a filtration reservoir and a microporous membrane filter with defined pore sizes in a removable nozzle holder (Figure 1). The sample is diluted with filtration buffer and then the diluted sample is passed through a microporous filtration membrane by applying a vacuum to the outflow side. The typical time for sample processing is less than three minutes. The filtration membrane allows passage of nucleated blood cells but retains CTCs. The membrane with captured cells is ejected from the device for further manipulation. This configuration supports a range of potential applications, including immunocytochemistry, gene expression analysis, and culture of isolated cells. Additional details and methods for use were described by Desitter and colleagues[8].

Efficiency of Isolation

Published results indicate that the ScreenCell® Cyto device can efficiently isolate fixed H2030 non-small cell lung cancer (NSCLC) cells spiked into whole blood from a healthy donor[8]. To demonstrate this, 2 or 5 cells were micropipetted into 1 mL of whole blood followed by the manufacturer's suggested standard isolation procedure. In samples that received 5 cells per mL of blood an average of 4.56±0.71 cells were recovered (91% recovery), while an average of 1.48±0.71 cells were recovered from samples receiving 2 cells per mL (74% recovery). Control experiments yielded recoveries of 88% and 82% for 5 and 2 cells, respectively, suggesting that cell loss was due to the pipetting procedure rather than filtration.

The viability of cells following recovery was tested by directly filtering 50 live H2030 cells through the ScreenCell® CC device. Following filtration the microporous membrane filter was removed and the viability of the cells was tested by trypan blue exclusion. More than 90% of the cells were recovered and the viability of isolated cells was 85%±9%.

Isolation of CTCs from Clinical Samples

The utility of the ScreenCell® Cyto device for enumeration of CTCs, cytological analysis, and analysis of genetic mutations was demonstrated in a series of 23 patients with cutaneous melanoma, a cell type that does not express EpCAM[10]. Blood samples were obtained prior to surgical resection of the primary tumor and at first follow-up post-surgery. The median CTC recovery was 1 CTC per 2 mL (range: 0 to 11) peripheral blood drawn prior to surgery. Sixteen of the 23 patients were CTC-positive. Post-surgery the median remained 1 (range : 0 to 3), with 15 of 23 patients CTC-positive.

Microscopic examination of the isolated melanoma CTCs revealed typical malignant morphology, with anisocaryosis and a high nucleocytoplasmic ratio. The cells were morphologically identical to cells from the primary tumor and expressed mart1/melan-A, demonstrating the melanocytic lineage. The

expression of mart1/melan-A was heterogeneous in the CTCs, as it was in the primary tumor. These results show that the ScreenCell® device can enable access to EpCAM-negative cell types.

The ScreenCell® MB device can support personalized medicine, as illustrated by use of the device to isolate DNA from patient melanoma cells for genotyping. DNA isolated from CTCs captured with the device was subjected to whole genome amplification followed by sequencing to identify patients with the BRAF V600E mutation targeted by vemurafenib. Sequence data revealed an T to A transversion at nucleotide 1799 in some patients that is characteristic of the V600E mutation, suggesting candidacy for therapy with vemurafenib. This "theranostic" capability will be widely applicable in oncology.

This study in melanoma patients demonstrates that the ScreenCell® Cyto device can reliably isolate EpCAM-negative CTCs from patient samples for enumeration, immunocytochemistry, and genetic analysis. Similar results have been obtained with the major epithelial-derived cancers: non-small cell lung cancer (NSCLC), breast adenocarcinoma (BC), and colorectal cancer (CRC). The ScreenCell® devices can be used to isolate CTCs from patient samples in each of the major histologic types of cancer independent of surface biomarker expression.

Flexible Technology Platform

Captured tumor cells are amenable to analysis with a wide range of technologies to support personalized medicine research and practice. Technologies that are supported for downstream analysis include IHC, FISH, DNA sequencing, gene expression arrays, and cell culture. Examples of applications that can support monitoring of biomarker status are provided below.

As an example of the utility of ScreenCell® devices to monitor clinically-relevant biomarker status, HER2 expression was analyzed in BC CTCs isolated with the ScreenCell® Cyto device. IHC with anti-HER2 antibody demonstrated that the CTCs were strongly HER2-positive, as was the primary tumor. In other samples from a patient with HER2-negative BC primary tumor, HER2-positive CTC were isolated from the peripheral blood, suggesting continued tumor evolution during progression, as reported by others[11].

Biomarker monitoring using FISH is another example of a technology that can be monitored via CTCs isolated using ScreenCell® devices. In this example, quantitative visualization of the human epidermal growth factor receptor (EGFR) gene has been demonstrated in HT29 CRC cells spiked into whole human blood. Following isolation with a ScreenCell® Cyto device, cells were hybridized to FISH probe mix for EGFR and centromere 7 directly on the microporous membrane filter as described [8]. Quantitative fluorescence microscopy revealed multiple EGFR hybridizations per cell, indicating gene duplication, as well as more than 2 CEN-7 signals per cell, demonstrating chromosome 7 polysomy. These types of data can facilitate companion diagnostic application of CTC analysis.

The ability to analyze proliferation of viable CTCs has been lacking until now. This dimension of the biological properties of CTCs is particularly relevant to basic research into metastasis and for drug development. The ScreenCell® CC device is tailored to support culture of viable CTCs following isolation. NSCLC cultured cells were spiked into normal blood samples then isolated using ScreenCell® CC devices. Following the isolation procedure, the cells were cultured directly on the device microporous membrane filter in multiwell plates.

Proliferation capacity *in vivo* is also retained by cells isolated using this device. In a separate experiment, cells were isolated as above using the ScreenCell® CC device, then the microporous membrane filter was implanted subcutaneously in an immunocompromised mouse, ultimately developing into a

subcutaneous tumor. The possibility of routinely culturing CTCs from patient samples opens the door to development of potentially ground-breaking analytical methods for disease management.

Additional clinical and research applications can be envisioned. These include initial assessments of patients suspected of harboring malignant disease, based on the exquisite specificity of CTCs for malignant disease. Single cell DNA sequencing and other cutting-edge genomic technologies for personalizing therapy are also compatible with the ScreenCell® device. Finally, analysis of other cell types that appear at low levels in the peripheral circulation, such as fetal cells or circulating endothelial cells, is possible.

Conclusions

Over the last 40 years tremendous progress has been made in defining the molecular mechanisms of oncogenesis. These conceptual strides forward in understanding the inner workings of cancer are being applied to develop personalized medicine for cancer. In this schema, CTCs may have an important role in both the research lab and the clinic. Because of their role in metastasis and their accessibility, information from CTCs has the promise to not only assist in prognosis for many, if not all cancers, but also to help define individualized therapeutic regimens.

In order to achieve this vision, improved technologies for accessing CTCs routinely from patient samples are needed. While methods based on EpCAM enrichment have brought CTC information to the clinic, going forward there will be an important role in defining use of CTC information for newer technologies, such as ScreenCell®, that are broadly applicable to CTC isolation and can support downstream analytical techniques. The ease of use and flexibility of the ScreenCell® devices will aid in making the technology available to more labs and clinics. The additional access to CTCs will accelerate the use of CTC information in both settings.

References

1. Gallivan M.V., Lokich J.J.: Carcinocythemia (carcinoma cell leukemia). Report of two cases with English literature review. Cancer 53:1100-2, 1984
2. Cristofanilli M., Budd G.T., Ellis M.J., et al: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. New Engl J Med 351:781-91, 2004
3. Zhang L., Riethdorf S., Wu G., et al: Meta-analysis of the prognostic value of circulating tumor cells in breast cancer. Clin Cancer Res 19:5701-5710, 2012
4. Gerlinger M., Rowan A.J., Horswell S., et al: Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 366:883-92, 2012
5. Nadal R., Fernandez A., Sanchez-Rovira P., et al: Biomarkers characterization of circulating tumour cells in breast cancer patients. Breast Cancer Res 14:R71, 2012
6. Sieuwerts A.M., Kraan J., Bolt J., et al: Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. J Natl Cancer Inst 101:61-6, 2009
7. Punnoose E.A., Atwal S.K., Spoerke J.M., et al: Molecular biomarker analyses using circulating tumor cells. PLoS One 5:e12517, 2010
8. Desitter I., Guerrouahen B.S., Benali-Furet N., et al: A New Device for Rapid Isolation by Size and Characterization of Rare Circulating Tumor Cells. Anticancer Res 31:427-441, 2011
9. 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 circulatingtumor cells. Am J Pathol 156:57-63, 2000
10. Momburg F., Moldenhauer G., Hämmerling G.J., et al: Immunohistochemical Study of the Expression of a Mr 34,000 Human Epithelium-specific Surface Glycoprotein in Normal and Malignant Tissues. Cancer Res 47:2883-2891, 1987
11. Fehm T., Muller V., Aktas B., et al: HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. Breast Cancer Res Treat 124:403-12, 2010