

Circulating Tumor Cell Isolation: A Marathon Race Worth Running

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It took almost 150 years after the first report on the presence of circulating tumor cells (CTCs)² by Thomas Ashworth in 1869 (1) to reach the point at which their detection and molecular characterization is one of the hottest fields in cancer research (2). The clinical significance of CTCs has been evaluated in many types of solid cancers, and the Food and Drug Administration (FDA) cleared the testing of CTCs in metastatic breast, colorectal, and prostate cancer almost a decade ago (3).

In contrast to tumor biopsies, peripheral blood samples can be frequently and sequentially obtained for CTC isolation and downstream analysis. The non-invasive isolation of CTCs from peripheral blood and their further downstream molecular characterization at the protein, DNA, and RNA levels could now serve as a “liquid biopsy” approach, eventually offering additional information and, even more, a serious advantage over the conventional and well-established tumor biopsy approach (4, 5). Cell-free DNA circulating in plasma or serum of cancer patients has also been recently proposed as an alternative to the CTC liquid biopsy approach (6). By the use of extremely powerful and highly sensitive detection techniques, the presence of specific mutations in the plasma of patients with cancer could give valuable information concerning responses to specific molecular targeted therapies (7). However, there is a substantial difference between these two approaches; CTCs are viable cells that circulate in blood, and understanding their biology in a holistic way could give valuable information on metastatic spread, clarify their connection to cancer stem cells, and reveal active and possible targetable signaling networks. Cell-free DNA could give specific information as a circulating biomarker for the presence or absence of specific alterations indicating therapy response.

The prognostic relevance of CTCs has been evaluated in numerous studies so far, and a recent meta-analysis clearly indicated that the detection of CTCs is a reliable prognostic factor in patients with early-stage and metastatic breast cancer (8). Nowadays, CTC molecular characterization has a strong potential to be translated into individualized targeted treatments (9) and the number of ongoing trials that evaluate CTCs as markers for early prediction of treatment efficacy is continuously rising (10).

A tremendous effort is ongoing toward the development of novel state-of-the-art technologies for CTC isolation and molecular characterization (4, 5). CTC molecular characterization systems are mainly based on protein and image-based approaches or on molecular assays based on the analysis of the nucleic acids in CTCs. Nowadays, the application of extremely powerful next generation sequencing technologies in the area of CTC molecular characterization in combination with reliable single-CTC isolation opens new frontiers for the management of patients.

There is, however, substantial variability in the rates of positive samples determined by using existing CTC isolation and detection techniques. This lack of standardization of technology hampers the implementation of CTC measurement in clinical routine practice. Direct comparison of different methodologies for detecting CTCs in blood samples from patients with breast cancer has revealed substantial variations in the detection rates (11). CTC phenotypes have not yet been fully defined. According to the FDA-cleared CellSearch system, which has shown clinical relevance, CTCs are defined as epithelial cell adhesion molecule (EpCAM)⁺, cytokeratin⁺, and CD45⁻. However, there is ongoing discussion on the variable expression of cell antigens used for CTC enrichment with immunoaffinity-based isolation methods. Expression of EpCAM is variable across epithelial cancers and, more importantly, clinically relevant subpopulations of CTCs may go through an epithelial-to-mesenchymal transition or exist as cancer stem cells (12). We now know that CTCs are highly heterogeneous and that different analytical systems detect different cell populations on the basis of the approach being used for their isolation. The concordance between different analytical CTC detection systems is far below 100%, indicating that each system is actually identifying different and partially overlapping

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² Nonstandard abbreviations: CTC, circulating tumor cells; FDA, Food and Drug Administration; EpCAM, epithelial cell adhesion molecule; FMSA, flexible micro spring array.

fractions of the whole CTC population. Thus, statistically meaningful numbers of viable CTCs may need to be isolated, covering the whole spectrum, to characterize CTC heterogeneity, determine their cellular origin (primary vs metastatic tumor), and evaluate CTC response (signaling, proliferation, and apoptosis) after therapeutic interventions (11).

The main strategies for CTC isolation use separation on the basis of CTC density, size, and electric charges, and protein expression on the cell surface of CTCs. The first development of a microfluidic platform (the “CTC-chip”), for the separation of viable CTCs from peripheral blood on the basis of the interaction of target CTCs with EpCAM-coated microposts under precisely controlled laminar flow conditions (13), was followed by numerous publications on the isolation of CTCs using microfluidics (14). A variety of microfluidics and filtration devices have been developed for the isolation of CTCs on the basis of the different properties of CTCs that distinguish them from the surrounding normal hematopoietic cells: (a) physical properties like size, density, electric charges, or deformability; (b) biological properties such as cell surface protein expression, which involve immunomagnetic bead separation with positive or negative selection; or (c) a combination of both with filtration-based size separation, antigen cell sorting using flow cytometry, and density gradient centrifugation.

In this issue of *Clinical Chemistry*, Harouaka and colleagues describe the development and evaluation of a new flexible micro spring array (FMSA) device for the enrichment of viable CTCs independent of antigen expression but dependent on their size (15). The same group previously described a similar parylene membrane filter-based portable microdevice for size-based isolation with high recovery rates and direct on-chip characterization of captured CTCs from human peripheral blood (16). However, in contrast to most previously described microfiltration devices, the present FMSA device is based on flexible structures at the micro scale that minimize cell damage and can preserve cell viability while maximizing throughput to allow rapid enrichment directly from whole blood without the need for sample preprocessing. With the use of this new device, viable CTCs could be isolated from clinically relevant volumes of whole blood (7.5 mL) in <10 min, thus opening the way for its use in a routine clinical setting, where it is expected that samples would be analyzed in an automated way. As reported, capture efficiency was very high (90%) and more than 80% of captured cells were viable, an extremely important issue for downstream research. Because the time required to isolate CTCs is quite short, and pressure is highly controlled, this device presents a promising step toward

viable CTC isolation and a basis for further developments focused on large-scale analysis.

However, there is still a long way to go and it is very early to derive any conclusions regarding the clinical utility of this device. Evaluation of this new CTC isolation device was mainly based on spiking experiments with cell lines. It is already known that these cell lines can be much more easily cultured than can patient-derived CTCs. When this EpCAM-independent but size-dependent CTC isolation approach was compared to the EpCAM-dependent but size-independent Cellsearch system, in a very limited number of real patient samples, the results were very different. This is a general finding, applying to most size-only-dependent microfiltration and microchip systems for CTC isolation. It is only through prospective studies performed in a large number of patients, specifically designed to compare the prognostic significance of CTC detection by using these completely different approaches, that the potential clinical utility of this novel CTC isolation system will be revealed.

This applies in general to all new and highly sophisticated state-of-the-art systems that are continuously being developed for the isolation of CTCs from peripheral blood. Before introducing these systems into clinical practice, their clinical utility should be shown. Moreover, standardization of protocols for isolation and detection of CTCs, cross-validation of findings between laboratories, and universal internal and external QC systems for CTC detection and enumeration are needed. Many questions remain unanswered regarding the biology of CTCs and the optimal methods to isolate and characterize them. The marathon race is still ongoing, but it is worth running!!

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