CTCs gain character(ization)

By Tracey Baas, Senior Editor

Despite the recent shuttering of circulating tumor cell diagnostic company On-Q-ity Inc., researchers at **Massachusetts General Hospital** think there is still reason to innovate in the space. The group has developed a microfluidic platform able to identify circulating tumor cells that

lack epithelial cell adhesion molecule.¹

Johnson & Johnson has rights to the technology and is funding development of the product, which is dubbed CTC-iChip, under a 2011 deal with MGH.²

Circulating tumor cells (CTCs) are rare cells that break off from solid tumors and flow in the bloodstream. The CellSearch CTC Test from Johnson & Johnson's Veridex LLC unit is the only FDA-approved product that detects the cells. According to CellSearch's label, a count of 5 or more CTCs per 7.5 mL of blood—the standard volume of blood drawn in the clinic—is predictive of shorter progression-free survival (PFS) and overall survival (OS) in patients with metastatic breast cancer or metastatic prostate cancer. A CTC count of three or more is associated with lower PFS and OS in patients with metastatic colorectal cancer.

However, CellSearch misses CTCs that are negative for epithelial cell adhesion molecule (EpCAM).

Isolating EpCAM⁻ CTCs is particularly important because cancer

Figure 1. Isolating CTCs. The circulating tumor cell (CTC)-iChip uses magnetic beads to label cells in whole blood. The blood sample is loaded onto the chip, on which lateral displacement, inertial focusing and magnetophoresis separate and collect the cells of interest from the bulk.

(I) Positive selection incorporates epithelial cell adhesion molecule (EpCAM)-targeting magnetic beads to bind CTCs, and (II) negative depletion incorporates common leukocyte antigen CD45-binding and granulocyte marker CD15 (fucosyltransferase 4 α 1,3 fucosyltransferase myeloid-specific; FUT4; SSEA-1)-targeting magnetic beads to bind white blood cells (WBCs).

During lateral displacement, the sample moves through an array of microposts, spaced 32 μ m apart, which results in size-based separation and retention of nucleated cells. Red blood cells (RBCs), platelets and other components exit the device.

For inertial focusing, the nucleated cells enter an asymmetrical, curved channel and exit as a tight row of individual cells.

Finally, with magnetophoresis, a magnetic field (∇B) separates magnetically labeled cells from unlabeled cells and provides an isolated supply of CTCs in solution.

In total, the device can process about 8 mL of whole blood per hour, translating to about 107 total cells per second. (Figure based on Figure 1a in ref. 1.)

I Positive selection

II Negative depletion

cells downregulate expression of EpCAM as they proceed through epithelial-mesenchymal transition (EMT), a characteristic of metastasis, and there are multiple nonepithelial cancers such as melanoma.

Previous work from MGH developed CTC-iChips with surfaces modified with anti-EpCAM antibodies to bind EpCAM⁺ CTCs.^{3,4} However, only 1–2 mL of blood could be processed an hour, only EpCAM⁺ CTCs could be isolated and the chip required a 3D image-scanning system because CTCs are immobilized on the chip surface.

Now, the MGH team has developed a next-generation chip with a significantly higher throughput and the ability to isolate CTCs without an immobilization step. The latter feature allowed greater capture numbers of CTCs in solution and opened up the potential for downstream molecular characterization of the CTCs.¹

The team engineered two forms of the device. The ^{pos}CTC-iChip isolates EpCAM⁺ CTCs, whereas the ^{neg}CTC-iChip isolates EpCAM⁻ and EpCAM⁺ CTCs (see Figure 1, “Isolating CTCs”). The first has the advantage of providing higher-purity populations of EpCAM⁺ CTCs. The second provides a way to capture the elusive EpCAM⁻ CTCs and has the advantage of isolating CTCs from any type of cancer without the need to rely on a known surface antigen to isolate CTCs. This feature opens up the possibility to use the CTC-iChip for virtually all cancers.

As proof of concept, the researchers spiked blood with one of five different cancer cell lines. The ^{pos}CTC-iChip isolated EpCAM⁺ cancer cells but not cancer cells that had undergone EMT. The ^{neg}CTC-iChip isolated cancer cells that had undergone EMT as well as the parental cancer cells that had not.

In a head-to-head comparison, the ^{neg}CTC-iChip had an order of magnitude lower purification than the ^{pos}CTC-iChip.

The team compared the ^{pos}CTC-iChip with CellSearch using 42 blood samples from individuals with prostate, breast, pancreatic, colorectal or lung cancer. Both systems did equally well with 6 samples that contained >30 CTCs per 7.5 mL of blood. For the 36 samples with <30 CTCs per 7.5 mL, the number of CTCs isolated with the ^{pos}CTC-iChip was significantly higher in 22 cases ($p < 0.001$). For the remaining 14 cases with <30 CTCs, neither system could adequately isolate cells.

CTCs isolated with the ^{pos}CTC-iChip were able to be molecularly characterized, as an RT-PCR assay correctly identified four positive samples from patients with cancer that had the EML4-ALK oncogenic fusion protein at their primary tumor.

The team then used the ^{neg}CTC-iChip to isolate CTCs from blood obtained from patients with metastatic breast cancer, metastatic pancreatic cancer or metastatic melanoma. The researchers also were able to use gene expression profiling to characterize single CTCs collected by the ^{neg}CTC-iChip from the blood of an individual with prostate cancer.

Results were published in *Science Translational Medicine*.

“Ongoing studies with the CTC-iChip are underway in the clinic

to monitor tumor genotype and resistance to treatment in metastatic cancer for targeted therapies,” said corresponding author Mehmet Toner, professor of surgery at MGH and **Harvard Medical School**. “Our longer-term goal is early cancer detection.”

“On the technical side, we are working to make the CTC-iChip a one-piece integrated device,” Toner said.

Currently, the first step of size-based depletion of red blood cells and platelets is done on one chip. The second step of inertial focusing and deterministic magnetic sorting is done on a second chip.

“We are working to develop a device that includes the CTC isolation features as well as components that will completely characterize the isolated cells,” said Robert McCormack, head of technology, innovation and strategy at Veridex. “Some possibilities are fluorescence *in situ* hybridization (FISH) to detect the presence or absence of specific DNA or RNA sequences, fluorescent antibodies to detect cell surface markers such as HER2 or estrogen receptor, or next-generation sequencing. We are presently in collaboration or setting up partnerships with molecular and diagnostic companies to make this happen.”

Toner added, “To increase purity with the ^{neg}CTC-iChip, we are developing better ways to label the white blood cells with magnetic beads for negative depletion. Most of the contaminating white blood cells in the product expressed CD45 but somehow the beads didn’t attach to them. We are already an order-of-

magnitude better than the published paper and comparable to ^{pos}CTC-iChip.”

Other options

Although J&J is one of the few companies developing next-generation CTC products, competition could come from other avenues for obtaining liquid biopsies that predict outcomes and potential for metastasis and therapeutic resistance. These include circulating tumor DNA (ctDNA)^{5,6} and, to a lesser extent, exosomes.^{7,8}

In patients with cancer, 1%–10% of circulating or cell-free DNA derives from tumor cells,⁹ so the challenge has been to design assays that are sufficiently sensitive to detect low levels of rare mutant ctDNA among the much higher background levels of wild-type ctDNA and total cell-free DNA.¹⁰

This April, a **Cancer Research UK Cambridge Institute** team used a method to isolate, amplify and sequence tumor-derived ctDNA fragments to monitor acquired resistance to cancer therapy.¹¹ The method identified mutations acquired over a one to two-year period that were associated with emergence of therapy resistance and concordant with mutations in two corresponding metastatic tumor biopsy samples.

The team next plans to work toward gaining clinical pathology accreditation in the U.K.

“ctDNA is simpler and likely cheaper. However, in the long run it is unlikely to capture all the complexity of the cancer for diagnosis and monitoring of all tumors,” said Toner. “There are many genetic

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—Bruce Booth, Atlas Venture

lesions that can only be found at the RNA level and many characteristics of CTCs, such as epithelial-to-mesenchymal transition markers, that are expressed at the protein level.”

Atlas Venture partner Bruce Booth agreed. “ctDNA has the advantage of being far easier and cheaper to capture and analyze using new PCR-like techniques. It also could give insights into primary tumor genomic changes that might not be apparent in CTCs,” he said. “But intact CTCs have the advantage that they could help physicians characterize potential metastatic cells and their potential differences from the primary tumor, which could alter therapeutic choice. It’s not clear whether circulating DNA is from primary or metastatic tumors. Also, if CTCs could be captured and cultured, chemosensitivity testing could be done to identify what therapeutics—or more likely, combination of therapeutics—are most likely to work against metastatic cancer cells in those patients.”

Atlas had invested in On-Q-ity, which was developing a microfluidic chip that captured CTCs based on affinity and cell size. Each chip used several hundred thousand micro-sized posts covered with antibodies to capture the CTCs. **But the company failed to attract sufficient funding to continue developing the technology.**

“The closest thing to a head-to-head comparison so far between ctDNA and CTCs is whole-genome sequencing of the ctDNAs and enumeration of the CTCs,” said Paul Dempsey, CSO of **Cynvenio Biosystems Inc.** “With the new techniques, a head-to-head comparison to determine treatment responses could also include molecular characterization of CTCs, such as deep sequencing.”

Regardless of approach, “I think one important aspect that will need to be incorporated is sampling ctDNA or CTCs over long periods of time as patients respond to therapy,” said Dempsey. “This will help us to identify longitudinal markers of disease progression, remission and/or relapse that will ultimately benefit the patient in terms of helping to select appropriate therapies.”

Cynvenio offers its LiquidBiopsy lab service for recovering and analyzing CTCs.

“Right now, I think the field is still in the earliest innings of the game of ‘show me the data,’” Booth said.

A third approach for analyzing tumors involves exosome sequencing. Exosomes are small membrane vesicles, about 30–100 nm, that are shed by normal and cancerous cells. They carry diffusible factors, such as cytokines, growth factors and extracellular matrix molecules, and

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Cynvenio Biosystems Inc.**

mediate local and systemic transfer of mRNAs, microRNAs and proteins.^{8,12}

These exosome-packaged molecules might also provide cancer biomarkers and could do so with less degradation of the RNA within the exosomes than RNA that is freely circulating in the blood.

Exosome Diagnostics Inc. has started clinical trials of its tests in colon cancer, melanoma and glioblastoma multiforme (GBM).

NX PharmaGen offers NeXosome, a prenatal and oncology diagnostics proteomics platform that permits analysis of protein biomarkers shed specifically from placental or tumor cells within protected exosomes.

Multiple patents covering various aspects of the technology are at different stages of

filing and are exclusively licensed to J&J.

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