Using microfluidics to isolate circulating leukemia cells

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February 2020—Microfluidic assays are being used to isolate circulating leukemia cells and manage minimal residual disease in some patients with acute myeloid leukemia and B-cell/T-cell acute lymphoblastic leukemia.

“There is a lot of popularity in liquid biopsies, but there’s still a lot of work to do,” said Steven A. Soper, PhD, foundation distinguished professor of chemistry, mechanical engineering, and bioengineering at the University of Kansas. Dr. Soper, who also holds a teaching appointment at Ulsan National Institute of Science and Technology in Ulsan, South Korea, was a co-presenter with Sunitha Nagrath, PhD (see story), at the 2019 AMP annual meeting.

Most molecular analyses that depend on liquid biopsy markers will fail if the input into that assay is of low quality, Dr. Soper said, which is why “the isolation is extremely important.” Isolation removes potentially interfering blood cells and at the same time enriches the target cells such as the circulating leukemia cells (CLCs) and even circulating tumor cells (CTCs) for solid tumors.

Microfluidic platforms provide the capability to perform assays or use processing strategies that cannot be performed on the benchtop “and can provide some very elegant results,” Dr. Soper said. “I say this with a heavy ‘but be careful,’” he added. While there are several “elegant” technologies in the microfluidics community for the isolation of CTCs or CLCs, or both, “many of them don’t translate well into the clinic.”

‘CLCs can be used for a variety of leukemic-based diseases to manage those diseases completely and fully.’ **Steven Soper, PhD**

Dr. Soper presented the results of studies using his laboratory’s microfluidic device to detect minimal residual disease in hematologic cancers by isolating CLCs. CLCs differ from CTCs morphologically and biologically, he said, most notably in size. CLCs are comparable in size to WBCs, so filtration-based isolation techniques won’t work.

Another challenge with CLCs, Dr. Soper said, is that the antigens targeted for enriching CLCs can be expressed by normal blood cells. “When we use anti-EpCAM [anti-epithelial cell adhesion molecule] antibodies to isolate CTCs, the purity is high, and the amount of interfering cells is low because of the simple fact that WBCs for the most part don’t express EpCAM.”

In the case of B-cell acute lymphoblastic leukemia, for example, CD19 is used as the enrichment antigen. However, many B cells express CD19 but are not leukemia cells. The inferior biological purity of CLCs compared with CTCs “creates a bit of a conundrum,” Dr. Soper said.

Acute myeloid leukemia (AML) is a heterogeneous disease. “There’s no common marker that’s ubiquitous across all patients in this particular disease state. For example, personalized, patient-specific PCR tests can be used for each particular type of patient to monitor prognostic indicators,” Dr. Soper noted. “Because there is not a common marker expressed across all CLCs in AML, it makes it very difficult to enrich CLCs from patients with AML.”

With AML, “we don’t have one antigen that we can target to cover a large cohort of patients characterized with AML. We have to use a combination.” Dr. Soper’s laboratory’s microfluidic assay targets CD33, CD34, and CD117 antigens and in so doing, “we’re covering about 70 to 90 percent of the population with AML. However, in the case of ALL, CD19 is used exclusively to cover most patients with this disease.”

Since some non-leukemic cells will express CD34, for example, “we need to use aberrant markers to identify the leukemia cells from the normal hematopoietic cells that also express CD34.”

The ability to detect minimal residual disease is analytical method dependent, Dr. Soper said. “For example, multiparameter flow cytometry is typically used to monitor MRD. That’s typically done from bone marrow biopsies or aspirates because the leukemia cell content in the bone marrow is about 100- to 1,000-fold higher than it is in circulation. You need sensitive techniques to enrich those leukemia cells out of blood, and use them as an indicator of relapse from MRD.” And the need for bone marrow aspirates limits the frequency of sampling that can be done. “As such, a patient may relapse much sooner than detected via flow cytometry analysis of a bone marrow sample and, as such, the prognosis for that patient may be poor,” he said.

Dr. Soper shared results of a study in which his laboratory’s microfluidic assay was used to detect MRD in AML patients who had undergone hematopoietic stem cell transplantation (Jackson JM, et al. *Analyst.* 2016;141[2]:640–651).

The microfluidic assay can be performed on a sample of three milliliters of peripheral blood, Dr. Soper said, and is equipped with three different microchips surface-decorated with antibodies targeting CD33, CD34, or CD117 antigens. “We phenotypically sort these cells out because unique genetic alterations can occur in each one of these phenotypes.” Aberrant markers specific to the patients—CD7 and CD56—are used to identify the CLCs from the isolated fraction of cells.

The biological purity and analytical abilities of the microfluidic assay allow for the capture of sufficient numbers of cells to do molecular profiling of those cells, he said. “We don’t need to do single-cell analysis. We can elute off the chip using a photocleavable linker and then do any molecular analysis on them directly.”

The study results showed that “we were able to detect relapse two months prior to the clinically accepted standard of care,” which was PCR or flow cytometry analysis from a bone marrow biopsy, Dr. Soper said.

“PCR tests fail for 50 percent of AML patients,” he added.

Following treatment, relapse occurs in 15 to 20 percent of children with B-cell acute lymphoblastic leukemia (B-ALL), Dr. Soper said, and only half of those children who relapse are expected to be cured. MRD testing is unsuccessful in about 10 percent of children, Dr. Soper said. “And PCR for MRD is 20 percent unsuccessful” (Conter V, et al. *Blood.* 2010;115[16]:3206–3214).

In a pilot clinical study of 20 B-ALL patients at Children’s Mercy Hospital, Kansas City, Mo., Dr. Soper and collaborators Keith August, MD, and Maggie Witek, PhD, used the same microfluidic assay but with CD19 as the selection antigen. “It’s the same chip,” Dr. Soper said, retooled with a different antibody to target a different type of cell.

“To identify the leukemia cells from the normal B cells that express CD19, we used a panel of markers, in particular terminal deoxynucleotidyl transferase [TdT], which does untemplated extension of nucleic acids with deoxynucleotides,” Dr. Soper said. “We also used CD34, which is a stem cell marker, and CD10, which is a membrane metalloendopeptidase.”

“In these cases, the leukemia cells are nucleated, but they express TdT, a kind of ubiquitous marker that’s found in leukemia cells from B-ALL patients,” he said. Since TdT is present in the nucleus and cannot be used as an enrichment marker, “we used CD19. Almost all B cells express some amount of CD19.”

Dr. Soper and his collaborators tracked patients during therapy and up to 85 days after the period of consolidation therapy. By looking at the phenotypic distribution of cells for expression of TdT, CD34, or CD10, “we could see phenotypic changes that occurred as a result of chemotherapy.”

Data from one patient’s liquid biopsy results, for example, showed that the number of CD19-expressing cells dropped over the course of treatment, indicating the patient had moved into MRD. “There were still some signs of CD19-expressing cells,” Dr. Soper said. Examination of the CD19 cells that expressed TdT revealed residual leukemia cells indicative of MRD.

“Because we’re not doing bone marrow biopsies—irrespective of prognostic indicators—we can continue monitoring these patients to make sure there are no signs of relapse.”

Dr. Soper tested seven genes with potential to be prognostic and diagnostic for B-ALL: *CD19, WNT5A, CCND2, IL2RA, SORT1, FLT3,* and *DEFA1*. “There is diagnostic capacity in the *WNT5A* and *CCND2* genes,” he said, adding that expression of *FLT3* was shown to be particularly prognostic and also useful in AML. Patients with overexpression of *IL2RA, SORT1, FLT3,* and *DEFA1* had increased risks of relapse and death (Garza-Veloz I, et al. *Dis Markers.* 2015;828145).

A longitudinal gene expression study found significant downregulation of CD19 at the RNA level with treatment progression, particularly in one B-ALL patient. “That’s going to have a profound impact on the amount of leukemia cells we isolate during the enrichment phases of the assay,” Dr. Soper said. The patient’s results also revealed downregulation of the prognostic gene *FLT3*.

“There are a host of cytogenetic abnormalities that can be used for B-ALL as a prognostic indicator,” he said. For example, an *ETV6/RUNX1* fusion transcript indicates that a B-ALL patient is less susceptible to relapse. “Instead of using a bone marrow aspirate, we can gather these cells from blood and do FISH analysis on them.”

Dr. Soper said his laboratory’s microfluidic device for FISH analysis minimizes the time and cost. “The workflow has become less daunting,” which is common with microfluidics, he said. “We can secure results in two hours at about one-tenth of the cost for the reagents required for FISH.” His microfluidic device for FISH analysis uses enriched CLCs obtained by liquid biopsy versus cells from bone marrow biopsy.

The microfluidic assay, reprogrammed with anti-CD138 antibodies (syndecan-1), has also been shown to successfully isolate circulating plasma cells for identification of multiple myeloma-like characteristics. “We were able to stage patients” as monoclonal gammopathy of undetermined significance, smoldering multiple myeloma, or active multiple myeloma, Dr. Soper said (Kamande JW, et al. *Integr Biol (Camb).* 2018;10[2]:82–91).

“CLCs can be used for a variety of leukemic-based diseases to manage those diseases completely and fully. Even from minimal residual disease, where the CLC burden is low, we can still isolate those cells and monitor for recurrence. We can also look at prognostic indicators, molecular markers, directly from those leukemia cells,” he said.

What makes his team’s technology transferable to the clinical laboratory, even if so many others are not? The difficulties are usually related to fabrication, surface chemistry, and sampling statistics. Of the latter, Dr. Soper said, “Typically, you need to sample large input volumes of blood based on sampling statistics to make sure you catch the target cell, and that becomes somewhat problematic for many microfluidic platforms.”

He and his collaborators fabricate all of their microfluidic systems for clinical applications in plastics, he said, because plastics accommodate high-scale production using the injection molding technique. They can make 1,000 chips a day at about $2 a chip with very high reproducibility, he said. “So this is very accommodating for translation into the clinical laboratory.”

Plastics are attractive for another reason: They have a diverse array of surface chemistries that they can take advantage of. “The surface-to-volume ratio in microfluidics may be about 1,000 times larger than it is in your microcentrifuge tubes. With these plastics you can take advantage of the fact that you can expose them to UV ozone radiation, and when you do that, you change the surface chemistry. You actually add carboxylic acid groups to the surface.” These groups create a functional scaffold from which recognition elements can be attached, like antibodies, “or you make the surface very wettable.”

His lab’s microfluidic consists of an array of microchannels about 25 microns wide and 150 microns deep. There’s an input channel that allows the blood to flow into the device and then disperse through the arrays of microchannels. They have a sinusoidal architecture that collects the tumor cells that have antibodies on them and generates a centrifugal force that pushes the cell against the wall. “The antibodies are poised on these walls. That’s exactly what you need to have happen—these cells, the target cells, need to interact with the surfaces where the antibodies are placed,” he said.

“The longer they roll against this surface, the better the chance you have at gathering up those tumor cells no matter what they are or where they’re coming from. That’s the nature of this device.”

The device minimizes the amount of sample preparation on the front end. “This will process whole blood directly without dilution or lysing the RBCs. Very, very important. Every time you impose a processing step on the front end, you run the risk of contamination or loss of the elements you’re interested in.”

After the blood is washed out, there’s no residual blood components left in the chip. “That’s because we engineered the surface to accommodate minimal amounts of nonspecific absorption artifacts,” Dr. Soper explained.

What is interesting, he said, is being able to capture cells that have low expression of the particular antigen. “This is exactly the scenario you’re typically encountering when you’re looking at patient samples. They’re not like cell lines. It’s not a static picture phenotypically and genotypically of that cell. There’s a diverse range of different types of cells with different expressions of the target antigen.” He and his team designed the device in such a way that it can capture cells that have expression levels targeting antigens down to about 700.

“To put this in a frame of reference for you, the CellSearch technology has a limit of detection in terms of the antigens per cell of about 17,000 to 20,000. It’s several orders of magnitude higher.”

The device is also scalable. The channel architecture is thin, 25 microns, but deep to keep the throughput high. “We can process several milliliters of blood in less than an hour by generating the correct number of sinusoidal channels in the device, and the appropriate flow rate.”

A large-scale clinical study using the chips is underway for PARP inhibitors for pancreatic cancer, Dr. Soper said. “But it’s using EpCAM to gather up CTCs for pancreatic cancer.” The challenge with using anti-EpCAM antibodies, he said, is that cells undergo EMT-type (epithelial-to-mesenchymal) transition; they downregulate EpCAM. “So what we’re doing there is adding not only the EpCAM marker to enrich these cells but also a different marker that’s targeting these EMT or mesenchymal-type cells that have undergone EMT. That increases the number of cells we gather up, and we then have to do molecular analysis in that clinical trial, so we’re doing targeted NGS on those CTCs.”

For B-ALL, he and his team are working with collaborators at Children’s Mercy Hospital, where the pilot study of 20 patients was done, to scale up the sample to several thousand patients. “We’re now scaling that up to use circulating leukemia cells as an indicator of relapse in those patients,” Dr. Soper said.

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