Wading deeper into liquid biopsy

Karen Titus

March 2019—The standard riff for talking about a promising new cancer test should be familiar to anyone within sneezing distance of a laboratory: *There's no one-size-fits-all assay*.

But if any test were to come close, it would be liquid biopsy.

Are clinicians eager to use it? Check.

Is it relatively simple to do (check) with fairly quick turnaround times (check)?

Does it work for solid and hematological tumors? Check and check. Across multiple specimen types—serum, urine, vitreous fluid, cerebrospinal fluid, stool? Quite likely.

Can it be used to characterize patients' molecular profiles, monitor therapy, assess tumor evolution, identify resistance mechanisms, and detect early disease and minimal/measurable residual disease? Half a dozen checks.

Even if liquid biopsy does fall short of a one-size-fits-all assay, it's doing a reasonable impression of a Swiss Army knife (if not Sergeant Troy's sword fantastic, for those of you who are Thomas Hardy fans). The assay is dense with meaning, its rise enticing and swift.

Dr. Maria Arcila (right) with Dr. Laetitia Borsu Valente, technical director for digital PCR and amplicon-based NGS assays, at Memorial Sloan Kettering Cancer Center. To date, the lung cancer group is the heaviest user of cfDNA testing in the clinical lab at MSKCC. (Photo courtesy of Jennifer Altman)

"It is amazing to see how rapidly this testing modality is being adopted," says Maria Arcila, MD, director of the diagnostic molecular pathology laboratory at Memorial Sloan Kettering Cancer Center. Just a couple of years ago, she says, she did not foresee the current high demand for liquid biopsy testing from clinicians. But with the FDA's approval of the first liquid biopsy test for *EGFR* in 2016, and newer and improved technology, applications are broadening, she says, "and one can only expect that the demand will keep growing."

Liquid biopsy overcomes several of the shortcomings of tumor tissue biopsy. Aside from being readily accessible and a minimally invasive source of DNA, it addresses the problem of tumor heterogeneity, since circulating tumor DNA is a mixture of DNA derived from multiple sites and the tumor as a whole. “It is therefore potentially more representative of the malignant disease relative to a single localized biopsy,” Dr. Arcila says. Equally exciting is its use in longitudinal monitoring of patients for disease recurrence or for development of resistance mechanisms once a targeted therapy has been given. “You couldn’t do that before,” she says, without subjecting the patient to serial invasive biopsies and the associated risk.

It also addresses the drawn-out logistics of scheduling, performing, and processing a biopsy, which can stretch several weeks. With the faster turnaround time of a liquid biopsy, “you’re doing the patient a huge service,” Dr. Arcila says.
There are other benefits as well, and newer applications are being explored. “Circulating tumor DNA is dynamic and could be used to discover tumor changes and its evolution in real time,” she says. Positive molecular markers in ctDNA postoperatively, for example, could be used to assess for the presence of residual disease or metastases.

Maybe none of that should come as a surprise—liquid is, by nature, a multifaceted element.

With liquid biopsy, that advantage joins another truth. Simply put, “Cancer is molecular,” said Mark Routbort, MD, PhD, professor of hematopathology, UT MD Anderson Cancer Center, in a presentation at the Association for Molecular Pathology annual meeting last November.

He’s been doing molecular sign-outs only since 2012, he told his audience, entering the field at the time when next-generation and massive parallel sequencing were emerging as a way to detect the activating locations, sequence variants, and copy number changes that are ubiquitous in human cancer—and not only in tissue. Around the same time came revived interest in the fact (first described by Mandel and Metais in 1948, he noted) that nucleic acids of normal and tumor cells circulate, and that it’s possible to detect elevated DNA in the serum of cancer patients. Echoing a point Dr. Arcila made in her own AMP presentation, Dr. Routbort said that sufficient DNA in a patient’s circulation most likely points to cancer.

Dr. Routbort

Little wonder that researchers have moved briskly to bring this knowledge to bear on clinical applications.

At Memorial Sloan Kettering, Dr. Arcila and colleagues have been using digital PCR to detect key genetic alterations in single genes such as *EGFR* and the resistance mutation T790M, as well as mutations in *BRAF, IDH1*, and *IDH2*.

Digital PCR, or dPCR, is a great technology, she says, for periodic monitoring of well-known genetic alterations. While many methods are available for assessing ctDNA, Dr. Arcila says dPCR and BEAMing have shown superior sensitivity. Based on published literature, dPCR sensitivity may be 0.1 to 0.01 percent. It’s also quite rapid and is an inexpensive technology, with no need for the specialized bioinformatics support that next-generation sequencing technology would require.

Sensitivity is based on the amount of DNA that can be recovered from a plasma sample, says Dr. Arcila, adding that the amount of cfDNA shed by a tumor varies from patient to patient. “That’s an important concept.” The rate of shedding of tumor DNA into the circulation depends on many factors, including the location, vascularity, and size of the tumor and the amount of necrosis, among others. “The more advanced the disease and the more metastatic sites the patient has, the more ctDNA they’re going to have,” Dr. Arcila says. (As a reminder, she adds, cell-free

DNA encompasses both normal and circulating tumor DNA.)

Digital PCR, she explained in her AMP talk, is a biotechnology refinement of conventional PCR, used to clonally amplify and directly quantify nucleic acids. The key difference between dPCR and other PCR techniques is partitioning. Rather than running a PCR reaction in an entire tube, the test is run as thousands to millions of independent PCR reactions in discrete microdroplet-based compartments. By dividing the sample into so many droplets, the likelihood of having more than one DNA molecule being amplified in an individual droplet is quite low. That makes the assay precise, quantitative, and highly reproducible. Dr. Arcila called it unparalleled precision.

Digital PCR is relatively straightforward, she says, though like any assay, it has limitations and there are pitfalls. "We note that changes in the extraction protocols, for example, can have a major impact on how the assay performs," she says, as can changes in temperature or humidity. Digital PCR is also not high throughput. "It is the type of assay one would do for mutations that are known to be hotspot variants and with well-defined roles in patient management."

Memorial Sloan Kettering launched its dPCR journey three years ago with a test for *EGFR* T790M for patients with non-small cell lung carcinoma. Cell-free DNA testing is used as a screening test at the time of clinical suspicion of secondary resistance to *EGFR* tyrosine kinase inhibitors; only if the results are negative is the patient scheduled for a biopsy.

"The most important thing to keep in mind is that there are limitations to a liquid biopsy," Dr. Arcila says. "Very low levels of ctDNA, together with low overall cfDNA recovery from a plasma sample, may lead to false-negative results." This may be particularly evident in certain scenarios, she says, such as assessing for resistance mutations, which are often subclonal compared with the original sensitizing mutation. "So to me, any negative result with cell-free DNA should be taken as a false-negative until proven otherwise, and close follow-up or rebiopsy may be warranted."

Liquid biopsy samples, as it turns out, are much more difficult and variable than tumor biopsies. With the latter, "one can physically assess tissue suitability and presence or absence of tumor before testing is performed," Dr. Arcila says. "With cell-free DNA, who knows? You just have to extract it, and you won't know how much ctDNA is in your cfDNA sample unless the result is positive." Guidelines and standardized methods for cfDNA qualification, quantification, and testing are not yet defined as they are for other molecular methods.

To date, "our lung cancer group is the center's heaviest user of cfDNA." Current lung molecular testing guidelines permit use of cfDNA testing if a biopsy cannot be obtained, she says, but other cancers don't yet have similar guidance. "All of the questions right now are related to how fast each individual group introduces it into their guidelines." What has been moving along at a brisk walk will likely turn into a trot, if not a run, once there are standardized protocols and methods, she says. "But there's still a lot of work to do."

In addition to the rapid dPCR tests, "our group has also developed a broad panel for cell-free DNA testing by next-generation sequencing with duplex unique molecular indexing called MSK-ACCESS," which will soon be used in the clinical laboratory. The assay covers selected exons of 129 genes, as well as select copy number alterations and structural variants. "At this time, many institutions are or will be developing their own cell-free DNA assays. I don't see it going backwards from here," Dr. Arcila says.

While options abound, including comprehensive approaches using large hybrid-capture panels and exome sequencing, most clinical laboratories are using more targeted approaches to allow high depth of coverage of the most clinically relevant regions, according to Dr. Arcila. With dPCR, it may be possible to multiplex up to 15 mutations, although in a clinical setting a multiplex of even five will be challenging. Sensitivity is excellent, as she noted, and it is cost-effective for rapid genotyping and serial monitoring for specific, critical mutations. It also provides absolute quantification of mutant to wild-type copies and has minimal instrumentation requirements.

Using NGS panels provides a more comprehensive approach to known and unknown alterations. But, Dr. Arcila cautions, this approach is more costly and has longer turnaround times and results are semiquantitative. It will also

require a specialized infrastructure to run the assays, as well as a team of experts to process and analyze results, including strong bioinformatics support.

That would be the bailiwick of Dr. Routbort, who's been diving into cfDNA and related bioinformatics at MD Anderson, where he is also medical director of laboratory informatics and director of computational and integrational pathology. For panel-based cfDNA assays, he said, there's been strong initial clinical evidence that serial monitoring of tumor-specific DNA may be significantly superior to imaging for predicting outcome and relapse.

With cfDNA, he noted, "What you see in circulation may represent a mixture of tumor genotypes in a heterogeneous or a clonally evolving neoplasm.

"Now, that can be either an advantage or a disadvantage," he continued. The circulation compartment may reveal either the most aggressive genotype or a mixture of genotypes, "so you may see things circulating that aren't visible in the particular tumor section" obtained by biopsy for sequencing.

Dr. Routbort then sounded a warning. "You ignore the compartment issues at your own peril," he told his AMP audience. And then, to much laughter, he noted that while it was hardly Theranos' only problem, the company's downfall arguably started with compartment issues—using capillary bloodstick samples versus those from central venous circulation led to testing inaccuracies.

The assumption is that tumor compartment(s) equilibrate DNA with plasma in a reliable manner, he said. While they may indeed be highly correlated, that doesn't translate into linearity. Individual patient biology may be the key to whether labs can reliably interpret a negative finding.

Plunging into the data, Dr. Routbort offered some compelling evidence of liquid biopsy's clinical value.

One MD Anderson group, as part of a DNA assay validation for thyroid carcinoma, looked at patients with *RET* M918T mutated medullary thyroid carcinoma. Some of the patients known to have *RET* M918T-positive tumors were negative ("completely undetectable," says Dr. Routbort) in the assay, and some had very high circulating variant allele frequencies, with a wide range in between. "It's orders of magnitude," he said.

Looking at outcomes, the researchers found that patients with positive detectable circulating *RET* M918T had poorer prognosis. "If it's negative at the time they're tested—and these were all patients with metastatic disease—then the prognosis is much better," Dr. Routbort said.

A colon cancer study had similar findings. Monitoring specific mutations, researchers found that a significant number of patients had a positive ctDNA spike that preceded radiographic detection. Monitoring was done every three months, with both tests. "In most cases ctDNA would lead the radiographic discovery of recurrence." Since recurrence is a major transition point for therapy in stage II colon cancers, it's reasonable, he said, to think that earlier detection would be helpful.

In addition, Dr. Routbort said, clearance of ctDNA after chemotherapy was associated with superior progression-free survival. Though these findings emerged from a limited study set, he conceded, it offers the intriguing possibility that clearance in ctDNA-positive patients might be as good, and possibly better than, imaging.

What's needed in terms of sensitivity? It depends on the organ involved, Dr. Routbort said. It appears the amount of ctDNA associated with brain tumors is low, whereas with colon cancer, some patients have "stunning amounts" of ctDNA. "But the bottom line is, if you want to be able to detect small numbers of copies of ctDNA molecules, it just takes some back-of-the-envelope math." One nanogram of DNA is about 150 human diploid genome copies, he reminded his audience, so 20 ng—at the lower end of what might be expected in a milliliter of plasma—would translate into about 3,000 genomic equivalents. An assay with 0.1 percent sensitivity would be able to detect about three mutant molecules in 20 ng of circulating cell-free DNA.

Should the goal be one tumor molecule? "That's a pretty tough task," Dr. Routbort said. It's possible the extra

sensitivity might even be problematic, since on a subsequent test that molecule could be absent. He sees labs trying to target sensitivity between 0.1 and 0.01 percent.

Using a panel-based technique can be more complicated. Echoing Dr. Arcila's talk, he said that panel sensitivity is limited by read depth, for starters. "To detect a low-prevalence mutation you have to have a lot of reads. But that only scales to a certain amount, because all of these techniques involve some library prep PCR or even sequencing PCR, depending on your methodology." This can create DNA damage or PCR-based sequencing artifacts, which replicate and lead to nonlinearity. Some labs are doing very deep, amplified NGS to increase sensitivity for solid tumor testing, but that doesn't address the issues of specificity (which is related to introducing errors) or linearity (related to PCR duplicates, which can be discarded, though not entirely).

Achieving higher specificity and linearity requires unique molecular indexes, or UMIs, he said. Labs' use of sample-specific indexes is already ubiquitous, he said. "So you can multiplex samples on a flow cell or in a chip," but UMIs add a step in which highly stochastic sequence tags are used at the earliest step of library preparation or ligation or pre-PCR step to disambiguate PCR errors, PCR duplicates, or DNA damage from true variants.

In early 2018, MD Anderson launched its LB70 assay. Developed in an alliance with Guardant Health, it's a 70-gene ccfDNA panel; MD Anderson built its own bioinformatics on top of the company's technology for reporting adaptation. It's about 160 kilobases, he said, with ultrahigh depth, and it uses digital NGS and UMIs. Sensitivity appears to be between 0.1 and 0.3 percent.

Ordering is limited to certain types of cancers for now. Rather than placing it among other blood tests in the EMR, the lab positioned it as a biomarker, similar to solid tumor testing. "So it's cancer specific," Dr. Routbort said. "We're currently running about 200 samples a month." The test covers sequence variants, copy number variations, and some targeted fusions.

The lab also began running another panel in early 2018, called the solid tumor genomic assay, or STGA (MD Anderson does not excel at creative test monikers, he joked), which is an amplicon-based assay with an average depth coverage of 1,000–2,000.

The lab uses an existing genomic analysis and reporting application called OncoSeek, which it built in 2012, when Dr. Routbort came aboard. The reason for building software from scratch was simple, he said: They wanted a vendor-/pipeline-agnostic framework that would easily accommodate incremental changes in the panels and could be used for integration, pathologist review, report generation, sign-out, and analytics. The first case was run in April 2012, and over the years they've run about 40,000 samples in the clinical lab, on both hematologic and solid tumor assays.

He walked the AMP audience through several examples of the lab's work, including fusions. These are called genomically, which requires inference of fusion transcript junctions and may limit sensitivity. "For the variant tab, in OncoSeek, that's where we look for our sequence variants," Dr. Routbort said. "We generally show all called variants initially, and then once we've identified driver mutations, we can apply a set of filters sequentially to focus on reportable somatic mutations and exclude known and probable germline findings, as well as platform-specific sequencing artifacts" and other false-positives.

Especially helpful, he said, is the ability to show the population frequency—basically, the frequency with which they see a variant within the particular platform. It can be a "powerful and helpful discriminator" in the absence of other knowledge. They've also implemented a so-called trend tab, which enables them to see what molecular alterations have been identified previously in a particular patient.

Dr. Routbort broke down the first 1,098 cases done on the LB70 by tumor type: thoracic, 44 percent; GI, 43 percent; head and neck (including thyroid), five percent; GU, three percent; melanoma, one percent; and other, four percent. Clinicians' motivations for ordering tests were fairly clear. "By far and away the biggest clinical indication is that there's no tumor tissue available" (80 percent). "So there's less utilization of this for serial monitoring at this point." Other uses were estimating treatment response, baseline sample, single use (16

percent); estimating treatment response, follow-up sample, single use (three percent); and treatment resistance (one percent).

The number of correlative cases in the database are, for now, limited, he said, mostly due to limited access to primary tumor samples. A less specific way of looking at correlation is to look at multiple measurements from the same patient. Using this approach, the measurement appears to be extremely precise, he said—a function more of the assay's linearity than its sensitivity.

He's also looked at the most common mutations on STGA versus LB70 in a lung non-small cell cancer cohort (solid n = 668, liquid n = 370). "I was kind of surprised to see that the correlation was this good, both in terms of the incidence and the set of mutations," Dr. Routbort reported. "Nearly all of the mutations are called on both platforms." The commonality "gives us a high-level view that these assays are testing the same underlying things.

"Our experience to date has supported that the overall pattern and incidence of common mutations and fusions seen on liquid biopsy closely mirror what we see in solid tumor testing patterns."

Hematologic cancers appear to be a promising target as well.

Amanda Winters, MD, PhD, and her colleagues are using digital droplet PCR for adult acute myeloid leukemia patients in a variety of settings, including post-transplant.

Minimal/measurable residual disease (MRD), as measured by droplet PCR, is being used as an exploratory endpoint in several clinical trials involving adult AML patients, says Dr. Winters, instructor, Department of Pediatrics, University of Colorado Denver, and pediatric oncologist, Center for Cancer and Blood Disorders, Children's Hospital Colorado. One is a retrospective study involving patients whose DNA had been obtained previously from bone marrow biopsy, looking at AML-associated mutations pre- and post-transplant. Droplet PCR MRD positivity is predictive of relapse, Dr. Winters says. "And in most of the cohorts that we've looked at [it] seems to also be predictive of overall survival as well." Each cohort has a limiting number individually, "but we're hoping, as we get more patients accrued, to get enough of a population to be able to make some statistical inferences as we move forward," she adds.

It's possible, she suggests, that the droplet PCR method could be used to augment chimerism, which is not an MRD tool per se—it looks at percent donor engraftment in the bone marrow post-transplant and serves as an early warning sign for AML relapse. "With PCR being much more sensitive, we anticipated and showed in our small patient cohort that it was more predictive of relapse than chimerism."

The study used 21 different assays targeting AML-associated mutations. "We actually have about 60 now total," Dr. Winters said, including *IDH1, IDH2*, and *NPM1* mutations. They also designed a variety of patient-specific assays for use in certain clinical trial cohorts.

A clinical version of the droplet PCR is run at Children's Hospital through the molecular core laboratory. In that setting, a microgram of DNA is used per sample; Dr. Winters and her colleagues use 100 ng of DNA per sample in their work. The data correlates between the two assays, she says, but the sensitivity is lower in the test using less DNA. "Sensitivity is an issue," she says. "A lot of times patients who have undergone treatment have very hypocellular marrows, and so getting enough of a sample to work with can be a challenge."

Predicting relapse is valuable clinically. Emerging data from flow cytometry-based MRD analysis suggest that detecting relapse before it occurs clinically might be useful in modifying therapy. "The issue with flow cytometry, with AML in particular, is that the cell surface markers can be quite mutable across diagnosis to relapse," says Dr. Winters. Following a known founder mutation is much more reliable and can help clinicians feel more confident in their treatment choices.

For their clinical trial patients, she and her colleagues have looked at combining MRD data derived from droplet PCR with flow MRD data, which was used to guide initial therapy. In some cases, therapy has been modified by removing one or two drugs the patient is receiving; if the MRD numbers are good, that might allow clinicians to

reduce toxicity of therapy. In other cases, therapy might be expanded.

Dr. Winters points to another possible role for droplet PCR. “Transplanting a patient with barely detectable AML is far preferable to transplanting a patient who has frank AML. They do much better if you transplant them with lower burden.”

It might be redundant to refer to circulating tumor DNA assays in the scenarios Dr. Winters describes. AML is by definition a disease of the blood, of course. “You’re basically looking at the disease in its natural location in the body,” and thus not looking for circulating tumor DNA per se. “The tumor is circulating, so it’s not the same as the liquid biopsies that you think of for solid tumors. There’s a little bit of confusion there sometimes.”

In another bit of terminology tidying up, she notes that as more sensitive methods are developed, the language is changing from minimal residual disease to measurable residual disease. “We still don’t know quite what 0.001 percent mutation actually means—if that’s clinically significant or not,” she says. “As we are trying to improve these assays, we also need to keep in mind that statistically significant doesn’t necessarily mean clinically significant.” But, she adds, “I do think that based on the levels we have seen in patients—depending on the mutation you’re looking at—that most of the time even the bare minimum of detectable mutations actually does mean something. But who can say if that’s going to be the case as we dive even one log-fold more sensitive?”

Dr. Winters

Tumor shedding doesn’t enter into the equation for leukemias, but peripheral blood sampling versus bone marrow sampling does. “All of the data we have so far has been exclusively bone marrow,” Dr. Winters says, “because we know that that’s more sensitive.” Patients with AML will peripheralize their disease to different extents—some may have 20 percent blasts in their circulating blood, she says, while others will have none but will have truly defined AML in their bone marrow. Different leukemias are more or less “sticky” in the bone marrow, she explains, and thus don’t peripheralize. “That gets a little tricky as we start to think if there’s a little bit less invasive way of getting the same answer.”

Not all mutations are equal when it comes to AML. Founder mutations are inherent to the disease and are unlikely to change. If such a mutation is present at one percent allelic frequency, it’s likely representative of the amount of AML present, she says. But AML also acquires passenger, or later, mutations; *NPM1* is a good example. Detecting it indicates AML is present. “But if you don’t detect it, that doesn’t tell you whether just that little subclone is gone, or whether the whole AML is gone. AML is sort of a hierarchy of clonal populations,” Dr. Winters says.

“So the earlier clone that you can track, the more reliable that is with respect to the status of disease.”

Like Dr. Arcila, Dr. Winters says she sees the field evolving swiftly and predicts genomics-based MRD will leave flow cytometry behind, since it appears to offer a more reliable and more durable MRD marker compared with phenotypic surface markers. “In that sense it seems like it’s going pretty fast.”

Dr. Winters also sees plenty of enthusiasm among her colleagues. The data are so promising that the head of the AML clinical trials team at Colorado has expressed interest in using digital PCR MRD testing prospectively. “The reaction to this is overall one of excitement,” she says, though she sounds a cautious note as well. “As clinicians, we’re always trying to temper being aggressive with treating therapy with not doing harm to our patients.”

It’s good to be careful, Dr. Arcila agrees. “Everything needs clarification,” she says. She laughs but is clearly not joking. “Cell-free DNA is just so new. And there are right now very few scenarios where cell-free DNA has actually

been incorporated as a clinical assay that's acceptable." Even a Swiss Army knife—the epitome of safety—is still a knife.

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