Liquid biopsy—much to do about something

Karen Titus

March 2016—Lynette Sholl, MD, isn't fully sold on that hottest of feverishly hot topics, liquid biopsy. "It's kind of a sexy colloquialism, I suppose," says Dr. Sholl, associate director, Center for Advanced Molecular Diagnostics, and associate pathologist, Brigham and Women's Hospital, Boston. "Is there an official definition?"

Although she had been tasked with writing a State-of-the-Art paper on liquid biopsy in lung cancer—the plan was to submit it for publication following the USCAP meeting in March—she's focused on clinical practice, not language. "We essentially refer to it as 'plasma cell-free DNA testing,'" says Dr. Sholl, who is also assistant professor, pathology, Harvard Medical School. "Our lab code is 'plasma EGFR.'"

Dr. Lynette Sholl and colleagues, including thoracic oncologist Geoffrey Oxnard, MD, of Dana-Farber Cancer Institute, are looking at ways to apply plasma cell-free DNA testing in lung cancer. They will begin this spring to use it in-house for EGFR relapse patients.

In the opposite corner of the country, in San Diego, Mark Erlander, PhD, and his colleagues at Trovagene are focusing on developing assays for circulating tumor DNA, in both urine and blood. You could call these liquid biopsies, but Dr. Erlander isn't a fan of the term, either.

"It's totally overused," says Dr. Erlander, Trovagene's chief scientific officer.

Clearly, Dr. Erlander and Dr. Sholl won't be collecting any medical marketing awards. Nevertheless, the science behind the label is vibrant. "Everyone's excited about this," says Karen Kaul, MD, PhD, chair of pathology and laboratory medicine, NorthShore University HealthSystem, Evanston, Ill. "It's a hot topic—whatever that means."

Liquid biopsies inspire plenty of electrifying words. Interest is "skyrocketing," says one pathologist. The field is "exploding," says another observer. The potential is "mind-boggling," adds a third.

Unless you're Shakespeare, thrilling language will take you only so far. The term liquid biopsy is tossed about as much as Pericles. But as Dr. Sholl points out, a precise definition is up for grabs.

Cell-free DNA is one broad category—but in and of itself, it covers more than tumor biology (think prenatal diagnosis). A subset of cfDNA, circulating tumor DNA, narrows things down, though in the oncology literature cfDNA and ctDNA are used interchangeably, says Dr. Erlander. "But ctDNA is the only way I can think about it," he says. The other term is simply too broad, to his mind. "If you run around talking about cell-free DNA, there are lots of different kinds. So what one are you talking about?"

"There are a number of takes on what we mean when we say 'liquid biopsy,'" agrees Dr. Kaul.

Dr. Erlander

A brief jog through history provides some clarity. When DNA- and RNA-based molecular pathology took off in the mid-1990s, Dr. Kaul says, it gave pathologists the ability to look for circulating tumor cells in blood. It didn't take long for physicians to envision how this might yield deep insights, along the lines of discovering the migration pattern of a rare, secretive bird: Imagine following a tumor as its cells entered the bloodstream, circulated, landed, and grew into a metastatic lesion.

"In theory, this was very, very plausible," says Dr. Kaul. "In practice, it was more difficult." Early methods allowed pathologists to identify cells present in tiny numbers. "It was pretty much a rare event analysis," Dr. Kaul recalls. "But it didn't tell us much about the capacity of that cell to form a metastatic lesion. I suspect there are a variety of cells that get into the bloodstream." Whether they can metastasize is another matter. "We don't really know what the markers are that convey that capability."

Two decades later, molecular pathology shows no sign of stagnating. Next-generation sequencing, in particular, has revolutionized pathologists' understanding of which mutations are important in cancers, and new tools are being applied to blood and urine samples. Hence, "liquid biopsy." Dr. Kaul adumbrates three different target types:

1. Circulating tumor cells, or CTCs, that are on the metastatic pathway. Next-gen sequencing allows pathologists to look at those cells using large panels (approximately 50 to 100 genes) to not only determine which mutations are present but also glean an estimate of how many cells have those mutations.
2. Microvesicles and exosomes. "This has become interesting in recent years," says Dr. Kaul. "These are membrane-bound particles that are secreted off cells." They contain microRNAs and DNA fragments, as well as a variety of proteins, growth factors, etc. "We're beginning to understand not only are these important in normal processes, like immune regulation, but they're also useful in cancers."
3. Cell-free DNA. As with CTCs, next-gen sequencing has allowed pathologists to take a closer look at cfDNA with a fair degree of accuracy, says Dr. Kaul. These small but detectable amounts of DNA are thought to

be stabilized by being wrapped around nucleosomes. As cells undergo apoptosis, the genetic material is released into the bloodstream and circulates free in the plasma. “The technology has been key,” says Dr. Kaul. Small fragments that would have been difficult to examine and detect in the past can now be assessed with a fair degree of accuracy. The field appears to be developing in ways similar to what has happened in prenatal diagnosis, she says, where it’s now possible to look at aneuploidy and even specific genetic alterations using cfDNA from the placenta—fetal in origin, and present in maternal circulation.

Dr. Kaul

Another technical breakthrough, says Gregory Tsongalis, PhD, has been improved collection systems that allow labs to preserve and stabilize small pieces of DNA in blood and plasma. “There are new tubes, new kits, and we’ve continued to refine the analysis. Over the last few years these things have gotten better and better,” says Dr. Tsongalis, professor of pathology and director, Laboratory for Clinical Genomics and Advanced Technology, Department of Pathology and Laboratory Medicine, Dartmouth-Hitchcock Medical Center and the Audrey and Theodor Geisel School of Medicine at Dartmouth, Lebanon, NH. “It’s exciting.”

In oncology, the push to apply these technologies clinically has come from treating physicians and patients as well as pathologists, says Dr. Kaul. “And it’s gratifying to be able to connect those dots and make this real as part of patient care.”

Dr. Sholl and her colleagues offer one glimpse into the work being done to align the potential with the veridical. Specifically, they’re looking at ways to apply cfDNA testing in lung cancer.

Their work draws its inspiration from the use of minimal residual disease testing in chronic myelogenous leukemia, and how it has transformed treatment and outcomes. This was driven by a druggable target, BCR-ABL rearrangements in the blood, and by a highly effective drug, imatinib. Plenty of studies showed “that if you were able to track the levels of the disease transcript over time, you could predict how the patients were going to do, and modulate their therapy accordingly,” Dr. Sholl says.

That paradigm wasn’t lost on general oncologists, who for years have hoped to see its use expand from liquid tumors to solid tumors—and who are thrilled with the idea of a less-invasive approach to monitoring disease. With the advent of sensitive sequencing and PCR-based methods, Dr. Sholl continues, “We have finally reached a point where we might begin to use blood-based sampling to manage patients more effectively, or actually understand the burden of their disease better than if we were relying only on tissue-based biopsies and the associated radiology.”

Non-small cell lung cancer is a more-than-ready candidate, Dr. Sholl says. The EGFR mutation is a discrete molecular target. More importantly, oncologists can choose from several effective drugs, including first-line therapies and those that work in the context of patients who’ve acquired resistance associated with T790M

mutation. While it's been clear for several years that knowing a patient's genotype is important at diagnosis, "now there's also a good reason to understand what their genotype is when they relapse," she says.

Her laboratory is using droplet digital PCR to genotype cfDNA. "Honestly, it's been around a long time," says Dr. Sholl. "But everything has to come together in space and time, in the right way, for things to be used optimally. Now we have a more pressing need for highly sensitive assays."

The droplet digital approach is, essentially, absolute quantitation, she says. In contrast, more familiar assays tend to provide relative quantitation and use a housekeeping gene, an identified target, and a ratio. "You never really know exactly how many copies of your target are present in your specimen, but you have a relative sense," Dr. Sholl says.

With ddPCR, "You're actually counting the number of fragments of your particular sequence of interest that are present in your specimen." Naturally, it's only as sensitive as the amount of material obtained. A small sample of blood might not be sensitive, she says. "But if you get a 10-cc tube of blood, then you can potentially pick up very, very low levels of circulating DNA sequence in that plasma specimen."

Her colleagues reported on their work in *Clinical Cancer Research* (Oxnard GR, et al. 2014; 20:1698–1705). The researchers assessed response and resistance in patients with EGFR-mutant lung cancer who were receiving erlotinib. Serial quantifications of plasma genotype, they found, let them detect resistance mutations up to 16 weeks before radiographic progression.

As with a plotless ballet, there are gaps aplenty. EGFR-mutated lung cancers are quite heterogeneous. Some tumors appear to shed considerable amounts of DNA into circulation; others do not. In the latter case, pathologists would either need to rely on a tissue biopsy or retest with plasma at a later date. "We're working on that, to try to figure out that biology," Dr. Sholl says. She thinks the varied levels might be, in part, stage dependent.

Data to date suggest that with advanced-stage patients who have clinically suspected relapse disease, the sensitivity of ddPCR for plasma cf-DNA is about 70 percent, says Dr. Sholl. "We do have to acknowledge the sensitivity isn't perfect," she says. "So it's important, when you're designing these assays, that you are tracking not only the resistance mutation but also the original activating mutation, so you know you've got that patient's tumor DNA in your sample."

Droplet digital PCR isn't the only testing option, although Dr. Sholl says she and others have found it to be reliable, fairly cost-effective, and easy to run from a technical standpoint. And while it's not optimized for looking at larger panels (30 or 40 genes), "If you have a couple of particular targets, it's very robust as a clinical assay," she says.

Their work has brought unexpected satisfaction. "That it works as well as it does, honestly, is a bit of a surprise," Dr. Sholl says. "Everything that we've seen so far in these plasma specimens is that the specificity is excellent. We haven't had any false-positive results." She accepts the inevitable tradeoff with sensitivity that's typical of these assays. "In this particular case we're happy to lose a little bit of sensitivity to make sure that positive predictive value is outstanding."

Patients continue to be followed and are enrolled in trials for third-generation EGFR inhibitors. Not surprisingly, Dr. Sholl says, "We're beginning to pick up resistance mutations in these patients, so we're hitting that same roadblock. What do you do now that the best drug we have isn't working anymore?" Her colleagues at Dana-Farber Cancer Institute recently reported in Nature Medicine (Thress KS, et al. 2015;21:560–562) on their work using ddPCR to study cfDNA in patients with advanced lung cancer who had developed resistance to AZD9291, an EGFR tyrosine kinase inhibitor. All were positive for T790M mutation prior to treatment; after resistance developed, three molecular subtypes emerged, including cases with the EGFR C797S mutation.

"Having the option to serially monitor these patients [using] blood opens up the opportunity to understand the mechanisms of relapse much better," Dr. Sholl says. In turn, that points to the importance of optimizing next-generation sequencing techniques in plasma. While such assays may not ultimately play a role in routine clinical care, it's critical to push that envelope, she says, in trying to understand relapse.

Trovagene is also taking aim at lung cancer, in keeping with the company's emphasis on clinically actionable biomarkers. "I know that's a cliché," Dr. Erlander says. "But that's what we're doing. Instead of going after broad coverage with lots of genes and higher level panels, our focus is on having highly sensitive assays, where there's no question they're clinically useful."

Lung cancer is the so-called poster child for using ctDNA, he says, given the convergence of technology, demand, and therapeutics. Patients with stage IV NSCLC with EGFR activating mutations will receive erlotinib as a first-line therapy. Time to progression is usually about 10 months; among those who progress, 60 percent will have a T790M mutation.

AZD9291 has been approved for treating disease in patients with the T790M mutation, and it may soon be joined by others. "We're aware of six other companies that have T790M drugs in development," says Dr. Erlander. "This is an area where there will be a lot of alternatives for the physician to choose from in the years to come."

For Dr. Erlander, the main question to answer will be, How do you determine T790M status? Urine could be the answer if tissue biopsy is not practical. He says Trovagene has urine assays to detect activating mutations EGFR L858R and exon 19 deletions and T790M. They might be used to determine EGFR mutation status, he suggests.

In addition, the company continues to conduct clinical studies monitoring patients on erlotinib to identify the emergence of the T790M resistance mutation. "We can detect T790M a couple months prior to radiographic progression," he says. "So we think this can be useful for the treating physician, because they could monitor the patient with a urine sample to see whether T790M is starting to go up."

He's used to skeptics. "Many people are surprised it works," he says. When he talks about his company's efforts, he continues, most people assume the target is bladder or renal cancer. "But we're looking at all solid tumors—what we call systematically derived ctDNA."

Urine is a simple element to work with, he says. It's not as complicated as plasma, and it contains more DNA. "We can leverage it; it's very flexible."

Dr. Tsongalis sees urine as both more exciting and more challenging. "When you think about cell-free DNA [he prefers that term over ctDNA] in plasma, you get all comers," he explains, noting that some groups have shown that cfDNA from tumor may be a little more fragmented than cfDNA coming from normal cells. With the kidney potentially acting as a natural filter, he says, it's possible only the smaller, tumor cfDNA would be found in the urine. "If that's really true," he says, "it would be remarkable."

The ifs are surpassed only by the buts. Dr. Tsongalis sums up matters nicely when he asks, "It's great that we can detect this stuff, but what does it mean clinically?"

"There are still a lot of critical questions that need to be answered before we start doing this," he adds.

Dr. Kaul sounds similarly cautious when she considers the larger picture.

"In theory," she begins, "the DNA that we're seeing in the circulation should mirror that in the tumor. And it does. But again, we know that not all loci in tumor in the body are identical. Some tumor types have a lot of heterogeneity; others don't. Nor is it clear how the original tumor might differ from the metastatic lesion. Are there additional genetic or other types of alterations?

"These are examinations we need to be very aware of," she continues. "We need to do all these correlations and understand exactly where this DNA is coming from, how to best use that, and what it means."

She's not convinced the current research efforts will be sufficient. "I certainly would like to see more rigorous work being done," she says. The combination of opportunity and clinical need have driven small, narrow studies to answer specific questions relatively quickly, she says. "But I think it's important for us to step back and look more

broadly at larger numbers of patients, who span the spectrum of disease in a particular cancer, and who span a variety of cancers." Multicenter clinical trials with outcomes data will be necessary. Right now, however, she sees enthusiasm and opportunism (her words) outweighing more systematic approaches. That said, she remains optimistic. "I'm very enthusiastic this will improve patient care." The biological, technological, and clinical challenges are innumerable. What are the best targets for patient care, and which approach might work best?

Dr. Erlander sees all the pieces coming together—eventually. "It's all fragmented right now. Everybody's running around talking about how great their technology is and how it's changing the world," he says with a laugh.

From a purely academic standpoint, circulating tumor cells offer a clear window into how tumors metastasize. But it's not a straight shot to proving their value clinically. Exosomes are equally fascinating, Dr. Erlander says, and there's been a recent surge of interest in them. Looking at intact RNA might be extremely useful—with emphasis on the "might," he says. "We'll have to see how that plays out." Professionally, he's placing his bets on ctDNA, in no small part because of growing evidence that ctDNA is a surrogate for patient response, he says. "But that's not to say exosomes and CTCs won't play a role in the future."

Head-to-head comparisons will help sort matters out further regarding future use of CTCs and cfDNA, says Dr. Sholl, who adds the two approaches might complement each other. She, too, is intrigued by exosomes and microvesicles. "That's very appealing," she says. "It would be wonderful if we could get an RNA fraction for expression analysis, and pick up fusion transcripts. But can you translate it into clinical practice cost-effectively?"

Dr. Sholl suspects that the most successful technologies will be the ones that clear two obvious hurdles. "It's a function of what's quick and easy," she says.

Then there's the matter of tumor timelines, so to speak. When might it make the most sense to turn away from tissue? The focus on late-stage cancer has been a natural, given that sicker patients have more circulating tumor DNA. "But even more importantly, that's where the drugs are," Dr. Erlander says, and where the most intense decision-making occurs. But he foresees a day when ctDNA makes an earlier entrance.

Dr. Sholl reassures her pathologist colleagues when she talks about her work. "Our plan is certainly not to replace diagnostic biopsies," she says, noting that she's seen fears of that in the pathology community. But it makes sense, she says, to look at the potential of a plasma-based biopsy in the pretreatment setting when, after diagnosis, subsequent genotype testing is impossible because of insufficient or wasted tissue. "You don't want to do another tissue biopsy on those patients," she says.

Dr. Erlander likewise suggests that blood or urine ctDNA biopsies might be useful diagnostically in a subset of patients. In up to 20 percent of NSCLC cases, he says, a tissue biopsy doesn't provide adequate material for EGFR mutation testing. "Circulating tumor DNA would be able to offer an alternative in those cases." Pointing to an abstract presented at the European Lung Cancer Conference in 2015, he adds that up to 25 percent of lung cancer patients received first-line treatment prior to biomarker assessment. The reasons included insufficient tumor sample, poor patient health, long turnaround times, and patients' desire to start therapy.

Eventually, he'd like to see these assays used even earlier, for screening purposes. He's realistic about the difficulties. "The fundamental question there, which people don't talk about, is that it requires an incredible amount of sensitivity. Because what you're really saying is, 'Look, I need to find one fragment of DNA that has a mutation on it, in a sea of wild-type DNA of 100,000 fragments that are wild-type.'" Those are challenging numbers, to say the least. "And if you build that tool, do you then get enough cell-free DNA in blood or urine? You're looking for a needle in a massive barn of hay." But in the next breath he says, "It's exciting. Everybody's thinking about it. But you want to be smart about it."

Dr. Kaul is less convinced. "Wouldn't it be great if we could use this to screen people? It would be fabulous," she says, before dousing that idea in a cold shower of questions. "What tumors? At what point in development? Do they have to have a certain histology?

“We don’t know all these factors that influence the amount of DNA that gets from tumor into the circulation,” she continues. “Just looking at tumors as pathologists, we know that some have lots of apoptosis and others don’t. Some like to invade the bloodstream; others don’t. I suspect we need to define what features of a tumor correlate with us being able to detect cell-free DNA in the circulation to understand how to use this.”

She’s not skeptical, exactly. Dr. Kaul prefers the word “study,” as in, “We need to study this. Is this technique good enough to screen patients? We don’t know. We don’t know if enough tumors are going to be spilling their DNA into the circulation to use it as a screen. We have to do our homework. It may be a long shot. And yes, I am editorializing here,” she says with a laugh.

Like the others, Dr. Kaul is excited about the potential use of cfDNA in monitoring patients and selecting treatments. “But there’s a lot of basic biology we need to understand more fully.” Right now, liquid biopsies are like the American colonies under the purview of George III and his ministers. Everyone’s aware that something interesting is happening, but with only a foggy notion of how things will turn out. “It’s time to take a look at the meaning of all of this,” she says.

Dr. Txongalis

Dr. Tsongalis says he’s most intrigued about the potential for monitoring patients. Cancer patients come back routinely for follow-up visits, which often include some type of imaging study and additional biopsy or FNA. “If you could minimize what the patient has to go through by using a blood test, in my mind, I’m not sure there’s anything better or more useful than that.”

In his own laboratory, at Dartmouth, the focus has been on pancreatic cancer. The reason is simple: It’s aggressive and deadly. He echoes Dr. Sholl when he talks about how exciting it was to see the early steps succeed. “For us, the proof of principle—that we can even detect these things with some of the tools we have in the lab—was the first kind of, ‘Oh my gosh, this could really work’ moment,” he recalls. Another such moment came when he and his colleagues began to study tumor biology in mice by looking at cfDNA in plasma. “It was exciting, and continues to be.” In fact, he says, the question he fields most often from clinical colleagues, at least initially, hasn’t been, “When can we use this?” but “Can you really do this?” The sense of wonder is palpable, he says.

“There is no doubt about us being able to detect cell-free DNA that is specific for pancreatic tumor,” he says. “But we get back to the fundamental questions: What does this mean? Is this patient going to have a higher rate or quicker time to progression? Is this associated with tumor that has much more metastatic capabilities?

“Those are the questions we’re focusing on right now,” Dr. Tsongalis says. But every answer launches more questions. “Oh, absolutely. Absolutely. That’s why I keep focusing on potential. I’m not comfortable saying this is going to work yet.”

At NorthShore, Dr. Kaul says she and her colleagues have sent out “only a few” cases for cfDNA testing, when there was no tissue available for molecular testing. Again, the questions play on an endless loop. How is the assay validated? What data are available? And, can they rest easy with the result? “If it’s a situation where they see the mutation,” says Dr. Kaul, “then it’s there. You can bank on a positive result to a certain degree. But if it’s negative? Then you’re left wondering if the method was sensitive enough to detect it.”

This spring, Dr. Sholl and her colleagues hope they can start answering some of those questions for themselves, when they launch in-house clinical plasma cfDNA testing for EGFR relapse patients.

One question they may struggle to answer is what to call the test. “I’m happy with calling it a plasma-based mutational analysis. Or whatever,” she says. ‘Liquid biopsy’ seems a little funny.”

Perhaps she simply needs a little time—specifically, a few weeks, when she planned to be writing that aforementioned State-of-the-Art paper. “I don’t know if ‘liquid biopsy’ is the right term or not,” she says. “I don’t tend to use it. But I guess I’m going to be stuck with it when we publish that paper.”

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