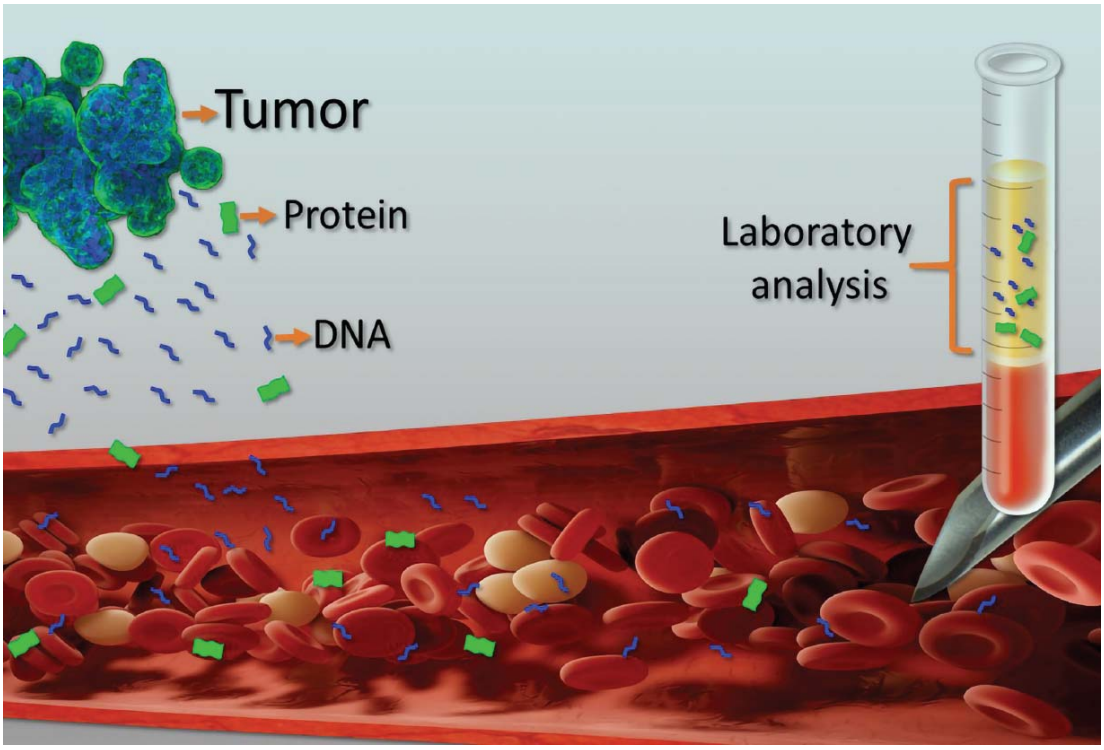
GEN Genetic Engineering & Biotechnology News
Vol. 40, No. 8, August 1, 2020

pp. 44–46, 48, 49
DOI: 10.1089/gen.40.08.14

Liquid Biopsies Generate Ample Insights from Sparse Clues

Duplex sequencing, target enrichment, and other technologies reveal incipient and residual disease as well early signs of drug resistance

Jon Kelvey



Among the more interesting biomarkers found in liquid biopsies are the molecular fragments shed by tumors. Circulating tumor DNA (ctDNA), for example, is being subjected to increasingly sophisticated analyses that can tune into vanishingly weak signals while filtering out enormous amounts of background noise.
Jill George/NIH

Like many other professional gatherings in the bioscience world, this year's Liquid Biopsy Summit was canceled due to the COVID-19 pandemic. It was to have been the fifth such event organized by the Cambridge Health Institute, which had arranged for liquid biopsy's leaders to present their latest advances June 15–17 in Seattle.

The cancellation of any bioscience event is unwelcome news, but the loss of this year's Liquid Biopsy Summit may be felt especially keenly because it comes just when the liquid biopsy field is burgeoning. According to Grand View Research, the field attained a value of $24 million in 2016 and is projected to grow to more than $2 billion by 2030.

In partial compensation for the loss, *GEN* presents this article. It is a virtual summit of sorts. It highlights what would have been discussed at the real summit by several key presenters. The emphasis here is on emerging technologies that are helping the liquid biopsy field overcome its biggest challenges: weak signals, subtly variable fragments, and vexing interpretive puzzles.

Tiny differences in tiny samples

"If you [take a liquid biopsy], you are sampling a liquid to detect something," says Jesse Salk, MD, PhD, the CEO of **Twin-Strand Biosciences**. "A blood draw is a liquid biopsy. Taking a urine sample—that's a liquid biopsy. Taking a sample of cerebrospinal fluid—that's a liquid biopsy. You could say a complete blood count is a liquid biopsy."

But when it comes to a liquid biopsy designed to detect signs of a particular cancer, or a rare mutation in a tumor, the signal-to-noise ratio can cause real problems for existing next-generation sequencing (NGS) techniques, according to Salk, who notes that limitations in both optics and biochemistry typically lead to background error rates of 0.1–1%"That's not a problem at all," he says, "if you're looking at the genetic difference between person A and person B while focusing, for example, on the gene that codes for green eyes versus brown eyes."

The background error rate, however, becomes a big problem when you're looking at tiny differences in a tiny sample. "Suppose you're trying to detect one leukemia cell carrying one leukemia-defining mutation mixed in with 100,000 normal cells," Salk suggests. "If your background error rate is 1%, you can't do that."

What TwinStrand has developed is a high-accuracy NGS technique called duplex sequencing, which uses both strands of a DNA molecule in the sequencing process to check for errors. Duplex sequencing, Salk maintains, can reduce the error rate to about one in 10 million. "We can," he continues, "detect incredibly low-frequency variants and mutations." What he had planned for the

summit was a presentation on two applications of duplex sequencing: the detection of residual disease in patients being treated for blood cancers, and the detection of emerging drug resistance in those patients.

In patients with acute myeloid leukemia, the front-line treatment, Salk says, is chemo-therapy. But determining whether the cancer is truly gone or whether there are residual cancer cells is difficult with existing technologies, and giving additional, cytotoxic treatment carries its own risks for patients who might not actually carry any residual disease.

“Historically, we have done bone marrow biopsies because it gives us a higher concentration of cells, but we know they are pretty unpleasant,” Salk acknowledges. “We would love to be able to take a blood sample and look for one in a million cancer cells left.” He asserts that if duplex sequencing were performed instead, it would be possible to detect leukemia-related mutations at levels of one in a million. Doing so, he argues, could improve the development of new therapies and better inform treatment decisions. For example, clinicians could identify people who should start or continue taking anti-cancer therapies. “Clinicians could,” Salk emphasizes, “prevent people from getting toxicities from unnecessary treatments when their cancer is already cured.”

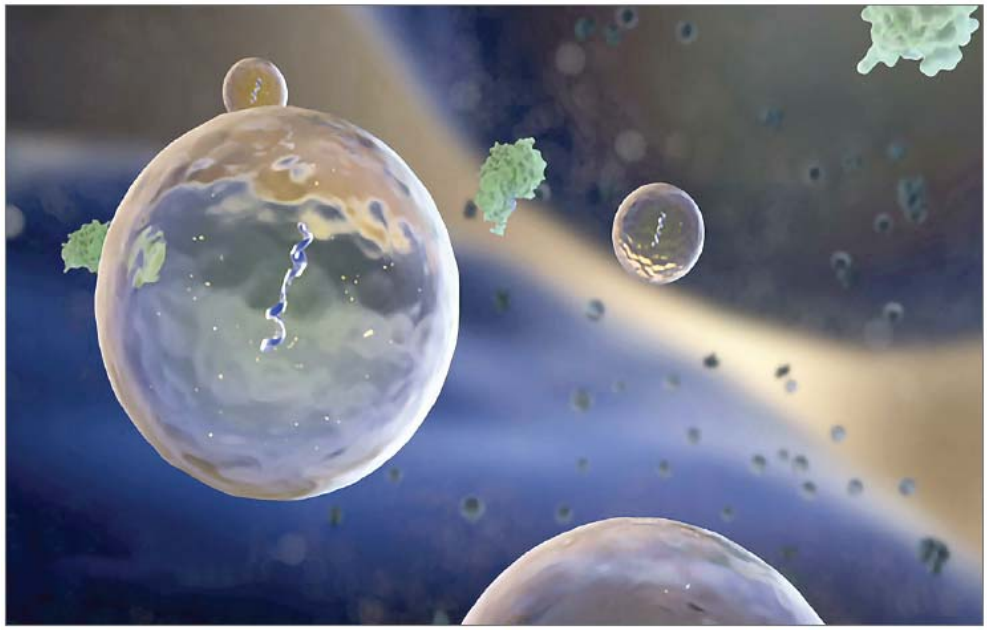
Duplex sequencing could also help clinicians detect cancer drug resistance as soon as it emerges because the genetic changes that characterize drug resistance in a tumor are understood. At present, Salk says, these changes seldom inform assessments of drug resistance. Instead, the typical clinical sign that a tumor has become resistant to a chemotherapy drug is that a patient relapses.

“What if,” Salk asks “you could see that happening really, really early—like months before somebody apparently relapsed. What if you saw their leukemia count come back then?”

With duplex sequencing, he says, clinicians could see resistance occurring at “low, low frequency.” He adds that in clinical practices where there are multiple generations of drugs already approved, “you just switch somebody to a different one to prevent their relapse.”

TwinStrand Biosciences is currently offering duplex sequencing kits and bioinformatics analysis, but the company is also collaborating with other companies, discusses Salk, in some cases helping the other companies identify patients most suitable to clinical trials.

“You really want to find the subset of patients who are likely to benefit and not the ones who are already cured or not at risk of relapse,” he advises. “They just slow down your trial. They flood out your signal.” Working with the right patients, he points out, can “really help these trials move along quickly so these drugs can get approved and get to patients.”



According to Captis Diagnostics, extracellular vesicles (EVs) have several advantages over other liquid biopsies such as circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs). EVs in blood contain circulating biomarkers such as DNA, RNA, lipids, and proteins for potentially comprehensive biomarker analyses. (This image, from the NIH, shows extracellular RNA encapsulated in an EV.)

One means for many ends

At **Claret Bioscience**, research has focused on capturing genetic information that is typically lost during analysis of the cell-free DNA (cfDNA) in a liquid biopsy, relates Varsha Rao, PhD, the company's director of clinical research and development.

"When DNA is fragmented, it has different types of ends," she says. "There can be blunt ends, 5′ ends, 5′ overhangs, and 3′ overhangs, depending on the nuclear activity. This information is usually lost because you have to do end polishing. You fill in 5′ overhangs or you cleave 3′ overhangs, and then you basically generate blunt-ended molecules."

Rao was headed to the Liquid Biopsy Summit to present on Claret's Single Reaction Single-Stranded LibrarY (SRSLY) technology, which uses a single-step phosphorylation/ligation reaction to produce complex libraries from small samples of DNA. This approach avoids end polishing and preserves the native ends of DNA fragments.

"We believe that DNA fragmentation is important in trying to understand the biology of any disease," she declares. "Our aim is to generate technologies that will retain the DNA ends."

Claret's technology also captures many shorter fragments of DNA, providing more information than other techniques, asserts Rao, who adds that there are a lot of fragments between 30 and 120 base pairs that are lost in conventional methods. "Our technology addresses the shortcomings in current approaches that suggest you have to ignore your short fragments, and that you have to do end repair and lose information about DNA fragmentation points."

Claret is currently offering SRSLY library preparation kits in 12- or 96-reaction formats. According to Rao, the company is just getting started. "We just recently came out of stealth mode," she explains. "We've been selling the kits since November and gaining a lot of attention."

Magnetic enrichment of short fragments

When **Apostle** entered the liquid biopsy space, the company resolved to develop technology that could capture information that eluded existing technologies. Soon, the company began exploring what it characterizes as a unique solution to a common problem.

Apostle has developed an automatable, high-resolution DNA size enrichment workflow, named MiniEnrich, on a magnetic nanoplatform, named MagTouch. Together, these technologies can capture subtle differences in the fragment sizes of circulating free DNA. In general, the small pieces from diseased tissues are more diverse or varied compared with those from normal tissues.

Apostle's CEO, David Ge, MD, PhD, notes that any liquid biopsy technology needs to detect a small signal (generated by a diseased tissue) within a huge amount of noise (generated by healthy tissues). In addition to this basic requirement, Ge points to more advanced challenges: "When nucleic acids come from diseased organs or tissues, they are for the most part shorter—or

sometimes even longer—than the nucleic acids from normal tissues. In general, the small pieces from diseased tissues are more diverse or varied compared with those from normal tissues."

The cfDNA molecules of interest are not the sections of genomic DNA that are so large that one might use conventional technology to sequence them. Instead of focusing on these sections, which are hundreds to thousands of base pairs long, Apostle distinguishes between cfDNA fragments that are much shorter. These cfDNA fragments often range from 165 to 175 base pairs if they come from healthy tissues, and they are typically about 20 base pairs shorter if they come from diseased organs.

The very subtle difference between short and even shorter fragments could be very helpful—if it could be measured efficiently in a clinical setting. "What we tried to do," Ge says, "is invent a new way to isolate those circulating, small fragments of genetic materials."

At present, cfDNA enrichment technology usually involves spin columns or magnetic nanoparticles. Seeing an opportunity to use magnetic nanoparticles more effectively, Apostle developed its MiniEnrich technology. "We invented a new way to bind and enrich the functional groups to interact with the nucleic acid fragments, on top of a new magnetic bead core," Ge details. The result, he says, is a technology that can recover DNA fragments as small as 20 base pairs. He adds that MiniEnrich technology provides the resolution necessary to utilize the length of fragments as a means of detecting disease.

Ge is willing to entertain different options for MiniEnrich's introduction to the market. The technology could be developed into a standalone product, or it could be integrated into another company's existing product line. Either way, he hopes that Apostle will bring a finished product to the market before 2021.

A peek inside extracellular vesicles

If the Liquid Biopsy Summit hadn't been canceled, **Captis Diagnostics** CEO Hong-Zhang He, PhD, would have discussed his company's focus on a very different medium for conducting liquid biopsy. Rather than detect or analyze non-encasulated cfDNA in the blood, Captis harnesses information contained in nanoscale extracellular vesicles (EVs) from tumor tissues.

"EVs have several unique advantages over tissue biopsies and other liquid biopsies such as circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs)," He tells *GEN*. "EVs in blood contain circulating biomarkers such as DNA, RNA, lipids, and proteins for potentially comprehensive biomarker analyses."

Captis has developed a lipid nanoprobe that can label EVs for magnetic enrichment and isolate EVs from plasma within 15 minutes. According to He, the

lipid nanoprobe “does not require expensive and bulky equipment, which is suitable for miniaturization, automation, and high-throughput instrumentation.”

Captis published a proof-of-concept study of this technology in 2017, in *Nature*, but He said that the company has more work to do before its approach is ready for the clinic. “In the next phase,” He notes, “we will validate the analytical sensitivity/specificity, the clinical sensitivity/specificity, and the clinical utility of the developed assay in large clinical trials evaluating patients with advanced cancer.”

Making sequencing a snap

NGS is essential to liquid biopsy approaches, but according to **Avida Biomed’s** head of clinical research, Grace Zhao, PhD, the technology has several drawbacks—it’s complicated, time consuming, and often quite expensive, especially when it is used to search for cancer-specific mutations and biomarkers through methylation analysis. “Nowadays, if you need whole genome methylation analysis, you might pay $3000 for cfDNA analysis,” she says. “Even if you need smaller coverage, you might pay up to $1000. It’s still too expensive if, say, you want to do early screening.”

So, Avida’s focus as a company, according to Zhao, has been to make NGS as simple and inexpensive as possible. “We named our technology Point-n-Seq™ because we want to make NGS as easy to use as a point-and-shoot camera,” she points out.

Point-n-Seq, as Zhao would have discussed at the summit, is a platform for combined methylation sequencing and mutation analysis of cfDNA signals of cancer in blood plasma. It may be used to assess all disease stages, but it may be most valuable as a means of enabling early detection, which requires high sensitivity. Typically, only minuscule signals are emitted by early-stage tumors.

“Current bisulfite-based technologies have a very low recovery rate. If you put in, say, 10 nanograms of human DNA, which is around 3000 copies of the genome, you may get 100–200 copies back,” she explains. “If your recovery is so low, you’ve lost a lot of signal. You’ve lost your sensitivity.”

Point-n-Seq, by contrast, can handle nanogram-level inputs and perform a combined methylation and genetic alteration analysis without splitting a DNA sample. And by focusing on small panels sufficient for one cancer type, Avida can keep the cost of sequencing low, the workflow efficient, and the fidelity of the signal high.

“If you’re looking at a huge panel, your background noise is very high,” Zhao points out. “The target capture step in Point-n-Seq for targeted methylation sequencing is before C-to-T conversion and before any amplification, which enables highly specific target enrichment even in very focused panels. If we do a

very focused panel, it is cheap and fast, and the background noise can be very low."

The result is an NGS tool that has a turnaround time of 1–2 days instead of 5–10 days, Zhao asserts, and at 1/10th the usual sequencing cost.

"Our goal is to make our platform technology readily available to researchers and clinicians doing cutting-edge work, and we're open to partnering with collaborators," she indicates. "If a big pharmaceutical company looks for an assay to screen for patients—say, it wants to stratify the patients at the early relapse stage—we believe that our assay can provide such a tool."

Challenges ahead

The emerging technologies in the liquid biopsy field, like all emerging technologies, present new opportunities and challenges. As Salk notes, liquid biopsy technologies that enhance sensitivity not only probe deeper and detect more fine variations in genetic samples, they also generate data that can be hard to interpret.

"Mutations accumulate at low frequency during life that look completely like they come from cancer, but they are actually just a part of aging," he says. "One has to be very careful now that we are able to detect these, to not automatically assume that this means someone has cancer. With great sensitivity comes great responsibility."

And then there is the more immediate and universal challenge posed by the COVID-19 pandemic, which is complicating operations in every field. The bioscience field—as the cancellation of the Liquid Biopsy Summit demonstrates—is no exception.

"The COVID-19 situation has had a very lasting impact on our operations," says Ge, who adds that the impact goes beyond work-from-home arrangements. "We were working to get to the next stage in clinical studies with a larger sample size," he explains. "That has been paused as well, as every hospital is fighting COVID-19 at the moment."

Still, Ge believes that the challenges presented by COVID-19, like the liquid biopsy field's technological challenges, will shake out with time. "I personally believe it will be over in no time, within several months," he says. "It will be over, and we will get back on track very soon."

Smarter Immuno-Oncology Trials Using Tumor-Informed ctDNA Analysis

By Alexey Aleshin

Alexey Aleshin, Senior Medical Director, Natera

Immune checkpoint inhibitors are being increasingly investigated through clinical trials for a range of cancers. However, only a minority of patients benefit from these interventions, and current biomarkers do not reliably predict treatment response.

A prospective Phase II clinical trial, described in *Nature Cancer*, assessed circulating tumor DNA (ctDNA) as a biomarker of response to immune checkpoint inhibitors in patients with advanced solid tumors being treated with pembrolizumab. It demonstrated that sensitive ctDNA assessment, using a tumor-informed assay—namely, the Signatera assay from **Natera**—can predict response to pembrolizumab in a histology- agnostic manner.

Signatera provided a readout on the trial in as short a time as six weeks, while complementing standard radiologic imaging to differentiate pseudoprogression from true progression, potentially enabling patients who are deriving clinical benefit to continue therapy, while sparing others from unnecessary toxicities and costs.

GEN