10TH ANNIVERSARY SERIES

Applications of liquid biopsies for cancer

Austin K. Mattox, Chetan Bettegowda, Shibin Zhou, Nickolas Papadopoulos, Kenneth W. Kinzler, Bert Vogelstein*

Liquid biopsies have the potential to detect, characterize, and monitor cancers earlier than is possible with conventional approaches.

INTRODUCTION

Finding minimally invasive methods to assess cancers has long been a central goal of oncology research. In the past decade, there have been major advances in our ability to examine tumor-derived material in the circulation and other biofluids, including urine, saliva, and cerebrospinal fluid. This has been possible due to the development of sensitive assays capable of detecting rare cancer-specific analytes immersed in a vast excess of analytes derived from normal cells. The analytes used for liquid biopsy include circulating tumor cells (CTCs), cell-free tumor DNA (ctDNA), proteins, metabolites, exosomes, mRNA, and miRNAs. Each analyte has its own advantages and disadvantages that must be considered when choosing a marker to answer specific clinical questions. For example, whereas CTCs are relatively rare in early cancers, they provide a particularly powerful approach to detect a variety of cancer-specific abnormalities in advanced cancers, such as androgen receptor splice variants. Moreover, studies of CTCs have led to remarkable insights about cancer biology that could not have been achieved using other analytes, such as the importance of cell aggregates in seeding metastases (*1*, *2*). In this eighth installment of *Science Translational Medicine*'s 10th anniversary Focus series, we discuss the current status of liquid biopsies and their applications for cancer detection.

TYPES OF LIQUID BIOPSIES

Many recent liquid biopsy studies use DNA as the analyte, partially due to the ease of DNA isolation and the availability of massively parallel sequencing technologies to assess tumor-specific alterations. Although DNA mutations occur in normal cells at a rate of five mutations per genome per cell division, only clonal proliferations of cells, containing tens of millions of cells with the identical mutation, can contribute enough mutant DNA templates to be detected at frequencies above 0.01% in plasma. This is in part because the half-life of circulating cell-free DNA is less than 1 hour, mandating a continuous outpouring of mutant DNA templates to be detectable in a randomly collected plasma sample. In addition to point mutations and small insertions and deletions, other genetic aberrations such as DNA fragment sizes, copy number changes, translocations, and epigenetic changes can be detected. When cancers release sufficient DNA into the circulation (or other bodily fluids), they can be detected with sensitive digital technologies such as massively parallel sequencing, wherein each individual DNA molecule is assessed independently.

Most cell-free DNA in plasma is derived from dying cells, namely, leukocytes (*3*). In general, there are between 3 and 9 ng of cell-free DNA per milliliter of plasma from normal individuals and patients with early-stage cancer. However, in patients with advanced cancer, there can be more than a 10-fold increase in the amount of cell-free DNA per milliliter of plasma, but the fraction of mutant DNA templates is still less than 10% of the total templates. The origin of the vast excess of nonmutant cell-free DNA in patients with advanced cancer remains unexplained. Empirically, we know that there is generally more mutant cell-free DNA in the plasma of patients with larger and more advanced cancers than in patients with smaller and less advanced tumors. However, the amount of mutant DNA found in plasma varies widely even in patients with the same tumor type, size, and stage, excluding the simplest explanations.

Protein biomarkers, such as carcinoembryonic antigen and carbohydrate antigen 19-9, were among the first analytes used to assess cancers through blood tests. Such biomarkers have been approved for assessing tumor burden in patients already diagnosed with cancer, particularly during therapy in patients with advanced cancer. Recent studies have indicated that protein biomarkers, when carefully applied, may also prove valuable for the detection of early cancers (*4*). With advances in mass spectrometry, we expect that a new generation of protein biomarkers for cancer will soon be available. Mass spectrometry should also enable the discovery of metabolites that can provide clues about the presence of cancer not possible with either proteins or DNA (*5*). Multi-analyte tests that include evaluations of DNA, proteins, metabolites, and RNA could theoretically be used to detect early cancers in a highly sensitive manner. Practically, however, there are challenges associated with using several platforms within a single test while maintaining sufficient specificity, throughput, and cost-effectiveness.

APPLICATIONS OF LIQUID BIOPSIES

To date, there are four clinical scenarios in which liquid biopsies are being evaluated.

At initial diagnosis

Because precision medicine for patients with cancer is now largely based on the mutations harbored by tumors, it is essential to identify these mutations at initial diagnosis for individuals being considered for adjuvant therapies, such as chemo- or radiotherapy, to prevent the cancer from recurring. Liquid biopsies are not optimal for this purpose; they are always less preferable than the evaluation of DNA from the primary tumor. The fraction of neoplastic cell DNA in liquid biopsies, particularly from patients with early-stage cancer, is usually low (often <0.1%) and often undetectable. In contrast, the fraction of neoplastic DNA in conventional solid tumor biopsies is usually >10 times higher, particularly after macrodissection of selected areas of the tumor. It can be difficult to confidently detect low mutant fractions and the mutations identified in the plasma of such

Ludwig Center and the Howard Hughes Medical Institute at the Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins School of Medicine, 1650 Orleans St., CRB1, Room 520, Baltimore, MD 21128, USA.
*Corresponding author. Email: vogelbe@jhmi.edu

cases can be artifactual and not present in the actual primary tumor (*6*).

One postulated advantage of liquid biopsies is that they reflect a random sampling of all the alterations in a tumor, whereas single biopsies from primary tumors are inadequate to reveal heterogeneity within the tumors. We do not think this advantage is compelling for two reasons. First, the heterogeneity within primary tumors is generally confined to passenger mutations. Targeted therapies are always tied to driver gene mutations, and these mutations are usually homogeneous throughout the primary tumor (*7*). Second, if the heterogeneity in primary tumors was reflected in independent metastases, then therapies directed against such a driver gene would not be helpful. Unless a targeted mutation is present in all metastatic lesions, the therapy will be of limited use.

On the other hand, liquid biopsies at initial diagnosis may be useful for prognostication. Another potential advantage of liquid biopsies over solid tumor biopsies is that, in unresectable cancers, the only solid tumor biopsy available may be a fine-needle aspirate. There may be insufficient tissue available from this aspirate for DNA sequencing, and a liquid biopsy may be the only noninvasive source of DNA available. Similarly, it can be problematic to obtain formalin-fixed paraffin-embedded sections of primary tumors, causing potential delays in the initiation of therapy. Regardless, it would be imprudent to use mutations detected in liquid biopsies for choosing first-line therapy unless the frequency of the mutant allele is high enough to warrant complete confidence that the mutation is likely to be derived from the tumor itself, rather than from leukocytes or technical errors. Otherwise, mistakes are bound to occur (*6*).

After surgery

In many patients without evident metastatic disease, the value of chemotherapy following complete surgical excision is not clear. Patients will nearly always suffer side effects from such therapies, but patients without residual disease or occult metastases cannot possibly benefit. The standard of care is to treat virtually all patients with a certain stage of cancer [e.g., stage III colorectal cancer (CRC)] with adjuvant therapy, even though ~70% of these patients with stage III CRC will not live longer as a result of the treatment. Similarly, standard of care dictates that patients with a lower stage of cancer (e.g., certain stage II CRC patients) do not receive adjuvant therapy, even though 20% of these stage II CRC patients have micrometastatic lesions that will eventually cause them to relapse. Suppose there was a way to more reliably know which of these patients should be treated with adjuvant therapy regardless of their stage? This would not only reduce suffering from the toxic effects of therapy but also save time, effort, and money by focusing care on the patients that most need it. Moreover, it could considerably simplify future clinical trials for new adjuvant agents; only patients who have micrometastatic disease need be enrolled.

Liquid biopsies taken after surgery are promising in this context. There are already studies showing that patients who have circulating tumor DNA or CTCs following surgery are very likely to relapse, whereas patients without circulating tumor DNA relapse less frequently (*8*). It would seem reasonable to consider treating most patients with a positive test for circulating tumor DNA with adjuvant therapy as long as the identified mutation was present in the primary tumor. On the other hand, it would not be prudent to assume that patients with a negative test for circulating tumor DNA will not relapse. At the present state of the art, the sensitivity for detecting occult disease is far from 100%. A negative test should therefore be considered as another feature guiding the need for therapy rather than the sole feature used for decision-making.

After additional therapies

Liquid biopsies are able to detect early recurrences prior to the tumors becoming radiographically or clinically apparent, potentially giving clinicians a larger window of opportunity during which treatment regimens could be altered (*8*). Once a patient relapses, liquid biopsies may reveal new mutations not present in the primary tumor that could guide choices for second-line therapy. In lung cancers with anaplastic lymphoma kinase (ALK) mutations, for example, an identical new mutation occurs in many of the residual lung lesions and could dictate the best next-generation ALK inhibitor to use (*9*). In many cancers treated with other agents, however, a liquid biopsy will reveal heterogeneous new mutations, one or two in each of several different metastases. No single drug will be able to target all these mutations. The only mutations found in all metastatic lesions will be those identified in the primary tumor through evaluation of the original solid tumor biopsy. Targeting another of these clonal mutations from the primary tumor is thus the best choice for second-line targeted therapy.

Screening for cancer

Using liquid biopsies before cancer is clinically detected is discussed last because it is by far the most difficult application, but it also has the greatest potential to reduce morbidity and mortality from cancer. The three applications noted above are designed for patients already known to have cancer, i.e., they are diagnostic tests. Cost and specificity are less of an issue for such patients; sensitivity is most important, as patients untreated on the basis of a false negative test are likely to die. The converse is true for screening tests; the test has to be cost-effective if it is to be applied to millions of patients at relatively low risk for cancer. Unless the test is exquisitely specific, then the number of false positives will greatly outnumber the number of true positives, engendering anxiety in patients and needless additional, sometimes invasive, procedures. Much of the controversy around current screening tests such as mammography and prostate-specific antigen is based on the relatively low positive predictive value (high ratio of false positives to true positives). A related problem is overdiagnosis—the detection of cancers that are indolent and never would cause morbidity or mortality if they had remained undetected.

Another issue that is especially important for screening applications of liquid biopsies concerns communication. How should information about the test be relayed to the patient? No liquid biopsy on the horizon is going to be 100% specific. On the basis of current publications, we envision that many average-risk patients testing positively with a liquid biopsy will not actually have cancer. How can patients be informed of a positive test in a way that causes the least degree of anxiety? What will the follow-up of such tests be, and what are the least invasive ways of following up a positive test in situations where the source of the tumor cannot be identified with certainty? Conversely, no liquid biopsy in the near term is going to be 100% sensitive, especially for early cancers. How can we make sure that patients do not think that they are at zero risk for cancer if their tests are negative, thereby unintentionally stimulating them to discount other primary or secondary prevention measures?

THE NEXT DECADE OF LIQUID BIOPSIES

Although many challenges abound for liquid biopsies in screening applications, the

opportunities are equally bountiful. One often misunderstood but critical point is that screening tests do not have to detect cancers that are very early and amenable to surgical resection to save lives and reduce morbidity. All they have to do is to detect cancers earlier than they would be detected otherwise. Studies using conventional chemotherapeutics, oncogene-targeted agents, immune checkpoint inhibitors, or T cells have all shown that therapies are much more effective when the tumor burden is low rather than high. As just one example, conventional chemotherapeutic agents can cure 47% of patients with micrometastatic CRC but nearly zero patients with bulky disease. The development of early detection tests is therefore intertwined with the development of new therapeutics: The earlier cancers are detected, the better any drug will work. As previously discussed, new therapeutics will require robust biomarkers to stratify responders from nonresponders, thereby treating patients most likely to benefit and preventing unnecessary harm to those patients least likely to be helped. Liquid biopsies are likely to provide such markers for many types of cancer.

Another often unappreciated point is that screening tests do not have to be 100% sensitive to make a difference. Suppose a liquid biopsy–based screening test was able to detect 20% of patients with solid tumors of diverse types and that half of these patients could be cured. Would such a test be useful? A common answer to such a question would be "No, this sensitivity is not high enough." But another answer to this question is provided by comparison to an analogous therapeutic. Suppose a new drug were developed that could cure 10% of all cancer patients—not just put them into remission but actually cure them, with minimal side effects. Would that be considered a major advance? This comparison illustrates the double standard that is often used to judge new diagnostic tests versus new therapeutic agents (*10*).

Overdiagnosis may be a problem faced by new early detection tests, but we currently face the opposite problem: underdiagnosis. Most of the 600,000 patients who die each year from cancer in the United States die only because their cancers were not detected early enough to be cured by surgery or other currently available treatments. How to balance the current, intolerable underdiagnosis with potential overdiagnosis is a challenge that further research will hopefully solve. However, a solution to this problem will only be possible if reliable early diagnostic tests for major cancers are developed and used. With technologies advancing rapidly, and with the accelerating interest in liquid biopsies from both academia and industry, we look forward to the day when liquid biopsies that detect cancers earlier become a routine part of preventive medicine.

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Competing Interests: B.V., K.W.K., and N.P. are founders of Thrive and Personal Genome Diagnostics. K.W.K. and B.V. are scientific advisory board members for Sysmex Eisai, Cage Pharma, NeoPhore, and Morphotek; B.V. is also an advisor to Nexus. N.P. is a scientific advisor for NeoPhore. S.Z. is a founder of Personal Genome Diagnostics and holds equity in Personal Genome Diagnostics, Thrive, and NeoPhore. These companies and others have licensed technologies described here from Johns Hopkins University; B.V., K.W.K., and N.P. are inventors on some of these technologies and receive equity or royalty payments. C.B. is a consultant for DePuy Synthes.

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