



Liquid Biopsy Cancer Biomarkers – Current Status, Future Directions

Liquid Biopsy: A Brief Overview

Liquid biopsy offers a noninvasive or minimally invasive means of assessing physiological or pathophysiological processes that would otherwise require tissue biopsy or other invasive procedures. Most cancer diagnostic pathways currently require a fine needle aspiration at best, or an excisional biopsy at worst. Liquid biopsy, in this case, could circumvent the need for needle biopsy, endoscopy, or surgery by instead analyzing a blood sample for circulating tumor cells or circulating free nucleic acids. Since invasive diagnostic procedures are associated with a risk of hemorrhage, hematoma, tissue damage, seeding tumor cells in surrounding tissues, liquid biopsy is a safer alternative.

One could also imagine using liquid biopsy to track tumor phenotype and burden over time. Physicians currently use serial CT, MRI or PET scans to assess treatment response and disease progression. These imaging studies are expensive, time-consuming, and expose patients to external radiation, intravenous contrast and/or radioactive tracers. Moreover, imaging studies do not provide any information about tumor phenotype, which can change with treatment. Today, tumor phenotype information is currently gathered through tissue biopsy. However, it is hardly ever feasible to perform serial tissue biopsies given the costs and risks to patients. Liquid biopsy, on the other hand, could provide a virtually continuous assessment of cancer burden and phenotype at relatively low cost. The patient would be at no more risk than that posed by simple blood draw. Furthermore, liquid biopsy methods may be able to detect cancer recurrence before tumor is visible on imaging or large enough for traditional tissue biopsy.

While the potential advantages of liquid biopsy are clear, the field is relatively young and rapidly evolving. Several liquid biopsy applications are available, with many more on the horizon; however, some potential uses still remain theoretical. We will discuss the current state of liquid biopsy, with a particular focus on cancer genomics. We also discuss some of the keys to success in working with cell-free DNA (cfDNA), and we will finish with future directions of this exciting field.

Current Applications of Liquid Biopsy

Prenatal testing, transplantation medicine and oncology are driving the science of liquid biopsy, and have produced some visible clinical applications.

Prenatal Screening

The most well-defined clinical applications for liquid biopsy are in prenatal medicine. There are obvious advantages to being able to test fetal tissue for genetic/chromosomal abnormalities during early gestation; however, obtaining a tissue sample using chorionic villus sampling or amniocentesis is associated with fetal morbidity and mortality. Liquid biopsy, on the other hand, offers the possibility of prenatal testing using a sample of the mother's blood.

Early successes of liquid biopsy in prenatal screening were whole fetal cell tests for trisomy 21, 18 and 13. Fetal cells that have entered the maternal bloodstream could be collected for liquid biopsy and karyotyped to identify chromosomal abnormalities. More recently, cfDNA testing has been used to detect the trisomies listed above, and also to identify ABO blood group, sex chromosome aneuploidy, chromosomal deletion mutations and single-gene mutation disorders.¹

Transplantation Medicine

Another key application of liquid biopsy is in transplantation medicine. It is often necessary to assess the functioning of the organ or tissue after transplant. Ideally one would avoid sampling the transplanted tissue directly—though tissue biopsy is sometimes necessary—liquid biopsy can be used as an alternative would be advantageous.

One of the key considerations in post-transplant surveillance is to ensure adequate but not excessive immunosuppression. The presumed ideal is to suppress the organ recipient's immune system only to a degree the transplant is not rejected. Minimal immune suppression allows the patient to fight off infection, detect and manage nascent tumor cells, etc.

To this end, liquid biopsy potentially allows for surveillance of tissue rejection. For example, researchers collected plasma and urine samples from 100 kidney transplant recipients during the first three months after transplantation surgery. Total cfDNA increased markedly during acute transplant rejection, and this elevation generally preceded clinical diagnosis of the rejection episode.² Conversely, total cfDNA return to baseline after immunosuppression halted the rejection.² Indeed, total and transplant specific DNA in urine or plasma can successfully be used for optimizing immunosuppressive therapy and can delay the kidney biopsy.^{3,4}

Oncology

We have known since the 1970s that patients with cancer have much higher levels of normal circulating cfDNA/circulating nucleic acids than healthy individuals.⁵ Even at that time, researchers showed plasma DNA levels decreased after chemotherapy.⁵

From a theoretical perspective, the field of oncology has the most potential applications for liquid biopsy. One could envision a role for early detection and cancer screening, diagnosis, treatment monitoring and posttreatment surveillance. While we will discuss the potential roles of liquid biopsy and all of these facets of oncology, not all of these applications are equally mature. Indeed, the most commercially advanced application of liquid biopsy in oncology is in treatment selection and monitoring.

Most of the immunotherapies or targeted therapies for cancer are not administered to all patients with a certain type of cancer. For example, some patients with metastatic non-small cell lung cancer (NSCLC) will be eligible to receive first-line treatment with gefitinib, an oral EGFR-tyrosine kinase inhibitor; others will not. This is because gefitinib is far more effective in patients with cancers that possess certain mutations in EGFR. Hence, the FDA has approved gefitinib as first-line treatment for patients with these types of metastatic NSCLC only. They simultaneously approved a companion diagnostic, the Therascreen EGFR RGQ PCR kit, to identify patients with the requisite mutation.

The Therascreen companion diagnostic requires formalin-fixed, paraffin-embedded, non-small cell lung tumor samples. Unfortunately, it is not always possible to get a lung cancer tissue biopsy for EGFR mutation analysis. Indeed, tissue samples are either unavailable or indeterminate in as many as a third of all patients with lung cancer.⁶ Liquid biopsy has emerged to fill in the gap left by inadequate tissue biopsy specimens. Tumors release tumor cells and tumor DNA into the circulation, which can be isolated and tested from liquid biopsy samples. First Europe, then China, then the United States approved liquid biopsy testing for EGFR mutations prior to gefitinib treatment for NSCLC.⁷ Other liquid biopsy based mutation tests are soon to follow such as cfDNA tests to detect BRAF V600 mutations⁸ and KRAS mutations,⁹ among others.

The Future of Liquid Biopsy in Cancer

While the liquid biopsy market in cancer is most mature in the area of tumor characterization and treatment selection, other applications of liquid biopsy for cancer are gaining traction.

Treatment Monitoring

Tumors initially susceptible to specific tyrosine kinases inhibitors are gefitinib, erlotinib, crizotinib and ceritinib often mutate and develop resistance to the drugs. Just as liquid biopsy has supplanted tissue biopsy for initial tumor characterization, there has been a move to use liquid biopsy for resistance testing as well.^{10,11} Liquid biopsy offers a non-invasive means to detect the change in tumor status and, in turn, change the treatment strategy.

The potential benefits of liquid biopsy tumor characterization are not limited to small molecule inhibitor applications. Immunotherapies such as checkpoint receptor blocking antibodies that target cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death-1 (PD-1) are available to patients with certain tumor histology.¹² Liquid biopsy approaches may be applied in a similar fashion to select and monitor cancer immunotherapy.¹³

Screening and Early Cancer Detection

One of the central dogmas of cancer care is that early detection of cancer results in better outcomes. Indeed, people with early-stage cancers have higher remission and survival rates than those with late-stage or malignant cancers, regardless of cancer type. However, it is impractical and likely unwise to perform serial radiologic testing or invasive procedures for cancer surveillance. Liquid biopsy offers the possibility of periodic cancer screening through routine blood draws or urinalyses.

Testing applications focus on the earliest premalignant genetic changes that differentiate cancer from healthy tissue. To be effective, any population-wide screen would have to have very high specificity.¹⁴ Unfortunately, certain known cancer-associated mutations tend to occur with age even in people who never develop cancer.¹⁵ Thus, the key to progress of liquid biopsy in early cancer detection is identifying highly specific, circulating markers. While there are approximately a dozen genetic screening tests available for the early detection of cancer in otherwise healthy individuals, the widespread applicability of these tests are hampered by sensitivity issues. Nonetheless, research interest in this area is robust.¹⁴

Posttreatment Disease Monitoring

Minimal residual disease is the persistence of cancer cells in the body despite treatment. These cells are below the resolution and detection levels of imaging studies, and are considered a principal cause of cancer and recurrence. The concept of liquid biopsy for minimal/measurable residual disease is relatively well-developed in leukemia, where increasingly sensitive sampling techniques allow clinicians to detect minimal residual disease at lower and lower levels.¹⁶ While the premise is the same for solid tumors, progress in that area is less developed.

As with cancer screening and early cancer detection, the clinical application of liquid biopsy is limited by sensitivity and specificity of current tests.¹⁶ These limitations are currently more pronounced for detecting minimal residual disease for solid tumors than blood cancers.¹⁴ Nevertheless, several companies currently market liquid biopsy assays for both solid tumor surveillance and leukemia/lymphoma minimal residual disease.

Types of Cancer Biomarkers

Liquid biopsy analysis tends to focus on three main types of biomarkers, namely circulating tumor cells, exosomes and cell-free nucleic acids.

Circulating Tumor Cells

Circulating tumor cells are perhaps the most easily understood of the liquid biopsy-derived cancer biomarkers. A primary solid tumor sheds cells that move into the bloodstream, which can then be detected in a blood or plasma sample. To date, success using circulating tumor cells is most notable in breast, lung, and prostate cancers.

One of the main technical barriers to using circulating tumor cells to detect or monitor solid tumors is the relative rarity of the cells in the bloodstream. Tests can detect one or two circulating tumor cells in relatively low volume blood samples¹⁷; however, some sort of sample enrichment is generally required to ensure adequate sensitivity.¹⁸ These enrichment techniques generally require a combination of physical and biological processes such as filtration based on physical size, electrical and immunomagnetic properties.¹⁸ Once isolated, cells can be expanded using in vitro culture techniques prior to analysis. Collecting intact, live target cells remains a challenge. Once collected, circulating tumor cell samples can be analyzed using RT-PCR, qRT-PCR, flow cytometry, immunofluorescence, and ELISA techniques. High throughput technologies are expected to help in various stages of processing.

Circulating tumor cells can be used for detecting DNA mutations, creating RNA expression profiles, performing epigenetic analysis and profiling proteins. Unfortunately, the biomarker relies on whole cells sloughed off by tumors, which generally occurs after the tumor is relatively mature (i.e. at a later stage of the disease). Therefore, this approach is not useful for early-stage detection and cancer screening.

Exosomes

Exosomes are membrane-bound particles, between 30 and 150 nm in size that are actively released by healthy and cancerous cells. Cells release hundreds of thousands of exosomes each day, such that 10^{12} exosomes can be collected from a single milliliter of blood.¹⁹

The contents of exosomes are highly concentrated, and contain various mixtures of nucleic acids, peptides/proteins and lipids among other molecules. Cells selectively sort and package the contents of exosomes based on the physiological status and requirements of the cell. Likewise, cancer cells can sort oncoproteins and other molecules into exosomes that could potentially be used to detect cancer.²⁰

Being membrane-bound particles, exosomes are relatively stable in bodily fluids. Moreover, exosomal membranes contain specific assortments of protein markers. Under normal conditions, these markers are important for cell-to-cell signaling, but can be exploited under assay conditions to identify exosome populations (e.g. exosomes released by precancerous or cancerous cells).^{21,22,23}

Circulating Free Nucleic Acids/Cell-free DNA

The term circulating free nucleic acids encompasses a variety of substances including circulating tumor DNA (ctDNA), cfDNA, methylated DNA, micro RNA (miRNA), messenger RNA (mRNA) and long non-coding RNA (lncRNA). Most cfDNA fragments measure between 140 and 200 base pairs, which is consistent with DNA released after cellular apoptosis.¹⁴ However, shorter and longer cfDNA fragments have been identified that correlate with certain cancer states.²⁴

ctDNA can be found in three-quarters of patients with various forms of solid tumors.²⁵ ctDNA is released into the bloodstream by apoptotic and necrotic tumor cells. Thus, it can be used in earlier-stage cancer detection than circulating tumor cells. The fraction of ctDNA as a portion of total circulating cfDNA may approach 50% in late-stage disease²⁶, but is a potentially measurable 1% in early-stage disease.²⁴ Perhaps more importantly for near-term clinical application, ctDNA can detect cancer relapse early.^{27,28} Unlike with circulating tumor cells, it is not possible to perform functional studies or protein profiling on ctDNA; however, DNA mutation analysis and epigenetic testing are possible.

Limitations of Liquid Biopsy

While the potential applications for liquid biopsy in cancer screening, detection, and treatment response are many, there are some limitations to the technology. While it is reasonable to assume that researchers in this field will be able to achieve adequate sample enrichment, specificity, and sensitivity, other limitations appear to be inherent to the process.

Liquid biopsy absolutely requires tumors to produce and release substances that can be collected from venipuncture. Small, early stage tumors may not produce enough usable material and thus frustrate even the most meticulous efforts to detect them. While many solid tumors develop a robust arterial blood supply, some do not, and venous/venule collection of blood may not be sufficient for detection through liquid biopsy. In addition, the blood brain barrier may thwart the use of liquid biopsy as a tool for checking and monitoring cancers of nervous system. It is conceivable technological advancements may overcome these limitations; however, it is likely that first-to-market clinical applications are those focused on tumor types that are not constricted by these seemingly inherent limitations.

Sample Quality is Critical Limitation in Liquid Biopsy

One of the lessons learned from the early days of liquid biopsy in clinical practice is that sample quality is critical for accurate results. This is perhaps best exemplified in liquid biopsy for cfDNA. Even under ideal conditions, the half-life of DNA fragment is under two hours.¹⁸ Total analyte is also quite small—a milliliter of plasma may contain only 10 ng of circulating DNA.²⁹ Cellular components must be completely removed from a sample without any cell lysis, which would contaminate samples with a relative ocean of nucleic acids.³⁰ In short, users must have a reliable way of collecting and extracting, stabilizing or rapidly analyzing cfDNA from fluid samples.

Some authors have shared their experiences with liquid biopsy handling, identifying certain tubes (PreAnalytiX PAXgene or Streck BCT) as superior over others (EDTA).³⁰ However, the field currently lacks an integrated, multicenter tested workflow that meets the needs of pre-analytical, analytical and post-analytical phase sample handling. Adjustments and specializations may be required for a particular biomarker of interest. In other words, optimal sample acquisition and handling will likely be far different for circulating tumor cells than it is for ctDNA, miRNA, exosomes, etc. Downstream readout technologies such as massively parallel sequencing for ctDNA will also need to be rigorously tested and optimized for clinical applications.³¹

The development and testing of these technologies for clinical applications is likely too large for a single academic or industry participant.³¹ This is perhaps why there only two sets of tests that have gained FDA approval so far. The CellSearch system (Menarini Silicon Biosystems, Inc.) is an FDA approved tool to quantify circulating tumor cells for three metastatic tumors. The Cobas EGFR Mutation Test v2 (Roche Molecular Systems, Inc.) is a ctDNA approach to detecting EGFR mutations.³¹ Nevertheless, scores of liquid biopsy products, from reagents and consumables to complete analytic systems and software packages are in various stages of development.

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