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Implementing liquid biopsies in clinical trials: State of affairs, opportunities and challenges

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Abstract

A primary goal of personalized medicine is to develop tumor specific biomarkers to aid in treatment selection and to better evaluate response to targeted therapies. The assessment of circulating blood markers as surrogate real time biopsies of disease status, termed 'liquid biopsies,' has been under investigation. There are many different types of liquid biopsies each with different functionalities and limitations. These include tumor markers, circulating tumor cells (CTCs), cell-free DNA, and extracellular vesicles including exosomes. Multiple clinical trials have evaluated liquid biopsies as prognostic biomarkers with positive results. Additional studies are underway to evaluate liquid biopsies as predictive biomarkers, pharmacodynamic biomarkers and surrogate efficacy endpoints for treatment response evaluation. There are several challenges and barriers in implementation of liquid biopsies into clinical trials and subsequently into routine clinical practice which will be addressed in this review.

Keywords

liquid biopsy; circulating tumor cells; exosomes; cell-free DNA; clinical trials

A challenge in solid tumor oncology is the lack of easily accessible tumor tissue for serial biopsies as metastatic sites may have discordant biological characteristics from the primary tumors.^{1–3} The assessment of circulating blood markers as surrogate real time biopsies of disease status, termed liquid biopsies, has gained enthusiasm in the last several decades. There are many different types of liquid biopsies each with different functionalities and limitations. These include tumor markers, circulating tumor cells (CTCs), cell-free DNA, and extracellular vesicles, particularly exosomes. In this review, we will highlight how various liquid biopsies are currently being utilized in clinical trials.

Circulating tumor cells (CTCs)

The best-studied and most well-established liquid biopsy approach is CTCs. CTCs are defined as nucleated cells expressing cytokeratin and lacking hematopoietic differentiation.

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However, there is no one accepted definition of a CTC and multiple assay types and enrichment procedures have been used. Newer approaches include the use of technologies, such as flow cytometry, in which CTCs are positively labeled with an antibody-fluorochrome conjugate, and molecular approaches, such as reverse transcriptase-polymerase chain reaction (RT-PCR). For all three of these detection approaches - IHC, flow cytometry, and RT-PCR - an enrichment step prior to the detection analysis is advisable given the rarity of CTCs within blood.⁴ Cell separation technology continues to evolve with advancement of technologies for characterizing rare CTCs including single cell analysis and genomic profiling.⁵

Extracellular vesicles (EV)

In addition to CTCs, there has been growing interest in liquid biopsy via analyses of extracellular vesicles (EV). EV are a diverse, heterogeneous group of nanoparticles that are defined by a lipid bilayer membrane and are released or possibly secreted by almost all cells. They are classified into three groups: exosomes, microvesicles, and apoptotic bodies, with corresponding sizes of 30 to 120 nm, 100 to 1,000 nm, and 100 to 5,000 nm, respectively. EVs can contain a variety of nucleic acids, metabolites, lipids, and proteins. EVs are believed to have roles in cell-cell communication, as well as the delivery of “cargo” from one cell to another. Such properties make EVs prime candidates for biomarkers for many disease states, not the least of which is cancer.^{6–8} A recent review of EVs as biomarkers lists over 20 different reports of potential cancer biomarkers associated with EVs, and over 25 different non-cancer disease biomarkers.⁹ A major challenge for EV research and implementation remains a lack of common methodology to detect and isolate EVs.

Cell-free DNA (cfDNA)

Another type of liquid biopsy is through quantification and sequencing of cell-free nucleic acids in the blood. Cell-free DNA (cfDNA) was first identified in 1947,¹⁰ and it has been known for over three decades that cancer patients have greater amounts of cfDNA in circulation relative to healthy individuals.¹¹ Cell-free DNA is thought to be derived from apoptotic and/or rapidly dividing cancer cells, not specifically from circulating tumor cells, although CTCs and cfDNA are both elevated in patients with advanced cancer.¹² Advances in next-generation sequencing technology along with improved computational approaches and rapid reduction in sequencing cost have facilitated a growing ability to obtain tumor sequences and profile tumors via cfDNA. Targeted panel sequencing assays of cfDNA are available in clinical practice in lieu of metastatic tumor biopsy sequencing, such as Guardant360 and FoundationACT.^{13,14} To date, most research approaches of cfDNA sequencing have focused on tracking specific mutations^{12,15–21} or sequencing targeted panels of cancer-related genes.^{14,22–25} These approaches offer high sensitivity to detect alterations via small amounts of tumor DNA but depend on knowledge of a tumors’ alterations or multiple mutations in frequently altered genes.

Prognostic biomarkers

Multiple clinical trials have evaluated liquid biopsies as prognostic biomarkers. The data is strong across multiple solid tumor types and with multiple assay types.^{26–30} Cristofanilli et al using the CellSearch assay demonstrated that elevated CTCs were strongly associated with worse clinical outcomes in metastatic breast cancer.²⁶ Multiple studies have reinforced that liquid biopsies can be prognostic in both metastatic and early stage cancers. Sparano et al most recently reported that elevated CTC counts 5 years post treatment completion in non-metastatic breast cancer correlated strongly with future disease recurrence.³¹ Whether treating these patients with additional therapy when they have no overt distant disease improves long term cure rates is not currently known but will be investigated in future trials. For cfDNA, genome-wide copy number analysis in cancer patients from plasma via shallow or low-coverage sequencing of cfDNA is scalable and cost-effective^{32–36} and has recently been shown to be highly prognostic.³⁶

Predictive biomarkers

Several studies have also raised the question whether liquid biopsies can serve as predictive biomarkers. Overall the validated use of any of the liquid biopsies as predictive biomarkers is not currently available for routine clinical use. The largest study was the S0500 trial in metastatic breast cancer in which patients were randomized to change their systemic therapy if their CTC numbers remained elevated after 1 cycle of chemo. This study demonstrated there was no benefit from early switch to a different treatment based on CTC numbers.³⁷ While this study dampened enthusiasm in CTCs as predictive biomarkers, the study leaders suggested that this group of patients may have very resistant disease and that CTC may help identify patients most likely to benefit from innovative clinical trials. This study also highlighted the need for further molecular characterization of CTCs in order to determine the best next systemic therapy option, suggesting that CTC enumeration alone may not be sufficient for clinical decision making. An illustration of this principal applies to prostate cancer studies where androgen receptor (AR)-V7 splice variants on CTCs have been shown to be associated with resistance to AR blocking therapy such as enzalutamide.³⁸ For this indication, CTCs may serve as predictive biomarkers for AR based therapy response pending additional validation.

Biological mechanisms and resistance pathways

Clinical trials are using liquid biopsies to better understand biological mechanisms and resistance pathways. Research in this realm is currently ongoing with all three types of liquid biopsies. CTCs offer the potential to interrogate proteins, including immune checkpoint molecules and other immunotherapy pathways. Several studies have demonstrated that PD-L1 is detectable on CTCs in patients with multiple cancer types including breast, bladder, and lung cancer,^{39–41} and the growing list of immunotherapy indications for PD-L1 positive tumors emphasizes the need to evaluate CTCs as a surrogate biomarker. Exome sequencing of cfDNA is feasible in patients with larger amounts of tumor-derived DNA in circulation.^{32,35,42,43} Blood can be collected serially and tumor evolution can be tracked through exome- or genome-level sequencing as patients are on

therapy, potentially identifying novel genomic mechanisms of sensitivity or resistance. Future trials may use findings from liquid biopsies as eligibility criteria or to “enrich” the study population for the target of interest. For example, the ongoing TAPUR study includes liquid biopsy findings as part of eligibility criteria and serial cfDNA as part of the response evaluation to targeted therapies and immunotherapies.⁴⁴

Pharmacodynamics

The utility of liquid biopsies as pharmacodynamic biomarker in early phase oncology trials has been explored. The ideal pharmacodynamic biomarker would provide evidence of the direct pharmacologic effect of a drug and can complement traditional pharmacokinetics.^{45–47} A number of studies have used CTC assays as a pharmacodynamic marker based on specific protein targets on CTCs including EGFR, HER2, VEGF, AR, IGF-R and others (Reviewed in reference⁴⁷). Additional studies have looked at DNA damage and apoptosis in response to chemotherapy, most helpful in the development of PARP inhibitors.^{48–50} However, none of these pharmacodynamic biomarkers have had full analytical validation. For example, although the CellSearch Assay has FDA approval for CTC enumeration, it is not approved for assessment of additional markers. Improving imaging capabilities in visualization of a variety of markers on the same rare cell in a consistent validated way is in development.⁵¹

Treatment Efficacy endpoints

One of the unmet needs in oncology clinical trials is whether a less expensive, less time consuming blood test can potentially replace cumbersome imaging studies such as bone scans and computed tomography. Aside from convenience, conventional imaging modalities can have significant limitations including difficulty evaluating bone predominant metastatic burden. For example, many breast and prostate cancers in the metastatic setting are primarily present in the bones. In prostate cancer, tumor marker PSA alone has not been sufficient as a sole treatment endpoint because it only partially reflects treatment effects.⁵² Efforts have focused on investigating the utility of liquid biopsies as surrogate efficacy endpoints for treatment response evaluation. In the phase II IMMC-38 trial, results of CTC numbers using the CellSearch Assay were more prognostic than PSA declines and were predictive of overall survival.²⁸ CTC enumeration was subsequently included in the phase III registration trial of abiraterone but was not the sole efficacy endpoint. The use of liquid biopsies as a surrogate efficacy endpoint is clearly not ready for prime time for most cancers but we anticipate that this area will be rapidly progressing in the coming decade.

Challenges and limitations

Despite the clinical and experimental promise of CTCs, exosomes, and cfDNA as liquid biopsies, like any new medical diagnostics, there are multifaceted challenges to bringing these new approaches to ongoing therapeutic trials and eventually to routine clinical practice. These challenges can be grouped into four broad challenges: 1) the reproducibility and sensitivity of each technology; 2) the effectiveness of the technology to determine a positive from negative/background signal in patient samples; 3) the utility of the biomarker

to an actionable clinical question, as a validated predictive biomarker, or to replace other currently used tests.

For liquid biopsy to be truly clinically applicable, the approach must be reproducible broadly and adequately sensitive to direct clinical decision-making (Challenge 1). For CTCs, one generally accepted criteria is to exclude cells of hematopoietic origin, typically through negative selection via antibody-fluorescent molecule conjugate targeting CD45 cell surface marker, an approach that depends both on antibody and technical approach. For example, Tong et al demonstrated that six different anti-CD45 clones, from seven different antibody manufactures, can produce significantly different results on the exact sample blood sample from a normal patient, with variability attributable to specific region of the CD45 molecule targeted by the antibody and whether other antibodies are already bound to the cell.⁵³ In addition, next-generation sequencing of analyzed patient samples, in two different, Clinical Laboratory Improvement Amendments (CLIA) –licensed, College of American Pathologies-accredited laboratories demonstrated striking discordance.⁵⁴ This raises questions about reproducibility for cfDNA analyses via different platforms and suggests some common set of standards must be enacted.

Determining positive results with adequate positive predictive value and above background is even more confounding (Challenge 2). For CTCs, Yang et al. used an anti-CD45 magnetic bead conjugate to remove cells of hematopoietic origin prior to CTC enumeration and demonstrated that cancer patient blood obtained during/right after surgery had significantly reduced hematopoietic cell removal.⁵⁵ This suggests that CTCs may not be applicable during all phases of treatment due to variation in background signal. Analyses of CTCs is also impacted by limitations of fluorescent labels on antibodies, including autofluorescence and photobleaching, which can impact the ability to distinguish a positive fluorescent signal from the background. For cfDNA and exosomes, the dynamics are less well-studied but it is clear that both are present in significantly greater amounts during systemic illness or infection, not necessarily from tumor origin, potentially impacting detection and analysis.⁵⁶

Perhaps the greatest challenge has been the application of these exciting technologies. The number of CTCs has been associated with prognosis for breast and other cancers for over a decade, yet utility in practice will depend on test results leading to a change in care that benefits patients. As highlighted above, SWOG S0500, failed to confirm that directing therapy based on CTC enumeration (by CellSearch technology) impacted survival. CTC enumeration, while prognostic, may not necessarily add substantially to known clinical biomarkers, such as poor response to therapy early on in their course and, as such, has not been widely implemented. For cfDNA, detectable mutations in circulation have been associated with therapeutic response, for example to aromatase inhibitor versus fulvestrant in advanced breast cancer,²⁰ but still requires prospective validation. Table 1 attempts to summarize some of these challenges.

Conclusions

In conclusion, liquid biopsy approaches including CTCs, cfDNA, and EVs, offer the potential to detect, monitor, and characterize cancer in diverse settings, from screening to

metastatic disease. While promising, several critical but not insurmountable barriers from bringing liquid biopsies to clinical practice remain. Importantly, standardization of technological approaches, sharing of methodologies, and guidelines for implementation will help move the entire field forward in the context of ongoing technological advances.⁵⁷ In the future, we anticipate the continued rapid growth of liquid biopsies and the expanded incorporation into various clinical trials that will address the unmet needs of current solid tumor treatment paradigms.

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Table 1

Technology	Advantages	Limitations
Circulating tumor cells (CTCs)	-multiple biomarker characterization on same cell -prognostic in multiple cancer types and stages	-no consistent definition of CTC -enrichment step needed due to rarity of cells
Cell Free DNA (cfDNA)	- prognostic in multiple cancer types and stages -does not rely on marker characterization on a given cell type	-origins of cfDNA unclear - which cells or organs are preferentially releasing -reproducibility of clinical assays
Exosomes	-may give important info about cell to cell communication -may hold insight to the biological mechanisms of metastatic disease	-clinical studies are limited