Current and Emerging Clinical Applications for Liquid Biopsies in the Management of Solid Tumors

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Overview
This activity is designed to inform physicians about recent and anticipated advances in the use of liquid biopsies to manage patients with solid tumors.

Target Audience
This activity is directed toward medical oncologists, primary care physicians, nurses, and nurse practitioners who treat and/or manage patients with solid tumors. Surgical oncologists, radiation oncologists, pathologists, internists, fellows, physician assistants, and other healthcare providers interested in the use of liquid biopsy in the management of solid tumors are also invited to participate.

Learning Objectives
After participating in this CME/CE activity, learners should be better prepared to:

- Characterize the clinical potential of liquid biopsy in the era of precision medicine.
- Describe the biologic and technologic advantages and limitations of liquid biopsy in the management of solid tumors.
- Discuss the processes associated with the collection and analysis of circulating tumor cells (CTCs) and cell-free circulating tumor DNA (ctDNA).
- Summarize the main findings from studies investigating the use of CTCs and ctDNA in patients with solid tumors.
- Explain the role of next-generation sequencing in the molecular characterization of ctDNA.
- Describe the current obstacles to the widespread implementation of liquid biopsy.

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Introduction

Tissue biopsy evaluated by a trained pathologist is the current standard of care for the diagnosis of cancer and the confirmation of metastatic disease.[1-4] In recent years, however, the use of liquid biopsy to analyze circulating tumor cells (CTCs) or cell-free circulating tumor DNA (ctDNA) in the blood of patients with solid tumors has attracted much attention.[5,6] CTCs are intact epithelial cells that have been released into the circulation from the primary tumor and/or metastatic sites,[5,7-9] whereas ctDNA consists of small fragments of nucleic acid that have been released into the circulation from apoptotic and necrotic tumor cells.[5,10] The isolation and characterization of CTCs and ctDNA offer the potential to overcome some of the limitations of tissue biopsy while providing the same information.[3]

In brief, liquid biopsy has the potential to detect early disease, predict prognosis, monitor tumor progression over time, and track treatment efficacy or resistance without exposing patients to the risks associated with invasive tissue sampling.[1,3-6,11-17] Liquid biopsy is also more cost-effective and better suited for serial sampling than tissue biopsy.[3] Furthermore, liquid biopsy contains CTCs or ctDNA that have been released into the circulation from numerous sites and thus may be more representative of tumor heterogeneity than tissue biopsy.[3] Nonetheless, despite these advantages, the widespread use of liquid biopsy has been hampered by technical and analytical challenges and the lack of standardization, quality control, and validation.[3]

The main technical challenges associated with CTC analysis include the enrichment of CTCs among millions of normal blood cells and the detection of CTCs in enriched cell fractions.[14,16,18] Notably, CTCs are extremely rare (the frequency may be as low as 1 CTC per 106 to 107 leukocytes),[7-9] and they spend only a brief time in the circulation (the estimated half-life is between 1.0 and 2.4 hours).[5,19] The analysis of CTCs, therefore, generally begins with enrichment, a process that depletes most other blood cells to facilitate the isolation of single CTCs or clusters of CTCs.[5,18] Enrichment is based on strategies that distinguish CTCs from surrounding hematopoietic cells according to biologic properties (membrane protein expression) or physical properties (size, shape, rigidity, surface charges, and density).[5,18,20,21] Strategies based on membrane protein expression use antibodies directed against either epithelial-associated proteins (positive selection) or antibodies directed against antigens expressed by white blood cells (negative selection).[22] Positive selection, the most commonly used CTC enrichment method, is performed using antibodies directed against the epithelial cell adhesion molecule (EpCAM).[22] Although several different enrichment methods have been developed, only the CellSearch system — a semiautomated platform that uses anti-EpCAM antibodies conjugated to ferrofluids to enrich CTCs — has been approved by the FDA.[6,12,20,22,23] After enrichment, CTCs can be detected using molecular, immunologic, or functional assays.[5,18,22]

The value of CTCs to predict survival has been well established in breast, prostate, and colorectal cancers.[12] In fact, an early study in patients with metastatic breast cancer led to FDA approval of the CellSearch system and increased interest in the use of CTCs as an independent prognostic factor for survival outcome.[8] In the study, which included 177 patients with metastatic breast cancer, those with ≥5 CTCs per 7.5 mL were shown to have shorter median progression-free survival (PFS) than those with <5 CTCs per 7.5 mL (2.7 and 7.0 months, respectively); median overall survival (OS) was also shorter in the former compared with the latter group (10.1 and >18 months, respectively).[24] Another study in metastatic breast cancer — this one a pooled analysis of data from 1944 patients — found that the presence of ≥5 CTCs per 7.5 mL versus <5 CTCs per 7.5 mL at baseline was associated with decreased PFS and OS (hazard ratios [HRs] of 1.92 and 2.79, respectively).[25] Furthermore, adding CTC count and its change during treatment to an optimized clinicopathologic model significantly increased the prognostication of metastatic breast cancer, whereas adding the serum tumor markers carcinoembryonic antigen and cancer antigen (CA 15-3) did not.[25,26] Data from a prospective trial analyzing CTCs in 2026 patients with early breast cancer before adjuvant chemotherapy and 1492 patients after chemotherapy showed that prognosis was poorest in those who had ≥5 CTCs per 30 mL of blood (HRs of 4.51 and 3.60 for disease-free survival and OS, respectively).[27] An assessment of 212 specimens evaluable for CTC count from men with metastatic castration-resistant prostate cancer enrolled in SWOG S0421, a phase 3 trial investigating docetaxel plus prednisone with or without atrasentan, showed that median OS was 26 months for those with <5 CTCs per 7.5 mL and 13 months for those with ≥5 CTCs per 7.5 mL at day 0.[28] In a meta-analysis of 13 studies investigating the relationship between CTCs and tumor response in patients with colorectal cancer who received chemotherapy, high vs. low levels of CTCs were associated with poor PFS and OS (HRs of 2.500 and 2.856, respectively).[29]

The predictive and prognostic value of CTCs is also being explored in numerous other cancers, including gastric,[30] esophageal,[31] lung,[7,9,18,32] pancreatic,[33] ovarian,[34,35] melanoma,[36] and head and neck.[37] Researchers are also investigating the potential value of CTCs as a source to analyze protein and RNA expression, DNA mutations, and drug sensitivity.[12] At present, many questions remain, including whether CTC models can capture the heterogeneity of metastatic disease and treatment response.[12]

Both nontumor cells and tumor cells release fragments of cell-free DNA into the plasma of the blood.[1,38] The detection of ctDNA is challenging, as a typical fragment is small (between 160 and 180 base pairs) and must be distinguished from fragments of cell-free nontumoral DNA.[12] Furthermore, the proportion of circulating DNA that is tumor in origin varies considerably from patient to patient and, in many cases, may represent. less than 1.0% of total cell-free DNA in circulation, and in some cases, less than 0.01%.[39,40] Studies have found that tumors consisting of about 50 million malignant cells release enough DNA to detect ctDNA; fragments of ctDNA contain genetic material that is identical to that of the primary tumor.[1] Although ctDNA was initially detected using Sanger sequencing — a method associated with poor sensitivity, labor-intense protocols, low throughput, and the potential for polymerase chain reaction (PCR)-related bias — a multitude of newer, highly sensitive techniques with superior specificity and assay performance have applications in this field. In particular, advances in next-generation sequencing technology have led to the development of comprehensive, multiplexed test that nevertheless retain

the required sensitivity for ctDNA analysis.[2,41] A recent case series analysis of EGFR C797S mutations from 12,086 patients with non-small cell lung cancer (NSCLC) (13,987 ctDNA samples) showed that one-half of the plasma-positive cases were at mutant allele frequencies of <0.5% on a 70-gene next-generation sequencing panel.[42]

Data suggest that ctDNA is a sensitive and specific biomarker that is widely applicable to patients with different tumor types.[10] For example, in a study in 640 patients with various early and late stage cancers, ctDNA was detected at relatively high concentrations in most patients with metastatic cancer, but at lower (yet detectable) concentrations in those with localized cancers.[10] Specifically, ctDNA was detectable in >75% of patients with advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancer, but in <50% of those with primary brain, renal, prostate, and thyroid cancers.[10] In patients with localized tumors, ctDNA was detected in 73%, 57%, 50%, and 48% of patients with colorectal cancer, gastroesophageal cancer, breast adenocarcinoma, and pancreatic cancer, respectively.[10] Notably, ctDNA was also frequently found in patients who did not have detectable CTCs.10 In another study — this one in 30 women with metastatic breast cancer who were receiving systemic therapy — ctDNA was found to be a more sensitive biomarker than CTCs and CA 15-3, with detection rates of 97%, 87%, and 78%, respectively.[43]

Much has been learned from studies investigating the use of ctDNA to monitor disease progression, treatment efficacy, and mechanisms of tumor resistance. In one study, detectable levels of ctDNA were found to be correlated with cancer stage, increasing from 47% in patients with stage 1 cancer of any type to 55%, 69%, and 82% in patients with stage 2, 3, and 4 cancers, respectively.[10] Another study showed that the amount of ctDNA detected in patients with metastatic colorectal cancer following surgery generally decreased and was correlated with the extent of surgical resection; furthermore, patients who had detectable ctDNA following surgery usually relapsed within 1 year.[40]

A study in patients with advanced lung adenocarcinoma who had detectable EGFR mutations in ctDNA at baseline found that failure to clear plasma EGFR mutations after treatment was an independent predictor of lower disease control rate, shorter PFS, and shorter OS.[44,45] In a study investigating the development of resistance to the anti-EGFR antibody panitumumab in patients with chemorefractory metastatic colorectal cancer, ctDNA analysis showed that more than one-third of patients with tumors that were initially KRAS wild-type developed detectable mutations; of interest, the time of mutation detection was usually very consistent, occurring between 5 and 6 months following treatment.[46] Data from a study in patients with gastric cancer showed that ctDNA levels of TP53 mutations generally decreased after surgery and increased after disease recurrence.[47] A study in patients with pancreatic cancer showed that plasma ctDNA levels >62 ng/mL were significantly correlated with shorter OS, the presence of vascular encasement, and metastasis.[48]

Several studies have also investigated the use of ctDNA to identify specific mutations in patients with various solid tumors. For example, data from three meta-analyses showed that ctDNA is a highly specific biomarker for the detection of EGFR mutation status in patients with NSCLC, with specificity rates of 88.5%, 93%, and 95.9%; however, the respective sensitivity rates were only 64.5%, 67%, and 62%.[49-51] Another study — this one in patients with metastatic colorectal cancer — reported that the ctDNA specificity and sensitivity rates for the detection of clinically relevant KRAS mutations were 99.2% and 87.2%, respectively.[10]

In summary, liquid biopsy holds promise as an alternative to tissue biopsy for the management of solid tumors,[1,3-6,11-17] yet issues pertaining to standardization, quality control, and validation need to be adequately addressed before this strategy can be routinely implemented in clinical practice.[3]

Philip C. Mack, PhD, Adjunct Professor in the Department of Internal Medicine at the University of California-Davis (Sacramento, CA) offered his insights on current and emerging applications for the use of liquid biopsy in patients with solid tumors.

Moderator: What is the clinical potential of liquid biopsies in the era of precision medicine?

Mack: There are several aspects of liquid biopsies that lend themselves well to our current concepts of personalized therapy (aka precision medicine). First, as a minimally invasive option for biomarker assessment, this technology provides an avenue for patients in whom a traditional biopsy cannot be safely conducted, as well as for patients whose traditional biopsy was insufficient in yield for molecular analysis. Second, liquid biopsies increase the options for serial monitoring of disease evolution over time, which is particularly important for identifying appropriate therapy at the time of progression.

Moderator: What information can be obtained from analyzing CTCs and ctDNA? For example, how do the two liquid biopsy approaches differ, that is, what are the technologic and biologic advantages and limitations of each? Are the two approaches considered to be complementary to each other or exclusive? Have analyses of CTCs and ctDNA been performed in parallel in the same patient population?

Mack: Analysis of cell-free DNA is focused on the identification of point mutations and small insertions and deletions in DNA. Advanced applications that employ next-generation sequencing technologies can also assess copy number abnormalities and chromosomal fusion events. Several lines of investigation are also evaluating cell-free RNA as a surrogate for tumor RNA expression abnormalities. Now, in contrast, much more information can be obtained from CTCs, including analysis of protein expression levels and posttranslational modification, such as phosphorylation states and cellular localization; however, this comes somewhat at the expense of accurate quantification and a more complicated isolation process. Often, however, CTC analysis is used simply to enumerate CTCs, providing prognostic information. The two technologies can certainly be used in a complementary fashion, and some companies offer this service.

Moderator: Would you discuss the process of CTC isolation, enumeration, and molecular characterization?

Mack: Living intact tumor cells in circulation are very rare, outnumbered by five or six orders of magnitude by the white blood cell population. Most

established methodologies attempt to discern the CTCs using cell surface markers that identify them as epithelial cells of origin, including EpCAM and various cytokeratins. Further characterization is required for validation, including demonstrating that they are not hematopoietic in origin, usually by staining for CD45 and looking for CD45 negativity. Many technologies physically separate epithelial-positive cells and present them in a gallery for pathologic review; however, some newer technologies and platforms skip the actual isolation step and rely on highly advanced imaging.

Moderator: Early work by Cristofanilli and colleagues in metastatic breast cancer set the stage for the clinical application of CTCs in many tumor types. Would you describe their findings and provide examples of subsequent studies investigating the use of CTCs in other common solid tumors?
Mack: These pioneering works used the CellSearch system, a technology that provided a means for the precise definition and enumeration of CTCs in a standard tube of blood. The number of CTCs in a given quantity of blood was accurately documented, providing prognostic information regarding patient survival — initially for patients with metastatic breast cancer. The clinical application of CTCs has now been extended to many other cancer populations. The current goals of CTC analysis focus not only on counting cells, but also on molecular characterization in ways that can provide predictive information about likely responses to targeted therapies. Other areas of exploration include methods to capture CTCs that do not express typical epithelial markers, such as stem cell populations and cells undergoing epithelial mesenchymal transition.

Moderator: How is ctDNA collected and analyzed?
Mack: Cell-free DNA can be isolated directly from the plasma by DNA extraction. Tumor cells shed fragments of DNA into the circulation as they grow and die. Non-tumor cells also release DNA, so the challenge is to investigate the fraction of DNA in circulation that is tumor in origin. Many biological processes are involved, but in general, the more aggressive the disease, the more tumor DNA that will be in circulation.

Moderator: In your 2015 editorial in the *Journal of Thoracic Oncology*, you discuss the use of ctDNA to monitor disease progression in NSCLC and pose the following question: "What can we learn from a tube of blood?" Would you share the answer with us?
Mack: The answer is that, in many cases, we can learn a surprising amount from a tube of blood. Several research groups and commercial entities have developed exquisitely sensitive next-generation sequencing panels that provide a profile of biomarkers that can be used for treatment decision making. Any tumor-associated mutation that predicts for success with a targeted therapy can be identified in the large majority of patients with stage 4 disease using a liquid biopsy approach. Furthermore, because a blood draw is a routine, easy-to-perform procedure, we could use this technology to track the course of the disease — for example, by identifying emerging resistance mechanisms. This is particularly important in NSCLC, a tumor type in which drugs are now available to overcome many of the known resistance mechanisms that cause patients to relapse on targeted therapies. Additionally, liquid biopsies can provide a global picture of acquired mutations occurring not only at the site of tissue biopsy, but also at multiple metastatic lesions, thereby addressing — to some extent — the issue of tumor heterogeneity.

Moderator: In addition to NSCLC, which other cancers are especially well-suited to investigate the use of the ctDNA approach?
Mack: In general, the ctDNA approach can be investigated in any tumor type in which targeted therapies are assigned to patients based on the presence of DNA mutations. Tumor types in which emergent resistance mechanisms are treatable with next-generation therapeutic regimens (eg, prostate cancer, breast cancer, colorectal cancer, gastrointestinal stromal tumor, and others) are of particular interest; however, adenocarcinoma of the lung remains the flagship for this approach.

Moderator: What is the role of next-generation sequencing in the molecular characterization of ctDNA?
Mack: Most available tests are hot-spot mutation tests, meaning that they are designed to identify very specific mutations often using a PCR-based strategy. Next-generation sequencing brings a whole new dimension to this type of analysis. It is now possible to identify not only well-characterized major mutations, but also rare mutations that are often skipped by hot-spot analysis. Furthermore, using next-generation sequencing, it is possible to determine whether tumor-suppressor genes are compromised. These are not analyzable using hot-spot tracking because deleterious mutations could be located anywhere throughout the coding region, so a sequencing approach is required. Next-generation sequencing can also provide an accurate assessment of gene copy number abnormalities and identify the presence of fusion events. The ability to have such multiplexed information available from a single sample in a single assay has really propelled the field forward.

Moderator: What are the current challenges to meeting validation thresholds for ctDNA sensitivity, specificity, and reproducibility?
Mack: These are challenges that exist in all clinical tests, and it's essential for all applications of liquid biopsies that the appropriate levels of validation have been conducted. Analysis of circulating tumor DNA significantly magnifies these challenges because it is extremely limited both in quantity and as a percentage of the total DNA in circulation. It is typical for tumor DNA to represent substantially less than 1% of the total DNA in circulation. With a test that requires that level of sensitivity, it is important to be extremely careful that specificity is not compromised — false-positive results could derail the whole field. It is therefore important to use a service that provides as close to 100% specificity as is possible.

Moderator: What are the advantages and limitations of molecular analyses performed on blood samples versus tissue samples?
Mack: : The advantages of blood samples are very straightforward: they are easy to procure and can be collected serially and at key points in a patient's disease course without the significant safety concerns associated with traditional tissue biopsies. Additionally, they can provide information

on mutations associated with metastatic lesions and invasive areas of the primary tumor that cannot be matched by a single tissue biopsy. The main disadvantage is that not all tumors shed DNA at levels sufficient for detection in plasma, so the presence of mutations will be missed in a percentage of patients if only plasma is used.

Moderator: Would you summarize (insofar as is possible) what has thus far been learned about tumor biology and metastasis from the use of liquid biopsies?

Mack: To the degree that liquid biopsies represent a tool that can allow us to assess the dynamic evolution of a real cancer in an actual patient over time, they will be very important sources of information to move toward the goal of managing cancer as a chronic disease. No matter how clever a therapy may be, most metastatic cancers will evolve resistance to it. Being able to address the emergent resistant mechanisms in real time provides hope that we can exert some long-term control over diseases that are otherwise incurable.

Moderator: What do you see as the main emerging applications for liquid biopsies?

Mack: Currently, the greatest utility of liquid biopsies is in the identification of mutations associated with emergent resistance to previously successful targeted therapy.

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