Circulating Tumor DNA: Measurement and Clinical Utility

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Abstract
Circulating tumor DNA (ctDNA) is a component of the “naked” DNA found in blood. It can be isolated from plasma and represents combined genetic material from the primary tumor and metastases. Quantitative and qualitative information about a cancer, including mutations, can be derived using digital polymerase chain reaction and other technologies. This “liquid biopsy” is quicker and more easily repeated than tissue biopsy, yields real-time information about the cancer, and may suggest therapeutic options. All stages of cancer therapy have the ability to benefit from ctDNA, starting with screening for cancer before it is clinically apparent. During treatment of metastatic disease, it is useful to predict response and monitor disease progression. Currently, ctDNA is used in the clinic to select patients who may benefit from epidermal growth factor receptor–targeted therapy in non–small cell lung cancer. In the future, ctDNA technology promises useful applications in every part of clinical oncology care.
INTRODUCTION

The phenomenon of circulating cell-free DNA (ccfDNA) was described by Mandel & Métais in 1948 (1), but it would take until 1966 for changes in circulating DNA levels to be correlated with human disease in studies of systemic lupus erythematosis patients (2). This was the same year that Payton Rous won his Nobel Prize for work surrounding the discovery of Rous Sarcoma Virus. From a modern perspective, his work bolstered the evidence that cancer is a genetic disease, suggesting the power of ccfDNA to detect and monitor cancer. It took time for this hypothesis to be widely accepted. The first citation in Rous’s Nobel acceptance speech is a letter that he published in Nature expressing concern that the “surmise on surmise” of the somatic mutation theory of cancer is confusing the field (3). In 1977, Leon et al. (4) reported that many cancer patients had elevated ccfDNA. The quantity of ccfDNA correlated with burden of disease suggested that at least some of this DNA is tumor derived. The proof would require developing more advanced molecular biology techniques. Once those were in place, scientists could finally turn their attention to the content of circulating tumor DNA (ctDNA).

These efforts saw clinical application in 2016 with the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) approvals of the first “liquid biopsy” to identify patients who will benefit from epidermal growth factor receptor (EGFR)–targeted therapy in non–small cell lung cancer (NSCLC) when a tissue biopsy is unavailable (5, 6). Other commercial operations provide the results of ctDNA cancer mutation panels to clinicians, but studies confirming their validity or place in clinical practice are ongoing. This review focuses on ctDNA measurement in patients with solid tumors, along with current and future clinical applications.

Circulating genetic material is made up of exosomes, tumor educated platelets, circulating tumor cells, microRNA, and ccfDNA (7, 8). A variety of biological fluids contain ccfDNA, such as blood, lymph, urine, saliva, and cerebrospinal fluid. Our focus is on the distinct entity of ccfDNA in blood. The content of ccfDNA is predominately short, double-stranded fragments of nuclear and mitochondrial DNA. Their median length is approximately 160 to 180 base pairs (9), but fragments up to 10,000 base pairs have been recovered (10, 11). The primary mechanism of DNA release is still contested, but it has been observed to occur as a result of apoptosis, necrosis, and viable tumor cell secretion (12–14). Significantly, the commonly observed length of 166 base pairs matches the size of the DNA fragment associated with the nucleosome and is therefore consistent with an apoptotic mechanism. Most studies have shown that ctDNA is shorter than ccfDNA derived from noncancer cells (15–17). Levels of ctDNA can be influenced by increased production as well as circulating nucleases and decreased elimination in the kidney, liver, and spleen. The half-life of ccfDNA ranges from about 15 minutes to several hours, potentially allowing the assessment of real-time information on cancer (11, 18). The physical properties of ctDNA have informed processing methods.

PROCESSING CIRCULATING TUMOR DNA

An optimized technique for acquiring and preparing samples is critical to supply high-quality specimens for analysis. Before this was recognized, a variety of protocols were in use that may have resulted in suboptimal specimens. As a result, the literature on ctDNA must be interpreted cautiously. These issues have been expertly reviewed elsewhere (19–21), but a brief summary is useful.

Collection of ccfDNA is most often performed by phlebotomy and may consist of plasma or serum preparations depending on whether an anticoagulant is added or not. Serum samples generally yield more ccfDNA, but the additional material is derived from leukocyte lysis during clotting, which dilutes the ctDNA content (22). Consequently, plasma tumor DNA (ptDNA) is the preferred analyte for the majority of recently published studies. Additionally, freezing of
blood samples, heavy hemolysis of specimens, or delays greater than 1–2 h prior to centrifugation of plasma also have the effect of increasing ccfDNA through similar mechanisms of lymphocyte hemolysis. A variety of stabilizers are now in use to extend the available blood processing window from 1–2 h to several days (23), thus allowing multiple independent sites to recruit for central processing. Heparin is generally avoided as an anticoagulant as it has been observed to interfere with subsequent polymerase chain reaction (PCR) processing. Ethylenediaminetetraacetic acid (EDTA) or acid citrate dextrose (ACD) appear to have similar efficacy with regard to subsequent analysis. In general, double-spinning the blood is the preferred method to remove the maximum number of contaminating white blood cells from the plasma preparation. Although single-spin plasma samples can be used (24), the quantification of allelic fractions in such samples may be skewed owing to residual lymphocytes in the specimen. Studies conducted at multiple centers need to carefully standardize procedures for optimal sample quality. In a study of processing variability, some labs were unable to obtain ccfDNA from validated specimens (25).

Techniques of processing plasma to extract ptDNA include affinity column, magnetic bead, polymer, and phenol-chloroform methods. These methods vary in their ability to purify fragments of different sizes. Consequently, they may change the total quantity of ccfDNA isolated and skew the fraction of ctDNA (26, 27).

Many analyses have been performed in investigators’ labs where ctDNA may not be the only type of specimen processed. Because many analyses are sensitive enough to find low allelic frequency changes, a workflow must be implemented to protect patient samples from extraneous DNA contamination.

ANALYZING CIRCULATING TUMOR DNA

The techniques for analyzing ctDNA have become increasingly sensitive and specific. Early methods focused on total levels of ccfDNA. As next-generation sequencing techniques were developed, they were adapted to interrogate the content of ctDNA and improve sensitivity and specificity.

Initially, the available tools allowed only gross assessment of ccfDNA. Absolute normal ranges were difficult to define, with some estimates obtained in the same lab by the same technique varying by more than an order of magnitude (28, 29). However, Zanetti-Dallenbach et al. (30) demonstrated that within one analysis, ccfDNA can differentiate between benign and malignant breast disease. Others reported similar ability to discriminate, but ccfDNA did not correlate with the extent of disease (31, 32). As the techniques evolved to specifically look at the fraction of ccfDNA that is ctDNA, these less specific analyses declined. There are, however, ongoing attempts to jointly analyze total ccfDNA and specific ctDNA changes in a single platform (NCT02934360).

Molecular tools have been developed to analyze ctDNA by exploiting physical properties and content. Shorter fragment length and DNA methylation patterns can be used to discriminate between ctDNA and ccfDNA. Cancer-associated mutations (drivers and passengers), copy-number variations, and microsatellite alterations also have been used. Others have reviewed the variety of analyses in some detail (33). Here, we primarily discuss methods of detecting cancer-associated mutations. The percentage of ccfDNA that is ctDNA varies widely (between 0.01% and 90%), with the median at a few percent (11). Making detection even more difficult, the nature of cancer can give rise to subclonal mutations. Therefore, techniques are required that can consistently detect cancer-related molecular abnormalities with frequencies below 1%. Technology platforms have been developed that vary from genome-wide analysis to interrogation of single loci. The limit of detection for these platforms is quite variable. Genome-wide techniques generally work in the range of 5–10% allelic frequencies, whereas targeted sequencing and single-locus analysis are more sensitive, with a lower limit of a few hundredths or thousandths of a percent when optimized.
One of the early techniques that allowed semiautomated high-throughput analysis of individual loci was developed by Vogelstein, Kinzler and colleagues (12). It was named BEAM for the components involved in the technique: Beads, Emulsion, Amplification, and Magnetics (12, 34). This was an extrapolation of bulk PCR reactions where small cancer mutation signals are easily swamped by the normal signal from non-ctDNA. Amplification reactions are broken into “digital” reactions, where dilution and encapsulation in oil enable individual templates to be partitioned (35). These individual reactions are then read by flow cytometry, giving a digital readout. In several analyses of advanced colorectal cancer patients, the sensitivity of BEAMing to detect adenomatous polyposis coli variants approached 100% (11, 12). The next generation of digital techniques [COLD-PCR (36), SCODA (37), and NaME-PrO (38)] used preamplification processing to increase the detection of mutant alleles. This type of analysis was recently approved for diagnostic use in the United States and Europe as the Cobas and Therascreen EGFR assays.

Although these allele-based techniques improved the sensitivity of the assays, they are limited to a panel of hotspots that may not capture the diversity of mutations associated with individual cancers. Larger loci representing several kilobases to megabases can be analyzed using amplification [TAm-Seq (39) and Safe-SeqS (40)] and hybrid capture [Exome sequencing (41), CAPP-Seq (42), and digital sequencing (43)] techniques. The complexity of the processes involved in cancer progression and evolution of resistance calls for the greater power of these techniques to detect a variety of changes. Safe-SeqS makes use of unique identifiers or barcodes that are tagged to each DNA molecule prior to amplification to identify true variants and exclude the errors introduced in PCR amplification. This technique demonstrated >90% concordance between ctDNA and tissue biopsy (44). The improved sensitivity allowed 15 mutations to be assessed during therapy and response correlated with progression-free survival (PFS). Newer iterations of barcoding techniques include Duplex Sequencing (45) as well as bisulfite conversion and barcode sequencing (46) to improve the ability to distinguish true mutations from PCR and sequencing artifacts.

The natural progression of these technologies is to increase the coverage of the analysis to whole-exome or whole-genome scales. Several techniques have been adapted to whole-genome sequencing and detection of cancer rearrangements [Plasma-Seq (47) and PARE (48), respectively] and amplifications [FAST-SeqS (49) and mFAST-SeqS (50)]. PCR has the power to detect very few copies in a sample, especially when the template is unique. This advantage has been exploited in the clinical diagnostics for chronic myelogenous leukemia where the translocation t(9;21) (q34;q11.2) is pathognomonic for the disease. Because this rearrangement does not exist in normal cells, the background is essentially zero. However, most cancer types do not have conserved translocations. Personalized analysis of rearranged ends (PARE) uses massively parallel sequencing to identify unique rearrangements. In one study, six solid tumors were analyzed, and between four and 15 rearrangements were identified (48). Primer pairs created to detect these rearrangements were exquisitely sensitive to detect ptDNA in a background of ccfDNA. Although these techniques continue to be refined, many are being applied clinically.

ADVANTAGES AND CAVEATS

Cancer is recognized as a genetic disease, and it would therefore be difficult to overestimate the value of access to tumor-derived genetic material at all stages of clinical cancer care. Sensitive techniques could detect cancer before clinical presentation. Cancer diagnosis and treatment decisions surrounding adjuvant therapy, surveillance, recurrence, and progression could be made with more certainty. Both patients and doctors would have timelier access to information regarding response and prognosis.
In current practice, screening tests for cancer (mammography, colonoscopy, low-dose computed tomography, and Pap smear) are limited to the most prevalent diseases, and still their clinical impact is far from optimal (51). Cancer-derived antigens were explored to improve cancer screening. However, common examples, including beta human chorionic gonadotropin (bHCG), alpha fetoprotein (aFP), prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), CA 19-9, CA 15-3, and CA-125, suffer from poor sensitivity and specificity. PSA is an example of a protein biomarker whose primary effects may be increased biopsy rate and overdiagnosis rather than reduced mortality. Because genetic alterations define cancer, detection of ctDNA could enhance or replace other techniques for cancer screening and expand the list of cancers for which screening is indicated. Early attempts at this application suggested that caution is required, as individual cancer mutations can be found in healthy patients at low levels (52, 53). Commercial entities have developed liquid biopsy platforms that are intended as cancer screening tests for high-risk individuals. Several clinical trials are ongoing that intend to enroll several thousand healthy individuals to monitor ccfDNA and the rate of any malignancy for up to five years (NCT02612350 and NCT02889978). One of these trials (NCT02889978) aims to accrue an additional 7,000 patients with a new diagnosis of a solid tumor. Together, these investigations should begin to clarify whether there is a role for ctDNA in cancer screening.

Tissue biopsies are the current standard for molecular analyses. However, analysis of ctDNA has many benefits over tissue biopsy. Blood is readily available, and phlebotomy is less invasive and carries a much lower complication rate than tissue biopsy. Because ctDNA represents an average of the whole burden of disease, it may better capture the heterogeneity within and between tumors. De Mattos-Arruda et al. (54) performed ctDNA analysis in a patient with hormone receptor–positive, HER2-negative lobular breast cancer in whom they also simultaneously biopsied the primary tumor and metastases. The biopsies individually contained private mutations, but ctDNA analysis detected all of the mutations present in the biopsies. Further, tissue samples are occasionally insufficient to complete more than a limited number of tests, and the pressure on this resource is increasing. Routine molecular testing for NSCLC now includes EGFR mutation, proto-oncogene ROS1, anaplastic lymphoma kinase (ALK), and programed cell death protein ligand 1 (PD-L1). Diagnosis and staging are often combined, with endoscopic fine-needle aspiration of mediastinal or paratracheal lymph nodes that may yield limited tissue. Currently, this is the niche that the Cobas v2 fills in NSCLC. The concordance rate between tissue and ctDNA is ~76%, meaning that about a quarter of patients with an actionable mutation would be missed in the assay. This suggests that a positive outcome is more useful than a negative outcome for the test. Wu and colleagues (55) performed a post hoc analysis of ctDNA from the LUX-Lung 3 and 6 trials using Therascreen EGFR. More EGFR mutations were detected in ptDNA (61%) preparations than in serum tumor DNA (29%), and although both EGFR-mutation-positive and -negative patients had better PFS with atafinib than with a platinum doublet, there was a trend toward an overall survival benefit for mutation-positive patients. Prospective trials will be needed to confirm this result. Collecting ctDNA also avoids pathology workflows that require paraffin embedding of many specimens in the same reagents, where the phenomenon of inadvertent specimen transfer has been observed. A final disadvantage of tissue biopsy relative to ctDNA analysis is that the process of formalin-fixation paraffin-embedding (FFPE) covalently modifies DNA and can interfere with downstream analyses.

Although early studies of ctDNA in high-volume disease confirmed a high sensitivity for ptDNA (12), many clinical trials have attempted to quantify the concordance between tissue biopsy and ctDNA mutations. As noted above, the current EGFR mutation assays have about a 76% concurrence. Post hoc analyses from clinical trials support this assessment. Jenkins et al. (56) found a concordance of 61% in a T790M biopsy-positive cohort from the AURA extension
and AURA phase II studies. Using the same platform, Mok et al. (57) found a concordance of 88% from FASTACT-2 samples. Individual studies have raised provocative hypotheses. For example, one study suggested that ptDNA KRAS mutation status was correlated with overall survival and tissue biopsy mutation status was not (58), suggesting that a differential sensitivity of detection may help discriminate clinically relevant features. However, in a phase I trial expanding these analyses to 50 genes in advanced or metastatic solid tumors, the concordance between biopsies and ccfDNA fell to 55% (59). In some circumstances, only archival tissue is available. Tabernero and colleagues (60) demonstrated that the ctDNA may reveal mutations that were not seen in an archived biopsy as a result of either heterogeneity or de novo mutations. Collectively, these data suggest that actionable mutations detected in ctDNA may be helpful, but their absence does not yet reliably exclude a positive tissue biopsy or clinical benefit.

In many cancers, chemotherapy and radiation are given before and after surgery to extend disease-free survival or augment cure rates. As the result of screening, breast cancer is often detected and treated early. However, some patients have a better result with a smaller surgical field and neoadjuvant therapy is recommended. Because all surgical specimens go for pathology review, this is an opportunity to correlate ctDNA signature with pathological complete response. One such clinical trial (NCT02743910) looks to correlate the absence of ctDNA in HER2- and triple-negative breast cancer with pathological complete response. Similarly, after surgery, some patients benefit from adjuvant therapy, but current selection algorithms lead to overtreatment. Assays for minimal residual disease could help to guide adjuvant therapy. Early work has focused on the relative PFS in patients who have detectable or undetectable KRAS mutations in ctDNA (11). However, longer follow-up is likely needed to establish the validity and benefit of these assays.

Tumor antigens are regularly used as a means to assess progression of disease or tissue of origin if imaging or biopsy is equivocal. Again, their poor sensitivity and specificity make independent interpretation of antigen tests difficult. Further, the prolonged half-life of these markers makes quantitative changes difficult to detect on short time scales. Dawson et al. (61) demonstrated the ability to follow treatment response using ctDNA in 29 of 52 enrolled breast cancer patients. As compared with CA 15-3, ctDNA had a greater dynamic range that correlated with tumor burden. By using imaging, Frenel and colleagues (62) correlated resistance mutations with recurrence for patients on therapy targeting the mammalian target of rapamycin (mTOR) and mitogen activated protein kinase kinase (MEK) pathways. The dynamics of various mutations suggested that there may be different clones that respond differently under selective pressure. Several groups have even used ctDNA to document specific mutations in targeted pathways, which could lead to understanding and targeting mechanisms of resistance (63, 64). Radiologic techniques are also widely used to assess progression. In many cancer types, this assessment uses techniques such as computed tomography, which produce ionizing radiation and lead to a measurable increase in second malignancies. Assessing response and progression by ctDNA could reduce the need for this testing.

Advanced solid tumors are almost uniformly refractory to chemotherapy in relapse. This is thought to be the result of tumor adaptation as well as the selection of preexisting drug-resistant clones. For example, hormone-receptor-positive breast cancer is known to develop resistance to endocrine therapies by the emergence of hotspot ligand binding domain mutations in the estrogen receptor (65, 66). These mutations can also be detected in ctDNA (66–68). Chu et al. (67) also showed that ctDNA contained not only the mutations found in corresponding tissue biopsies but also mutations not captured by tissue biopsy. These mutations were found in different allelic frequencies, suggesting that multiple private somatic mutations may coexist within or between tumors. Detecting these mutations may be clinically important, as their presence predicts PFS as well as response to certain therapies in retrospective studies (69, 70).
Another important consideration for the metastatic patient is timeliness for treatment recommendation. In some settings, cancer can progress quickly, and declining performance status can limit therapeutic options. Parsons and colleagues (71) attempted to use tissue mutation analysis to recommend therapy within 28 days for triple-negative breast cancer patients. The study closed early when parameters for futility were met. Notably, mutational results could not be returned from tissue biopsy within the specified time frame in nearly half of the first 20 enrolled patients. However, 92% of these patients had mutations detected using ctDNA. This suggested that ctDNA may be a better strategy for recommending therapy for metastatic patients in a timely fashion, and many of these patients had subsequent ctDNA analysis to track disease progression and response to therapies. In support of this approach, Lebofsky et al. (72) found 97% concordance between mutations identified by metastatic biopsy and by ctDNA. Earlier detection of relapse may also improve outcomes where therapies have better efficacy in settings of a low burden of disease.

**CURRENT CLINICAL TRIALS**

The registry ClinicalTrials.gov (<http://www.clinicaltrials.gov>) reveals a number of actively recruiting studies in which ctDNA analyses are the primary outcome measure (Table 1). Many of these studies continue to explore concordance between ctDNA and tissue samples. Others are attempting to use ctDNA to screen high-risk populations or to predict mechanisms of therapeutic resistance. One clinical study (NCT03079011) from France is working to determine if ctDNA can be used directly in clinical decision making. As noted previously, ESR1 mutations arise after hormone therapy and portend a worse response; this study asks if patients with ESR1 mutations as measured by ctDNA would benefit from early discontinuation of an aromatase inhibitor with palbociclib and initiation of fulvestrant with palbociclib. The hope of such studies is that earlier intervention may increase PFS and perhaps overall survival. Additional, prospective trials with interventions based on qualitative and quantitative changes in ctDNA are required if we are to prove or disprove the clinical utility of ctDNA.

**CONCLUSION**

Clearly, the technology to detect ctDNA and the mutations therein has matured, and these data are increasingly in the hands of clinicians and patients to help guide clinical decisions. Understanding the biology of ctDNA has made it possible to possess a detailed genetic picture of an individual cancer in nearly real time. It is likely that ctDNA may even capture the heterogeneous nature of cancer better than a tissue biopsy. Advancements in understanding the collection and processing of ctDNA have led to more uniform practices, making analysis scalable for clinical trials. The correlative science of ccfDNA levels has given way to much deeper analyses. The resulting information may guide binary decisions, such as starting an individual targeted therapy, or may contain multiple simultaneous assessments that could be useful for patient care. Newer, sophisticated techniques allow accurate detection of cancers before they are clinically detectable, which will be necessary if we are to develop ctDNA-based screening tests. At each clinical decision point, ctDNA could guide therapy choices, allowing individualized care that minimizes both over- and undertreatment. Increasingly, all of these possibilities seem attainable.

In another sense, there is still much to learn. Most of our knowledge of ctDNA comes from post hoc analyses of studies designed for other therapeutic or prognostic endpoints. These have been extremely valuable in generating correlation and hypotheses regarding the utility of ctDNA. However, similar to oncology drug development and approval, large prospective trials will be needed before ctDNA will have an independent and routine role in the clinic. For the current
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Assembled from actively recruiting protocols on [http://clinicaltrials.gov](http://clinicaltrials.gov) using the search terms “ctDNA,” “circulating tumor DNA,” “ptDNA,” and “plasma tumor DNA.” Current as of April 2017. Abbreviations: AI, aromatase inhibitor; BC, breast cancer; ccfDNA, circulating cell-free DNA; CEA, carcinoembryonic antigen; ctDNA, circulating tumor DNA; CRC, colorectal cancer; DFS, disease-free survival; EGFR, epidermal growth factor receptor; GIST, gastrointestinal stromal tumor; MRI, magnetic resonance imaging; NSCLC, non–small cell lung cancer; pCR, pathological complete response; OS, overall survival; PFS, progression-free survival; PSA, prostate-specific antigen; TKI, tyrosine kinase inhibitor; UM, uveal melanoma.

...time, it remains an adjunct to tissue biopsy, especially when there is demonstration of an actionable mutation. In the interim, the technology to detect ctDNA and applications to clinical care continue to advance. There are now several commercial entities that provide genomic mutational data to physicians from patient samples, including ctDNA. The responsibility then falls to the clinician to interpret and apply the information. Often the relevance of the information obtained is unclear. As more clinicians begin using these services, more clinical questions will arise. It is hoped that answering these questions through further research and validation will lead to improved therapies and outcomes for cancer patients.

DISCLOSURE STATEMENT

B.H.P. is a member of the scientific advisory boards of Jackson Laboratories, Horizon Discovery, Ltd., and Loxo Oncology; has ownership interest in Loxo Oncology; and has research contracts with Genomic Health, Inc. and Foundation Medicine, Inc. Under separate licensing agreements between Horizon Discovery, Ltd. and the Johns Hopkins University, B.H.P. is entitled to a share of royalties received by the university on sales of products. The terms of this arrangement are being managed by the Johns Hopkins University, in accordance with its conflict of interest policies. J.D. is entitled to a share of royalties received by City of Hope on licensing inventions.

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