Circulating Tumor DNA in Early-Stage Breast Cancer: New Directions and Potential Clinical Applications

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Abstract: The use of circulating tumor DNA (ctDNA) in liquid biopsy as a biomarker is becoming the new paradigm for the screening and surveillance of breast and many other cancers. Liquid biopsies provide prognostic and predictive information without the limitations of tissue biopsies. Most early studies of the use of ctDNA focused on metastatic disease. However, recent advancements in ctDNA technologies have improved sensitivity and selectivity, allowing ctDNA to be detected in early-stage disease, including early-stage breast cancer. Despite a clear potential for utility, the implementation of ctDNA liquid biopsy in standard of care is significantly lacking. Researchers and clinicians are currently working to validate the clinical utility of ctDNA in diagnostics, prognostics, the surveillance of minimal residual disease, and the monitoring of therapeutic response. This review summarizes the current applications of ctDNA in early-stage breast cancer and discusses its potential uses in clinical practice.

Introduction

Breast cancer is the leading cause of cancer death in women worldwide.[1] Despite advancements in the treatment of breast cancer, a significant clinical obstacle is the lack of a reliable method to detect disease early and monitor response to therapy effectively. The 5-year survival rate is 99% for patients given a diagnosis of localized breast cancer with no nodal involvement vs 27% for those with distant metastatic disease.[1] However, despite recent progress in genomic detection and diagnostics, the methods currently in use for the early detection of breast cancer have remained the same for more than 20 years. Standard methods of detection and diagnosis are invasive, expensive, and not always accurate. Furthermore, because these methods are difficult to implement, rates of breast cancer mortality are higher in rural areas and developing countries.[2]

The current standard of care in screening is mammography

annually for women between the ages of 50 and 74 years.[3] However, mammography-based screening can lead to both false-positive and false-negative results, the excessive use of biopsies, and unnecessary exposure to radiation.[4] Suspected malignancy in a lesion requires biopsy verification, and biopsy remains the gold standard for the retrieval of diagnostic, prognostic, and predictive information. To date, the standard practice for monitoring patients who undergo breast cancer treatment with curative intent is periodic clinical examination and mammography to detect physical evidence of recurrence. The limitations of this approach point to the need for better methods of early detection. Recent advances in molecular testing and genomics have led clinicians and doctors to revisit standard methods of early detection in an effort to improve accuracy and facilitate monitoring. Better techniques for the isolation and detection of circulating tumor DNA (ctDNA) have allowed clinicians and scientists to begin implementing liquid biopsy in standard clinical applications.

Liquid biopsy refers to the collection of bodily fluids, often blood, and the associated genetic materials (RNA, DNA, and/or cells). It represents an innovative tool in precision oncology.[5] Liquid biopsy offers several notable advantages in comparison with traditional tissue biopsy. First, it allows the detection of small tumors, minimal residual disease (MRD), and micrometastatic disease that cannot be detected with traditional biopsy.[6] Second, liquid biopsy detects ctDNA that has been released into the bloodstream from multiple tumor regions and makes it possible to identify intratumoral heterogeneity as well as clonal evolution.[7] Third, liquid biopsy can detect small quantitative variations within the blood, which enables real-time surveillance. Despite these advantages, liquid biopsy is not without flaws. Detection limits still exist, and testing is further complicated by hematologic genetic alterations—that is, clonal hematopoiesis—within the blood.[7] The low levels of ctDNA found in early-stage disease, along with the lack of ctDNA shedding from some tumors, can further complicate detection.

Liquid biopsy is not yet considered a standard of care, and more support for its clinical utility is needed before it can be brought into widespread use. In the past 5 years, research has focused on using liquid biopsy for screening, prognostication, treatment monitoring, and clinical decision making. It is important to note that most of the research on liquid biopsy has focused on the metastatic setting.[8-13] However, ctDNA technologies have rapidly advanced in recent years, making possible the sensitive and selective detection and the accurate quantification of tumor-associated DNA. These advances in turn have allowed the focus to shift from the metastatic setting to earlier-stage disease. Here, we discuss the clinical applications of ctDNA liquid biopsy in early-stage breast cancer.

Circulating Tumor DNA Technologies

It has been known for decades that all cells, both normal and cancerous, shed cell-free DNA (cfDNA) into the circulation.[14] The amount of cfDNA in the blood has been shown to be elevated in patients affected by cancer, stroke, trauma, or autoimmune disease.[15] In the early 1990s, allele-specific polymerase chain reaction (PCR) was the first technique used to detect both normal and mutated cfDNA sequences in human blood.[16] Currently, a myriad of technologies exist, each with benefits and disadvantages. They comprise 3 broad categories: PCR-based techniques, targeted deep sequencing, and whole-genome sequencing. In the early 2000s, a technique known as beads, emulsion, amplification, magnetics (BEAMing) was the first PCR-based technique to become widely used.[17] During the past 2 decades, the optimization of digital PCR to increase sensitivity and reduce costs resulted in the development of the most commonly used digital PCR method, droplet digital PCR (ddPCR).[18] Both BEAMing and ddPCR separate DNA molecules in a large number of emulsified droplets to amplify and detect individual DNA fragments.[18] Digital techniques are extremely sensitive and can detect point mutations with allelic fractions as low as 0.01%.[19] However, these techniques require prior mutational knowledge of a tumor and cannot easily address tumoral heterogeneity in an unbiased fashion.

Cancer personalized profiling by deep sequencing (CAPP-Seq), tagged-amplicon deep sequencing (TAm-Seq), and the safe sequencing system (Safe-SeqS) are all PCR-based, targeted techniques that comprehensively screen for unknown mutations by means of deep, next-generation sequencing.[20] They develop a comprehensive list of unknown variants with outstanding detection limits but often have the drawback of relatively long turnaround times. Because no prior mutational knowledge is available, these techniques detect only a predetermined list of genetic mutations, depending on the "gene panel" that is selected for analysis. Although the predetermined panel does not provide a comprehensive view of an entire tumor, the analytical sensitivity is better than it is with whole-genome (or exome) sequencing. Whole-genome sequencing methods can provide a more comprehensive ctDNA profile based on the detection of rearrangements, somatic chromosomal aberrations, and copy number variations, but they have the potential drawback of decreased analytical sensitivity.[7] Each technique has merit, and selection of the most appropriate technology depends on the intended application, along with coverage, sequencing depth, sensitivity, time, and cost.

Early Detection and Diagnosis

Early-stage breast cancer is potentially curable, but early detection and diagnosis remain crucial for reducing cancer-related mortality. The detection of early-stage breast cancer is challenging because it is characteristically asymptomatic and detection with standard-of-care screening methods is limited. The detection of ctDNA, a tumor-specific subset of cfDNA, may be a noninvasive and quick method to identify and screen for early-stage disease. Currently, ctDNA is most often utilized to identify and track genetic variants in metastatic disease, whereas its utility in primary screening and/or early-stage disease is still under investigation.

The earliest study of cfDNA detection in the blood of patients with cancer, which was published in 1977 by Leon and colleagues, found correlations between cfDNA levels and tumor burden, treatment response, and prognosis.[21] Nearly 30 years later, the first report of using cfDNA in the diagnosis of breast cancer was published by Huang and colleagues.[22] The results demonstrated that the median level of cfDNA was 5-fold higher in patients with breast cancer than in healthy controls, and approximately 3-fold higher than in patients who had benign breast disease. The study concluded that the quantification of circulating cfDNA might be a valuable complementary diagnostic tool for the early detection of breast cancer in apparently unaffected individuals. More recently, a study of early-stage breast cancer observed that cfDNA levels were higher in patients with breast cancer before surgery than they were in patients with breast cancer after surgery, in those with precancerous conditions, or in healthy patients.[23] This study provided additional evidence for the direct correlation between cfDNA and the presence of cancer. Although multiple studies have documented an elevated level of cfDNA in the bloodstream of patients with cancer, identifying a diagnostic threshold remains challenging. Furthermore, various other processes and diseases can significantly increase the levels of cfDNA,[24-27] and evidence regarding the difference between levels of ctDNA in patients with benign vs levels in those with malignant disease is conflicting.[28,29]

Our group and many others are currently running studies to address these shortcomings in an effort to improve and advance the diagnostic utility of ctDNA in early-stage breast cancer. In 2018, Thrive developed a blood test called CancerSEEK for the early detection of 8 common types of cancer, including breast cancer. This test has been shown to identify the origin of a breast tumor accurately in up to 83% of samples.[30-32] Similarly, GRAIL designed the Circulating Cell-free Genome Atlas Study (CCGA) in 2018 to collect cfDNA from patients with a new diagnosis of cancer and from healthy participants, and to create a database and develop models for distinguishing cancer from noncancerous conditions.[33] In China, a clinical trial is currently underway to evaluate the possible clinical application of ctDNA detection in the peripheral blood of control patients, patients with benign breast disease, and those with breast cancer (NCT03973034). The study will use low-depth whole-genome sequencing to evaluate the diagnostic utility of ctDNA detection in all populations. The use of ctDNA detection for early screening may be of particular interest in patients with a family history of breast cancer. Guardant Health recently developed the LUNAR-1 and LUNAR-2 assays, which are being studied for the detection of early, residual, or recurrent cancer.[31] LUNAR-2 is currently being tested for the detection of early-stage colorectal cancer in high-risk populations. If these studies are successful, similar studies may look at the use of LUNAR-2 in people with a family history of breast cancer. The ability to screen for and detect cancer before symptoms occur may significantly reduce cancer-related deaths in this specific subset. These studies offer a promising future for the ctDNA-based detection of cancer in its earliest stages. However, several limitations must be considered.

A significant hurdle in the utilization of cfDNA for detecting and diagnosing breast cancer is the lack of a prior knowledge of tumor-associated variants. Most ctDNA detection methods rely on the identification and detection of tumor-specific variants. However, some studies suggest that certain distinct characteristics in ctDNA, such as unique molecular weights, can be used to diagnose carcinogenesis accurately.[34,35] Despite these distinct characteristics, an impediment to detecting early-stage breast cancer is the low concentration of ctDNA relative to the total concentration of cfDNA, along with the inability to identify ctDNA-specific characteristics accurately.[36] Future methods may rely on the analysis of potential somatic mutations in ctDNA in combination with cfDNA levels to improve the accuracy of early detection and screening. Finally, the utility of ctDNA for initial diagnosis is further complicated by the presence of clonal hematopoiesis of indeterminant potential (CHIP) mutations, which are gene mutations often associated with cancer in blood stem cells that can accumulate as a healthy individual ages.[37] CHIP mutations are a reported source of background noise in liquid biopsy specimens, and when incorrectly classified as tumor-derived, they can lead to inappropriate therapeutic management.[38] Strategies to identify CHIP mutations accurately will need to be incorporated into early diagnostics to avoid mistreatment. Although the discussed studies offer promising results, ctDNA as a screening test for early-stage breast cancer has yet to be firmly established in standard-of-care practices. However, if ctDNA could be accurately detected during

the asymptomatic phase of early-stage breast cancer, it might serve as an easy, noninvasive screening method for all patients at risk.

Predicting Prognosis and Recurrence in the Neoadjuvant and Adjuvant Settings

As previously mentioned, most methods of ctDNA detection rely on the prior identification of tumor-specific variants. A prior knowledge of tumor-associated variants increases the sensitivity and selectivity of testing when concentrations of ctDNA are very low. In 2014, Beaver and colleagues demonstrated the potential utility of liquid biopsy for the detection of early-stage breast cancer.[39] In this prospective study, blood samples were collected before and after surgery from patients who had early-stage breast cancer with a previously identified *PIK3CA* mutation and were then analyzed with ddPCR. The study demonstrated that ddPCR could be used to detect ctDNA before and after surgery in patients with early-stage breast cancer, and ctDNA levels could be correlated with prognosis. Several research groups later confirmed these findings in other early-stage cancers.[40,41]

Recently, several clinical studies measuring ctDNA across breast cancer subtypes in both the neoadjuvant and adjuvant settings have demonstrated the predictive utility of ctDNA in predicting overall outcome. In a sub-study of the NeoALTTO phase 3 trial, which was a randomized, neoadjuvant study of trastuzumab, lapatinib, or both in patients with early human epidermal growth factor receptor 2 (HER2)-positive breast cancer, Rothé and colleagues found that the absence of detectable *PIK3CA* and *TP53* variants before neoadjuvant therapy was associated with high pathologic complete response (pCR) rates.[42] Furthermore, the data demonstrated that the detection of ctDNA before neoadjuvant therapy in patients with HER2-positive breast cancers was associated with decreased pCR rates, and patients with undetectable ctDNA at baseline had the highest pCR rates. Similarly, in a retrospective analysis of data collected in the phase 2 I-SPY 2 trial (this reported sub-study investigated pembrolizumab [Keytruda, Merck] in combination with standard therapy in HER2-negative breast cancers), researchers found that high levels of ctDNA before neoadjuvant therapy were associated with a greater tumor burden and more aggressive biology, and the presence of ctDNA after treatment was associated with lower pCR rates.[43]

In 2019, a prospective, multicenter study used serial plasma samples to monitor patients with early-stage breast cancer, irrespective of hormone receptor status or HER2 status.[44] Primary tumors were sequenced to identify somatic mutations, and personalized tumor-specific assays were used to surveil these mutations. Plasma samples were taken every 3 months for the first year of follow-up, and subsequently every 6 months. The study demonstrated that the presence of ctDNA in blood samples predicted relapse, on average, 10.7 months before the development of clinical symptoms. Furthermore, Garcia-Murillas and colleagues were able to use ctDNA to detect extracranial metastatic relapse in 96% of patients. The authors concluded that the detection of molecular relapse has a high level clinical validity and that the use of surveillance methods based on ctDNA may improve progression-free survival.

Several large-scale studies are further assessing the prognostic value of ctDNA. The PREDICT DNA trial, led by the Translational Breast Cancer Research Consortium (TBCRC), is an active prospective study in which the absence of ctDNA in liquid biopsy specimens is being correlated with pCR in stage II/III breast cancers that are HER2-positive or triple-negative (NCT02743910). An ongoing clinical trial in France is assessing the prognostic value of ctDNA in patients undergoing neoadjuvant chemotherapy (NCT03357120). Serial blood draws are taken for 5 years after surgery, and patients are monitored for relapse. In China, an observational study is focusing on the detection of ctDNA 1 month after surgery in patients with stage I to III breast cancer (NCT04353557). These studies are all focusing on early-stage breast cancer.

Detection of Minimal Residual Disease

The presence of MRD, or any tumor component remaining after surgical resection, is a major determinant of recurrence and the eventual development of metastatic disease.[45] However, the detection of MRD, particularly after neoadjuvant therapy, is challenging. In the previously mentioned NeoALTTO sub-study, researchers found that ctDNA became undetectable in more than 90% of patients in the neoadjuvant setting.[42] Because the accuracy of detecting residual disease in the neoadjuvant setting is limited, nonmetastatic studies have focused on surveillance for recurrence rather than the detection of MRD. For example, Garcia-Murillas and colleagues used ddPCR to track tumor-associated variants in an effort to predict relapse in patients with nonmetastatic breast cancer.[46] Only 50% of the patients in whom relapse eventually occurred had detectable ctDNA in postoperative samples drawn 2 to 4 weeks after surgery. Rates of disease-free survival were significantly lower in the patients with detectable ctDNA than in those without detectable ctDNA. Despite these results, ctDNA falls below the limit of detection in more than 90% of patients after neoadjuvant therapy, regardless of residual disease status.[47,48] If ctDNA is to be used effectively for MRD detection, techniques in the clinical workflow must consistently

detect variant alleles with frequencies of less than 0.1% to avoid false-negative results.[49] Additionally, techniques should incorporate strategies to control for sequencing errors and artifacts.

In 2019, McDonald and colleagues developed a method called targeted digital sequencing (TARDIS) to improve analytical sensitivity in personalized ctDNA analysis.[50] TARDIS improved sensitivity and allowed the detection and surveillance of MRD. In the study, TARDIS detected ctDNA in 77% (17/22) of patients in the post-neoadjuvant setting, including 5 of 9 patients with a pCR (no evidence of tumor cells in resected tissue). In 2020, Parsons and colleagues further improved the detection of MRD through the development of an ultrasensitive patient-specific panel that involved tracking hundreds of variants.[51] The tracking of many (>100) variants, rather than a few, inherently increases assay sensitivity. Although this concept is not new, previous methods lacked sufficient specificity. Parsons and colleagues are the first to have used ctDNA to track hundreds of patient-specific variants with sufficient analytical specificity (~1×10^6). In patients with stage 0 to III breast cancer, recurrence was predicted with a median lead time of 18.9 months (longest lead time was 39 months) without compromising clinical specificity. This median lead time is significantly longer than the previously discussed median of 10.7 months in the study by Garcia-Murillas and colleagues.[44] An increased lead time may allow clinicians to make earlier informed decisions regarding therapeutic interventions to prevent metastatic recurrence.

Although the methods of MRD detection based on ctDNA have advanced significantly in the past few years, several limitations still need to be addressed. A significant hurdle in the technique presented by Parsons and colleagues is that most patients do not have a sufficient number of tumor-associated variants to leverage the large fingerprint approach properly.[51] Additionally, MRD detection methods rely heavily on the timing of follow-up sample collection. Infrequent or poorly timed sampling may increase the number of false-negative results. The ability to detect MRD reliably and accurately may significantly increase the chances of preventing relapse, ultimately leading to a cure.

Tumor Surveillance and Therapeutic Decision Making

The ability to identify candidates for whom a de-escalation of therapeutic strategies in the postoperative setting would be appropriate remains an elusive goal in the clinic. Although a significant portion of patients benefit from adjuvant therapy, most do not. Unnecessary treatment is associated with increased risks and complications, including death.[52] The utilization of ctDNA for long-term surveillance may provide an opportunity for clinicians to de-escalate therapy. Studies of the use of ctDNA to de-escalate or cease therapy definitively in patients with early-stage breast cancer are limited. However, a recent case study published by Hunter and colleagues demonstrates the possibility of using ctDNA for surveillance while therapy is discontinued in the metastatic setting.[53] Although the patient in this study had metastatic disease, a similar concept might be applied after surgery for patients with early-stage disease. The current standard of care for patients with metastatic HER2-positive breast cancer is indefinite, continuous HER2-targeted therapy. The inability to stratify patients by risk when decisions to discontinue therapy are being made results in unnecessary treatment and risk for a subpopulation of patients. Using ctDNA, Hunter and colleagues confirmed the continued absence of disease during cessation of therapy. The identification of patients for whom de-escalation of therapy is appropriate not only removes the risks associated with unnecessary treatment but also can significantly improve quality of life.

The ability to track response to therapy reliably in real time would make it possible for clinicians to find the most effective therapy and would create a significant paradigm shift in the treatment of breast cancer. However, most breast cancers lack a reliable biomarker for monitoring tumor response to treatment accurately in the early-stage setting. The serial monitoring of ctDNA provides an accurate assessment of tumor progression in real time, in addition to valuable insight into the efficacy of administered therapies. To date, most of the studies focusing on treatment response have been conducted in the metastatic setting. However, a few recent studies have demonstrated the role of treatment monitoring in early-stage disease. In 2019, Butler and colleagues used ctDNA to monitor a small cohort (n=10) of patients with breast cancer undergoing neoadjuvant chemotherapy.[47] All of the patients with a pCR to treatment (n=4) had undetectable levels of ctDNA, demonstrating the direct correlation between ctDNA detection and therapeutic response. In the same year, Chen and colleagues showed a direct correlation between ctDNA profiling and treatment efficacy and disease progression in patients with breast cancer.[54] The study identified multiple ctDNA mutations in patients with HER2-positive and HER2-negative breast cancer that reliably correlated with response to administered therapies. The study also demonstrated that ctDNA monitoring could be used to identify potential mechanisms of therapeutic resistance. More recently, in 2020, a study observed that in a group of patients with triple-negative breast cancer (n=25), a slight rise in ctDNA levels after neoadjuvant therapy was predictive of an incomplete

pathologic response, and the absence of ctDNA was associated with increased overall survival.[55] These studies provide convincing evidence to support using ctDNA for therapeutic decision making. However, before ctDNA monitoring is implemented in standard clinical practice, high-powered clinical studies across all breast cancers will be required.

Several clinical trials are evaluating the utility of ctDNA as a method for assessing treatment in nonmetastatic breast cancer. In addition to predicting prognosis, the previously mentioned PREDICT DNA clinical trial is using ctDNA to determine the response of HER2-positive and triple-negative breast cancers in the preoperative/neoadjuvant setting. A 200-person observational study based in China is investigating whether ctDNA detection can reflect response to neoadjuvant chemotherapy and potentially identify MRD (NCT03881384). DARE is a randomized phase 2 clinical trial that will use ctDNA-guided second-line adjuvant therapy for patients with estrogen receptor–positive, stage II or III, high-risk residual breast cancer (NCT04567420). Although a greater number of studies are focused on metastatic disease, in the past few years several clinical trials involving ctDNA have begun to concentrate on early-stage disease.

Conclusion

Although liquid biopsy and ctDNA assays for early-stage breast cancers are still in their infancy, in the last few years the number of technologies focused on early-stage cancer and lower detection limits has increased significantly. Currently, numerous ctDNA-based assays exist that detect early-stage disease, residual disease, and therapeutic response. However, ctDNA detection methods are still relatively new, and the full utility of these technologies has yet to be realized. Although applications in the clinical setting are increasing, several limitations and considerations will need to be addressed before ctDNA technologies can be used as a standard of care for patients with early-stage breast cancer. Once approved, these technologies and many others on the horizon will provide clinicians with a relatively inexpensive and noninvasive method for diagnosing and monitoring early-stage cancers.

Disclosures

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