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Role of circulating tumor DNA and circulating tumor cells in breast cancer: History and updates

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Abstract

Circulating tumor DNA, cell-free DNA, and circulating tumor cells have been at the epitome of recent research in breast cancer. These forms of liquid biopsies have been used in monitoring disease progression, estimating the risk of relapse, and response to treatment. Much has been done in relation to serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease. Some studies have also explored their use in monitoring treatment response. As the field of liquid biopsies expands, more prospective studies are needed to tailor management in an individualistic approach. In this literature review, the authors explore the multiple uses of circulating tumor DNA and circulating tumor cells in breast cancer.

Keywords

Circulating tumor DNA, cell-free DNA, circulating tumor cells, breast cancer, biomarkers

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Introduction

The management of breast cancer has been an ever-changing modality. Every day we move closer to the prospect of an individualized treatment method based on highly specific diagnostic models. Over the past 10 years, different studies have evaluated the use of circulating cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA) in breast cancer. The apoptosis of cells triggers the release of cfDNA into the circulation. cfDNA describes the broad term of fragments of DNA circulating freely in the bloodstream, including ctDNA which are fragments of DNA released only by the apoptosis of tumor cells. Their use has been helpful in monitoring response to treatment thus cutting down on wasted time, cost of treatment, and toxicity of nonbeneficial regimens. Even though serial imaging is still the most frequently used modality in detecting changes in tumor size and response to treatment, radiographic measurements often fail to detect tumor burden.[1] Many studies around the world have found that ctDNA likely has a dual role. First, the levels of ctDNA measured several days after the last chemotherapeutic regimens serve as a surrogate marker for total tumor mass. Second, an indicator of tumor responsiveness can be obtained by measuring the difference between basal levels before and peak levels after administering chemotherapy.[2] ctDNA has also been used as a surrogate of residual tumor burden post-surgery.[3] Expanding our knowledge regarding liquid biopsies is needed to keep up with the ever-changing field of medicine.

Discussion

Milestones in diagnosing breast cancer

Breast cancer (BC) is the most common cancer affecting females worldwide, constituting the leading cause of cancer death among this population.[4] Early detection and diagnosis

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offer the best chance for effective treatment and a better prognosis.[5–7] Breast self-examination (BSE) represents the first step in the diagnosis. BSE makes women aware of the normal breast structure and alerts them of a potential abnormality. Despite its importance, BSE remains an insufficient diagnostic criterion, and abnormal findings always warrant subsequent imaging.[6] The American Cancer Society recommends screening mammography (MMG) for women starting at 45 years of age as this screening method has been found to reduce BC mortality.[8] Nevertheless, MMG is not advised for women under 40 at moderate risk for BC because exposure to ionizing radiation may trigger tumor development.[9] Moreover, MMG may show false-positive results in these patients leading to over-recognition: a tumor recognized on MMG and confirmed by histology may not develop into symptomatic disease during the lifetime of the patient but may instead cause unnecessary interventions.[3,6,8] On the other hand, in women at high risk for BC, magnetic resonance imaging (MRI), a more invasive screening modality is performed to complement MMG. In cases where precise imaging of soft tissue could not be made, the use of MRI as an adjunct to MMG showed increased sensitivity. As a consequence, the American Cancer Society recommends the combination of MMG and MRI for women at a lifetime risk of BC greater than 20%.[6,8] Finally, although less sensitive than MRI, ultrasonography (USG) is considered in women at high risk who cannot have breast MRI as well as in younger women at moderate risk for high-density breast tissue.[6,8]

Tumor biopsy is currently the gold standard diagnostic method in BC screening.[10] As it is challenging for pathologists to distinguish between closely related diseases, immunohistochemistry (IHC) assists in the characterization of tumor cells. The size of the tumor can usually be determined by gross pathological examination of the specimen, but due to breast tissue elasticity, the surgical specimen might deviate from the in vivo shape hindering accurate measurement of small cancers.[8] Aside from morphological features, IHC is routinely performed for the detection of tumor markers, which predict response to treatment agents.[8] Tissue-based protein biomarkers such as estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki67 are used to choose an appropriate treatment and estimate prognosis.[8,11] However, in locations where the cancer is surgically inaccessible, a tumor biopsy may increase the risk of metastasis.[3,12] Moreover, since neither multiple site-testing nor longitudinal testing is feasible, a tissue biopsy is not amenable for repetition.[13] Due to its static and invasive character, tumor biopsy fails to reveal significant variations present within a single specimen.[8,14] In addition to tumor heterogeneity, another limitation to tumor biopsy consists of dynamic adaptations in the tumor that are induced by antitumor drugs.[8,10] These simply limit precision medicine. The last disadvantage encountered with tumor biopsy is that it is expensive and time-consuming.[11] Blood-based tumor markers such as CA 15-3 and carcinoembryonic antigen (CEA) are used to monitor therapy in advanced disease. Several studies show that high levels of CA 15-3 and CEA levels act as highly accurate evidence of recurrence of BC in postoperative asymptomatic patients.[15–25] Nevertheless, the use of circulating biomarkers as surveillance tools is still questionable in terms of sensitivity and specificity.[11] To date, no standardized definition of a clinically significant biomarker level increase for any serum marker has been described. Also, recurrence has been observed in cases of absent biomarkers. For these reasons, the use of biomarkers may give inaccurate results leading to futile investigations and unindicated treatments. Furthermore, even though circulating biomarkers have efficiently uncovered metastasis to distant organs such as bones or liver, they have little value in characterizing locoregional metastasis or lung-only metastasis.[11]

Liquid biopsy has emerged as a clinical tool that can overcome such restraints, allowing for a better representation of disease status. Since blood can be easily sampled, this approach is regarded as noninvasive and repeatable, conveying dynamic assessment of biomarkers.[10,26–28] At this point, research in BC has shifted from traditionally protein-based circulating biomarkers to newer, more promising DNA-based molecules including ctDNA.[17] Thus, liquid biopsy has analyzed circulating tumor cells (CTCs), cfDNA, tumor-derived extracellular vesicles (exosomes), and ctDNA.[13,14] ctDNA possesses a greater dynamic range which enables it to be more sensitive for monitoring tumor progression. It has also been shown to have a higher specificity compared to other biomarkers for malignancy, thus decreasing false-positive results. However, the shorter half-life of ctDNA requires careful timing in specimen collection. ctDNA measurement allows the determination of the genetic variations accountable for the resistance to specific treatment regimens.[11] CTCs and ctDNA provide different, yet complementary information. ctDNA and CTCs have become the real-time liquid biopsy biomarkers redirecting the diagnosis, prognosis of cancers, and dynamic surveillance of cancer therapies.[27,29]

From liquid biopsies to clinical data

Circulating tumor cells. During the development of a primary tumor, CTCs are shed into the bloodstream. CTCs have been found to be important findings in the peripheral blood of patients with prostate, pancreatic, colorectal, lung, and breast cancers.[13,29] The specific mechanism behind their release remains unclear.[11,13] CTCs have a short half-life; apoptotic or fragmented CTCs are often found in the blood owing to the unfavorable environment. Therefore, to reach their final destination, CTCs travel in clusters supported by macrophages and activated platelets, attached to the endothelium and directed by chemokines.[29] Afterward, CTCs exit the blood and populate secondary organs contributing to metastasis. For instance, in colorectal cancer patients, the difference between CTCs measurements in the mesenteric vein compared to peripheral veins showed that the liver picks up

tumor cells liberated from the primary lesion.[29] Observations show that CTCs are also released by the metastatic lesions and recirculate to the primary site via a process called tumor self-seeding.[29] Thus, CTC clusters have been linked with higher metastatic potential and poorer prognosis.[27] However, the low number of cells relative to the abundant hematopoietic cells makes the enumeration of CTCs statistically challenging. In addition, the complex surface and heterogeneity of CTCs create another obstacle in their detection. Therefore, many enrichment and detection methods have been developed and have been classified either following physical or biological properties.[13] CTCs' isolation based on biological properties involves positive and negative immunoselection methods. Sensitive and specific analysis of CTCs has been achieved through positive selection using antibodies against epithelial tumor antigens that are not expressed on the surrounding mesenchymal blood cells such as epithelial cell adhesion molecule (EpCAM).[13,29] In immunomagnetic sort, antibodies attached to a magnetic bead isolate CTCs' antigen. In CellSearch, the only FDA-approved assay for CTC detection, CTCs are initially identified by binding to EpCAM positive and are then further classified as cytokeratin (CK) positive, CD45 negative cells.[11,13] However, CTCs are subject to epithelial-to-mesenchymal transition (EMT), which increases the plasticity of CTCs and their capacity for migration and metastasis. Partial EMT gives rise to CTCs with increased ability to adapt to secondary sites and reduced expression of epithelial markers.[29] This is evidenced by several cancer cases with CTCs that lack EpCAM expression.[13] To reach maximal collection, new technologies that combine enrichment, detection, and characterization methods of CTCs are under development, but this project has slowed down after the introduction of CTCs into clinical diagnostics.[29]

cfDNA and ctDNA. All individuals have small quantities of fragmented cell-free DNA in their plasma.[30] This amount increases following various triggers such as stroke, trauma, autoimmune disease, and cancer. The etiology behind the release of cfDNA is not yet fully understood, but the DNA fragment length might provide some clues about the source of the cfDNA.[29] In healthy subjects, white blood cells can actively secrete DNA. Nevertheless, the most important source of cfDNA appears to be apoptotic cells.[10] Subsequently, since cancer cells have faster cell turnover and undergo more apoptosis and necrosis, it was not surprising to find an exponential increase in cfDNA. Therefore, in cancer patients, the fraction of cfDNA released from primary tumors, CTCs, and metastases is known as the ctDNA; this ctDNA contains specific tumor mutations and is affected by the tumor size.[14,28,29] Thus, ctDNA offers a snapshot of the tumor genomic make-up which is of use in identifying mechanisms of resistance to therapy as well as in monitoring response to treatment.[11,14,27,29] However, the short half-life of cfDNA creates an obstacle to the sampling process. cfDNA is filtered from the blood by the liver and the kidneys, causing its clearance and concentrations to be affected by any renal and/or hepatic dysfunction.[29] Another clearance method under debate postulates that ctDNA can be taken up by normal host cells and transform them into malignant cells, thus contributing to tumorigenesis. If proven to be true, ctDNA may be examined as a potential target for antitumor therapy. Recently, two different technologies have been designed to study ctDNA. The first of these is the targeted approach which refers to the PCR-based methods. This approach can only locate single nucleotide mutations in ctDNA using predetermined genes. The second is the untargeted approach, which includes whole-genome sequencing. This approach screens the whole genome for new mutations that might confer resistance to a treatment regimen.[14,29] Although digital PCR (dPCR) is a more sensitive modality, as the ctDNA mutation profile changes over time, using next-generation sequencing (NGS) is more desirable particularly in genes without hotspots.[29,31] Unfortunately, the fact that ctDNA is diluted in the background of cfDNA is an obstacle for the examination of ctDNA, especially when tissue-damaging therapies like surgery, chemotherapy, and radiotherapy are indicated.[11,14,27,29] Even though patients with localized cancers have been found to have ctDNA present at notable rates, that is, 48%–73%, these figures remain unsatisfactory. Cancer-associated mutations have also been observed in older people with a very mild risk of contracting cancer during their lifetime.[29] So, in terms of sensitivity and specificity, monitoring of CTCs and ctDNA during cancer therapy faces fewer challenges than early detection of CTCs and ctDNA in early cancer patients. Therefore, a greater emphasis is put on choosing the right set of cancer-specific aberrations to develop standardized ultrasensitive approaches[29] (Table 1).

Role of CTCs and ctDNA in BC

CTCs and ctDNA have been implicated in a few malignancies such as non-small cell lung cancer (NSCLC). Recently, "Cancer Personalized Profiling by deep sequencing" (CAPP-Seq) has been designed to detect mutant alleles at a sensitivity of 0.02% with 96% specificity.[29] CAPP-Seq is an ultrasensitive technique that targets mutations in ctDNA. In this method, a selector that consists of biotinylated DNA oligonucleotides targeting recurrent mutations particular to a cancer type is designed. The former uses a probe-based hybridization capture on tumor versus normal cells to ascertain specific mutations identified in an individual.[53] Although detection rates were 10 times higher in NSCLC patients with advanced disease, this method was able to detect ctDNA in up to 50% of patients with stage I NSCLC. Refinements still have to be done to CAPP-Seq for it to reach adequate sensitivity levels for the detection of early cancer stages.[29] Following a phase IV clinical trial, the first ctDNA test gained approval for use in the stratification of NSCLC patients. These patients were segregated based on the presence of EGFR mutation. In this study, ctDNA showed

Table 1. Analysis of ctDNA using different methods in early and advanced breast cancer.

Method	Target	BC stage	Study
Real-time PCR	Rearrangements	Advanced	McBride et al.[32]
PCR-Ligation	SNVs	Advanced	Bettegowda et al.[33]
ARMS-Scorpion PCR	Hotspot *PIK3CA* mutations	Early, advanced	Board et al.[34]
Digital PCR	Rearrangements, *PIK3CA* mutations	Early	Beaver et al.,[35] Garcia-Murillas et al.,[36] and Olsson et al.[37]
	HER2 amplification, *ESR1* mutations	Advanced	Gevensleben et al.[38] and Guttery et al.[39]
	SNVs (e.g., *PIK3CA* and *TP53* mutations), copy number alterations and rearrangements	Advanced	Dawson et al.,[1] Forshew et al.,[40] and Murtaza et al.[41]
MAP	*TP53* mutation	Early	Chen et al.[42]
PARE	Rearrangements	Advanced	Bettegowda et al.[33] and Leary et al.[43]
BEAMing	*PIK3CA* mutations	Advanced	Higgins et al.[44]
MSK-IMPACT	SNVs and copy number alterations based on a custom panel	Advanced	Cheng et al.[45] and De Mattos-Arruda et al.[46,47]
TAm-Seq	SNVs (e.g., *PIK3CA* and *TP53* mutations), copy number alterations and rearrangements	Advanced	Dawson et al.,[1] Forshew et al.,[40] and Murtaza et al.[41,48]
Safe-SeqS	SNVs	Advanced	Bettegowda et al.[33]
Modified FAST-SeqS	Prescreening tool for an estimation of ctDNA percentage	Advanced	Belic et al.[49]
Ion-AmpliSeq	Selected SNVs (e.g., *TP53, IK3CA, ESR1, PTEN, AKT1, IDH2, FGFR1, FGFR2, SMAD4* mutations)	Advanced	Guttery et al.[39] and Rothé et al.[50]
Whole-exome sequencing	Protein coding SNVs and copy number alterations	Advanced	Murtaza et al.[41,48]
Whole-genome sequencing	SNVs, tumor-specific rearrangements, copy number changes (e.g., *ERBB2* and *CDK6* amplifications)	Advanced	Dawson et al.,[1] Forshew et al.,[40] and Leary et al.[51]
Shallow whole-genome sequencing	Rearrangements	Early	Olsson et al.[37]
	Copy number alterations	Advanced	Heidary et al.[52]

ARMS: amplification refractory mutation system allele-specific PCR and Scorpion probes; BC: breast cancer; BEAMing: beads, emulsion, amplification, magnetics; MAP: MIDI-activated pyrophosphorolysis; MSK-IMPACT: Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets; PARE: personalized analysis of rearranged ends; SNV: single nucleotide variants; TAm-Seq: tagged-amplicon deep sequencing; Safe-SeqS: safe-sequencing system.

effectiveness in replacing tissue biopsy when the latter is not easily obtained.[29] In conclusion, liquid biopsy has already been introduced to clinical practice for screening and early detection of NSCLC as well as for monitoring therapeutic targets and resistance mechanisms.[29] Here, we explore the possible role of CTCs and ctDNA in early BC.

Monitoring disease progression. Although their place in current clinical practice remains uncertain, both ctDNA and CTCs have been shown to have a role in monitoring disease relapse during treatment. *ESR1* mutations in BC are predominantly detected within hotspots using dPCR. Currently, NGS discovered new *ESR1* mutations in metastatic BC. Molecular barcode NGS (MB-NGS), a novel technique of NGS, has allowed a highly sensitive detection of ctDNA, and the detection of PCR errors in a relatively wide range of genes, with a lower detection limit of 0.01%–0.001% compared to 0.1%–1% achieved by conventional NGS. A molecular barcode is a nucleotide sequence attached to a unique DNA fragment. While different barcodes represent different original DNA molecules, sequences with the same barcode imply PCR duplication from the same original fragment.[54] The primary role of the molecular barcode is thus to identify the duplication and exclude it from the analysis, hence significantly reducing false positives. MB-NGS would therefore represent a perfect tool for the mutational analysis of *ESR1*.[31] With the improvement of detection sensitivity, more and more studies are focusing on the significance of CTCs and ctDNA in BC. There is increasing stress on the prognostic information provided by CTC count in patients with early-stage cancer with neither clinical nor radiological evidence of metastasis.[29] Measurement of CTC before and after surgery or chemotherapy in these patients was associated with poor relapse-free survival (RFS) and overall survival (OS).[55] ctDNA showed efficacy in catching minimal residual disease in BC patients. Shaw et al.[56] followed up cancer patients over 12 years and found that some DNA amplifications identified in the primary tumor tissue were still detectable by liquid biopsy inferring the presence of micrometastasis.

In a study by Cavallone et al.[3] done in 2020, the prognostic and predictive value of ctDNA was assessed during neoadjuvant chemotherapy for triple-negative BC. A total of 26 patients with triple-negative BC had ctDNA measured from the primary tumor along with serial blood draws prior, during, and after neoadjuvant chemotherapy. On tumor sequencing, 121 variants were found at an average of five variants per patient, and ctDNA was detected in 96% of patients at baseline. This study was based on personalized dPCR assays based on whole-exome sequencing of each tumor. The researchers were able to find that the mean mutant allele frequencies at baseline were significantly correlated with clinical factors such as tumor size, stage, grade, nodal status at diagnosis, or surgery, and residual cancer burden calculator (RCB) score, but not with age. After receiving neoadjuvant chemotherapy, a slight decrease in ctDNA was correlated with incomplete pathological tumor response. However, the absence of ctDNA after neoadjuvant chemotherapy predicted long-term relapse-free OS.[3] In a secondary analysis of a randomized clinical trial by Radovich et al.[57] in 2020, it was also found that the presence of ctDNA and CTCs in patients with early-stage triple-negative BC after neoadjuvant chemotherapy was associated with disease recurrence. The study was based on findings from 196 female patients whose blood was collected for ctDNA and CTCs after treatment with neoadjuvant chemotherapy. Based on the analysis, detection of ctDNA was significantly associated with less distant disease-free survival as well as disease-free survival and OS.[57] Another study by Coombes et al.[58] in 2019 recruited 49 patients with BC after surgery and adjuvant chemotherapy. Based on the primary tumor whole-exome data, personalized assays targeting 16 variants were selected. Liquid biopsies were then collected every 6 months for up to 4 years. The study had promising results, as plasma ctDNA was detected in 16 out of 18 relapsed patients ahead of clinical and/or radiological relapse with a lead time of up to 2 years (median of 8.9 months). Of the 31 nonrelapsing patients, none had detectable ctDNA levels at any time across the 156 samples. This reflected a sensitivity and specificity rate of 89% and 100%, respectively. Detection of BC relapse with ctDNA was also found to be earlier than with imaging, CA 15-3, clinical exam, and liver function tests. The study explored three main BC subtypes: ER-positive and HER2-negative, ER-positive and HER2-positive, and triple-negative BC. This study showed the potential utility of ctDNA in detecting relapse in BC after surgery and adjuvant therapy earlier than other current clinical modalities. Furthermore, the long lead time ahead of recurrence detected by ctDNA offers the clinical value of potential therapeutic intervention before disease progression. However, the utility of ctDNA in detecting a second primary tumor remains questionable, since smaller localized recurrence might be ctDNA negative.[58] Similarly, Olsson et al.[37] assessed the utility of ctDNA in detecting occult metastasis in patients with primary nonmetastatic BC. The study was based on retrospective analysis of serial monitoring of ctDNA in the setting of long follow-up. The analysis was based on whole-genome sequencing of 21 primary BC tumors wherein an average of 92 chromosomal rearrangements were found per tumor. Out of those rearrangements, four to six were selected per tumor and included in the PCR analysis. It was found that ctDNA detection of occult disease occurred ahead of clinical detection of metastasis in 86% of patients, at an average lead time of 11 months with a range of 0–37 months. The study was interesting due to the association of ctDNA quantity with poor clinical outcomes including disease-free survival and OS.[37] A similar recent study by Velimirovic assessed the significance of early changes in ctDNA levels in metastatic BC as a prognostic indicator of radiologic progression and subsequent survival. This study aimed to compare ctDNA with traditional biomarkers such as CEA and CA 15-3, and recruited 84 patients with metastatic BC, whose blood was sampled at baseline and during treatment and assessed for CEA, CA 15-3, as well as ctDNA. It was found that the baseline ctDNA level was not associated with subsequent radiologic progression (OR 0.99, 95% CI, 0.96–1.01, $p=0.29$), while an increase in ctDNA levels during treatment was significantly associated with radiologic progression (Odds Ratio (OR) 2.04, 95% CI, 1.74–2.41, $p<0.0001$) with an average lead time of 6 weeks. Genomic progression, which was defined as ctDNA increase from baseline during treatment, was also found to be associated with progression-free survival (PFS) (median of 4.2 versus 8.3 months; HR 2.97, 95% CI, 1.75–5.04, $p<0.0001$). Genomic assessment showed a positive predictive value of 81.8%, a negative predictive value of 89.7%, a sensitivity of 75%, and a specificity of 92.9%. The utility of ctDNA compared to traditional markers was confirmed as rise in CEA and CA 15-3 was not proven to be prognostic of radiologic progression and PFS.[59]

Coombes et al.[58] recruited and followed 49 BC patients who underwent surgery and received adjuvant therapy. ctDNA was undetectable in all nonrelapsing patients. In contrast, metastasis was detected using ctDNA in 89% of relapsed patients around 2 years before clinically discernable relapse.[58] Similarly, a novel American Society of Clinical Oncology (ASCO) publication demonstrated the high precision of ctDNA when determining the prognosis of early BC patients receiving neoadjuvant chemotherapy (NAC). In this study, serial NGS was performed on plasma samples of 52 patients with early BC, 21 of which had positive baseline ctDNA.[60] As expected, positive ctDNA patients had worse OS. Interestingly, ctDNA measurements in the middle of the NAC course were highly predictive of the outcome of the completed NAC sessions, especially in ER-negative patients. Continuous tracking of ctDNA may support imaging tools when assessing tumors during NAC.[60] Little was known about using imaging along with CTCs and ctDNA. A subsequent study by Förnvik et al.[26] examined the effects of MRI breast compression on CTCs and ctDNA levels. More CTCs are shed from the primary tumor cells into the blood following MRI breast compression; however, the small increase in CTCs and ctDNA levels was not clinically significant.

So, MRI is viewed as safe alongside liquid biopsy and would not act as a confounding factor.[26] In summary, as a larger amount of ctDNA correlates with tumor size or burden, ctDNA aids in allocating treatment regimens initially by providing a holistic view of intratumor heterogeneity and subsequently by monitoring mutations. ctDNA carries prognostic values that can classify BC patients into low and high risk and accordingly receive personalized therapies.[61]

Zhong and colleagues investigated the individual and joint effects of CTC and cfDNA values on the clinical outcomes of metastatic BC in terms of PFS and OS. With CTC and cfDNA measured in 227 blood samples taken from 117 metastatic BC patients, it was found that patients with five or more CTCs had 2.58 times increased risk of progression and 3.63 times increased risk of death compared to patients with less than five CTCs. Moreover, a high level of cfDNA increased the risk of progression by 2.05 times and the risk of death by 3.56 times. When combined, CTC and cfDNA elevation increased the risk of death by 17 times ($p < 0.001$). Although the subtypes of BC were heterogeneous, the association of CTC with patient outcomes was still significant after adjusting for multiple factors. The study found the joint effect of CTC and cfDNA to be more prominent on OS than PFS. Although ctDNA analysis is more personalized and targeted to mutant alleles, cfDNA might have a place in clinical practice due to its cost-effectiveness compared to ctDNA. Furthermore, not many recurrently mutated genes have been described in BC other than *TP53, PIK3CA, ESR1*, and HER2.[62]

Therapeutic targets and resistance mechanisms. Although the association of CTC with worse prognosis in metastatic BC has been well-documented in the previous studies, Paoletti et al.[63] hypothesized that CTC enumeration is not sufficient in determining prognosis. This is especially true in ER-positive BC wherein tumors are not always responsive to endocrine therapy, most likely due to the harboring of ER-negative CTCs which can evade endocrine therapy. This led to the development of a CTC-Endocrine Therapy Index (CTC-ETI) based on CTC enumeration and the expression of estrogen receptor, BCL-2, HER2, and Ki67. The clinical implications of this index are being studied in terms of prognosis and personalized treatment.[63] Another important therapy target in BC is the HER2 oncogene. Although primary breast tumors can be characterized as HER2 negative or positive, studies have shown that CTCs can have different characteristics from the primary tumor, thus allowing for the escape of targeted therapy.[64] Moreover, in patients with primary tumors that are HER2-negative, HER2-positive CTCs can be found in the peripheral blood albeit at levels lower than in patients with HER2-positive primary tumors.[65] Other important therapeutic targets in the treatment of metastatic BC include immune checkpoint regulators like PD-L1. Therapeutic agents targeting PD-L1 have proven to cause remission in patients with metastatic BC, albeit at the expense of high toxicity.[66] To minimize unnecessary exposure, Phillips et al., attempted to study the expression of PD-L1 on CTCs with the purpose of stratifying patients into those who might benefit from anti-PD-L1 therapy and those who might not. It was found that PD-L1 is expressed on CTCs in more than 60% of studied patients who had hormone receptor-positive, HER-2 negative BC.[66] Yet another interesting emerging potential biomarker and therapeutic target are miRNAs (microRNAs), which are important posttranscriptional gene regulators. Since miR-10b has been implicated in metastasis of BC, Gasch et al.[67] attempted to study the heterogeneity of CTCs in terms of miR-10b expression. CTCs showed variable expression of miR-10b, but the clinical significance remains an area for future investigation.[67] Gene mutations have also been found to affect the action of therapeutic drugs on cancer cells. Mutations in the *PIK3CA* gene which activate the PI3K pathway have been implicated in resistance to HER2-targeting therapies.[68–70] Multiple studies have determined the presence of heterogeneity in *PIK3CA* status in CTCs from the same patient.[71–73] The implications of these mutations with regards to clinical application remain as a target of future investigation. CTCs have proven to contain a wealth of information into the understanding of the treatment of cancer, be it at the level of DNA, RNA, or proteins.

In a preclinical study of Oshiro et al.,[74] 25 of 110 BC patients with *PIK3CA* mutation revealed positive serum for the mutation. Those with high ctDNA showed an increased risk of recurrence and death.[74] *PIK3CA* mutations are frequently encountered in ER-positive BC patients.[29] Thus, apart from its role in monitoring advanced BC, CTCs and ctDNA might play an important part in the early stages of BC. Instead of waiting for metastasis to appear, early intervention with personalized adjuvant therapy might be beneficial for early-stage patients with detectable CTCs and/or ctDNA.[29]

Monitoring treatment response. CTCs and ctDNA have been shown to have a valid role in monitoring the progression of metastatic BC and OS. In a pooled analysis of individual patient data, 1944 eligible participants with metastatic BC were recruited. This landmark analysis showed that patients with elevated CTC levels pretreatment had decreased PFS and OS compared to patients with lower CTC levels at baseline. Moreover, an increase in the CTC count after the start of therapy, compared to baseline was associated with a decrease in OS.[11,75] In summary, this study demonstrated that CTC quantification improves prognostication in patients with metastatic BC. In another study, Murtaza et al.[41] demonstrated that genomic alterations in solid cancers can be studied in a noninvasive way by sequencing ctDNA released from cancer cells into the plasma. Acquired drug resistance correlated with an increase in ctDNA mutation levels, an activating mutation in *PIK3CA* after treatment with paclitaxel, a truncating mutation in the ER coactivator mediator complex subunit 1 (MED1) following treatment with tamoxifen trastuzumab and lapatinib, and a splicing mutation in GAS6, the ligand for tyrosine kinase receptor AXL, are all examples.[41] These examples could

demonstrate that genomic alterations and resistance to therapy can be studied by parallel sequencing of circulating cell-free tumor DNA in solid cancers. However, in a comparative study of CTCs and ctDNA in patients with triple-negative BC, Madic et al.[76] showed that CTC levels in patients with triple-negative BC could predict OS and time to progression, whereas ctDNA had no prognostic effect on the latter. Their search was based on *TP53* mutations in triple-negative BC to compare ctDNA and CTC prognostic value in patients with metastatic BC. In fact, with a sample size of 27 patients with *TP53* mutations, CTC levels were positively associated with OS ($p = 0.04$) and marginally associated with time to progression of the disease ($p = 0.06$). This illustration could not be applied to ctDNA. These studies seem to provide conflicting results on the impact of CTC and ctDNA on depicting OS and prognosis of BC and further studies with larger cohorts are warranted.

Using SWOGO500 clinical trial, researchers associated increased CTCs with poor prognosis in patients with metastatic BC. High CTC levels corresponded to five or more CTC per 7.5 mL. Patients with metastatic BC were categorized based on CTC levels. Those with a low level of CTCs at baseline received first-line chemotherapy as assigned. Patients with initially high CTCs were monitored after a first chemotherapy cycle and those with persistently high CTCs after chemotherapy were stratified to either continue the first line of therapy or switch to a more cytotoxic regimen. By analyzing results, conclusions were made that no difference exists between those who remained on first-line therapy and those who were switched to another regimen on the OS. However, CTCs were strongly prognostic, with a higher OS in those with initially low CTCs at baseline, compared to those who had persistent elevated CTCs after therapy.[77,78] In a similar view, patients with metastatic BC with HER2 positive, with a persistently high level of CTCs after therapy were subject to a trial of trastuzumab. In a randomized trial phase 2 study by Georgoulias et al.,[79] trastuzumab was shown to decrease the incidence of relapse of the disease in this population.

Using the same rationale, several large interventional studies have been designed to demonstrate that CTC enumeration could be adapted to improve treatment in BC patients. In fact, these large international studies are still under research, and thus study designs will be discussed looking for future promising results.[77] In the Treat CTC trial, which is a randomized phase 2 trial, patients with HER2 BC who have completed neoadjuvant therapy and surgery, and who had $\geqslant 1$ CTC/15 mL of blood were selected. Patients were then categorized into either Trastuzumab arm or observation arm and followed subsequently.[80] The primary outcome is to compare the CTC level at week 18 between the two arms. This study will allow researchers to also compare the recurrence-free interval between the two arms. In the DETECT III study, patients with HER2 metastatic BC and receiving several chemotherapy lines were tested for HER2+CTC. Patients who met these inclusion criteria were subsequently stratified into either the planned treatment or the planned treatment in addition to lapatinib, and tumor evaluation/size was then monitored. Finally, the CirCe trial included patients with initially HER2- BC at baseline, who had become HER2+in CTCs after metastasis. Using FISH for HER2 amplification, patients with HER2 amplified CTCs were treated by the anti-HER2 drug. In this single-arm study, the primary outcome is the response rate after the anti-HER2 drug.[80]

Clinical oncology guidelines on ctDNA and CTCs

The ESMO/ASCO Recommendations for a Global Curriculum in Medical Oncology Edition 2016 emphasized the importance of liquid biopsy in cancer biology, laboratory medicine, and personalized cancer medicine. Due to the heterogeneous nature of tumor cells, liquid biopsy is needed as an indicator of relapse and the emergence of new mutations.[81] Several studies have proved the role of ctDNA in different types of cancers. Very recently, Przybyl et al.82 prospectively examined the blood of patients with uterine leiomyoma (LM) and found that in contrast to leiomyosarcoma (LMS), LM does not shed ctDNA. This observation encouraged the use of ctDNA-based testing to better preoperatively evaluate the risk of LMS in patients presenting with a uterine mass and thus prevent the dissemination of malignancy during surgery for a presumed LM.[82] Kurtz et al.83 used ctDNA to predict treatment outcomes of B cell lymphoma after pre-existing methods failed to do so. The dynamics of ctDNA were studied in 217 patients treated for the disease: the pretreatment ctDNA levels detectable in 98% of patients were prognostic of outcomes including recurrence-free and OS; the first and second treatment cycles marked a rapid decrease in ctDNA by 2-log and 2.5-log, respectively.[83] Oikkonen et al.84 monitored ctDNA from patients with epithelial ovarian cancer before, during, and after treatment. Mutations conferring drug resistance were detected in seven of 12 patients. Following the detection of ERBB2 amplification in the ctDNA of a chemoresistant patient, changing the treatment resulted in a remarkable reduction of the tumor size along with normalization of the tumor marker CA-125.[84] ctDNA also guided clinical decisions for the treatment of melanoma. The prospective study of Braune et al.[85] noted a change in ctDNA within 30 days of starting or changing treatment in stage IV melanoma. In summary, ctDNA's potential routine clinical applications have been extensively studied in the era of personalized medicine. However, due to the variability of the decision support tools analyzing molecular markers, tumors with identical profiles are still treated differently which disregards the promising genomic medicine.[86] Therefore, even though ctDNA assays are becoming popular and favored for the management of several cancer types, the success of such approaches in BC requires more prospective studies to attain standardization and conformance.

Table 2. Studies on ctDNA and CTCs in breast cancer monitoring disease relapse and disease treatment.

Study	Type of study	Results
Role in monitoring disease relapse		
Cavallone et al.[3]	Prospective cohort	26 TNBC patients were followed with tumor and serial blood tests. After NAC, there was a slight decrease in ctDNA correlated with incomplete response. Absence of ctDNA predicted long-term relapse-free OS
Radovich et al.[57]	Secondary analysis of randomized clinical trial	This study involved 196 patients with TNBC who had residual disease after NAC Presence of ctDNA and CTCs in early TNBC after neoadjuvant chemotherapy was associated with recurrence Detection of ctDNA was significantly associated with less distant disease-free survival as well as disease-free survival and OS
Coombes et al.[58]	Prospective cohort	49 patients with early BC were recruited after undergoing surgery and adjuvant chemotherapy, with blood samples collected subsequently over a 4-year period. Detection of relapse after surgery and adjuvant therapy with ctDNA was earlier than with imaging, CA 15-3, clinical examination, and liver function tests
Olsson et al.[37]	Retrospective analysis	20 patients diagnosed with BC were enrolled. Serial monitoring of ctDNA was done for detection of metastasis: ctDNA detection of occult disease occurred ahead of clinical metastasis in 86% of patients with an average lead time of 11 months. There was an association of ctDNA quantity with disease-free survival and OS
Velimirovic et al.[59]	Prospective cohort	Blood samples from 70 metastatic BC patients were drawn at baseline and during treatment for ctDNA, CEA and CA 15-3 Baseline ctDNA was not associated with radiologic progression. Increasing ctDNA levels during treatment predicted radiologic progression with an average lead time 6 weeks Genomic progression was associated with PFS. The rise in CEA and CA 15-3 was not predictive of radiologic progression and PFS
Ye et al.[62]	Prospective cohort	117 patients with metastatic BC were recruited and blood samples were collected. Patients with five or more CTCs had increased risk of progression and increased risk of death vs. patients with less than five CTCs; high level of cfDNA increased risk of progression and risk of death; combined, CTC and cfDNA elevation increased risk of death by 17 times
Role in disease treatment		
Bidard et al.[75]	Pooled analysis	1944 patients with metastatic BC were recruited from 50 different European centers. Higher CTC level at baseline was significantly associated with a decrease in PFS and OS compared to lower CTC level Likewise, an increase in CTC count after therapy was associated with decreased OS compared to baseline
Murtaza et al.[41]	Pooled analysis	6 patients with advanced breast, ovarian and lung cancer were followed for 2 years. CtDNA mutation levels was found to increase with acquired drug resistance: *PIK3CA, MED1*, and *GAS6*
Madic et al.[76]	Comparative study	31 patients with TNBC and TP53 mutations were enrolled to compare ctDNA and CTC prognostic value in TNBC. CTC levels could predict OS and time to progression whereas ctDNA had no prognostic effect on time to progression

CEA: carcinoembryonic antigen; CTC: circulating tumor cells; ctDNA: circulating tumor DNA; MED: mediator complex subunit; NAC: neoadjuvant chemotherapy; OS: overall survival; PFS: progression-free survival; TNBC: 26 triple-negative breast cancer.

Limitations

The authors acknowledge pertinent limitations regarding this review article. It is worth noting that the studies included herein have been selected from a broad literature review. Moreover, this article does not draw any quantitative conclusions but rather describes the most recent studies done on the role of ctDNA and CTCs in breast cancer. Finally, articles described in this review have recruited subjects with different characteristics and from different geographical locations, and the results presented might not be generalizable. Deductions done in this paper are not based on any statistical analysis rather than a consensus of thought done by all authors. Even though this is not a systematic review, the authors have conducted an extensive and broad literature review about the topic addressed. The different terms "circulating tumor DNA," "circulating tumor cells" and "breast cancer" have been used in different combinations on the PubMed database search engine. Articles prior to the year 2022 were included in this article when needed. Duplicates were removed.

Conclusion

The role of ctDNA and CTCs in the monitoring of disease progression, response to treatment, and the prediction of OS has been demonstrated in multiple studies (Table 2). With

worldwide trends shifting toward precision and personalized medicine, ctDNA and CTCs have been found to contain a wealth of information and promising results when it comes to creating a more individualized approach to breast cancer. However, larger cohorts are still needed to validate these findings in a practical clinical setting than can be applied to a broader range of breast cancer patients. Suggested future areas of investigation include the role of ctDNA in detecting new primary tumors as well as establishing a quantifiable prognosis based on liquid biopsy including lead time to recurrence, OS, and PFS.

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