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Analysis of circulating tumor DNA in breast cancer as a diagnostic and prognostic biomarker

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Abstract

Despite all the advances in diagnosis and treatment of breast cancer, a large number of patients suffer from late diagnosis or recurrence of their disease. Current available imaging modalities do not reveal micrometastasis and tumor biopsy is an invasive method to detect early stage or recurrent cancer, signifying the need for an inexpensive, non-invasive diagnostic modality. Cell-free tumor DNA (ctDNA) has been tried for early detection and targeted therapy of breast cancer, but its diagnostic and prognostic utility is still under investigation. This review summarizes the existing evidence on the use of ctDNA specifically in breast cancer, including detection methods, diagnostic accuracy, role in genetics and epigenetics evaluation of the tumor, and comparison with other biomarkers. Current evidence suggests that increasing levels of ctDNA in breast cancer can be of significant diagnostic value for early detection of breast cancer although the sensitivity and specificity of the methods is still suboptimal. Additionally, ctDNA allows for characterizing the tumor in a non-invasive way and monitor the response to therapy, although discordance of ctDNA results with direct biopsy (i.e. due to tumor heterogeneity) is still considered a notable limitation.

Keywords

Circulating tumor DNA; Breast cancer; Liquid biopsy; Disease progression; Biomarker; Response to therapy

Background

Hundreds of thousands of women are affected by breast cancer each year [1,2], and despite tremendous advances in diagnosis and treatment of breast cancer, some patients present with metastatic disease at the time of initial diagnosis [3,4], and the risk of recurrence persists decades after initial occurrence [5–10]. Meanwhile, radiologic screening for breast cancer in younger adults is fraught with controversy and many guidelines advise against this screening [11,12], and detecting primary or recurrent breast cancer relies on invasive biopsy of the tumor tissue. Therefore, new non-invasive and inexpensive alternative diagnostic methods are urgently needed.

Conflict of interest

The author claims no conflict of interest with regard to this manuscript.

Breast cancer may not be identifiable through clinical and radiologic studies until the disease burden is significant. Micrometastasis can occur early in malignancy [13–15], and migrate as circulating tumor cells (CTCs) from the initial tumor to bone marrow [15,16]. In addition to circulating tumor cells, deoxyribonucleic acids (DNAs) released from the tumor cells may enter the bloodstream in form of cell-free tumor DNA (ctDNA) [17]. Identifying and measuring ctDNA could allow for not only early detection but also genomic mutation analysis of the tumor, leading to a more tailored therapy of breast cancer.

In the era of personalized therapies that are selected based on the genetic characteristics of the breast tumor, obtaining tumor DNA in earlier stages of the disease through non-invasive methods can potentially lead to high success rates in chemotherapy. Genomic profiling of the tumor tissue through direct biopsy has limitations as a single biopsy can capture just a snapshot of the tissue that is subject to selection bias and may not perfectly represent the tumor alterations. Analyzing ctDNA through peripheral blood samples may lead to earlier diagnosis and better therapy of breast cancer, but these hypotheses are to be confirmed by empirical evidence.

In this review of literature, we summarize the existing evidence to answer the following questions: Is circulating ctDNA detectable in breast cancer patients? What are the methods for detecting ctDNA in breast cancer patients? Is there a diagnostic, prognostic, and/or predictive value for ctDNA in breast cancer? Are there recurrent genomic mutations identified in ctDNA from breast cancer patients?

Circulating tumor DNA

In addition to an individual's own DNA, two forms of human circulating DNA can be detected in the blood: fetal DNA in pregnant women, and tumor DNA in cancer patients. The original work on isolation of fetal DNA in maternal blood was focused on the rare fetal nucleated cells [18,19]. Since then evidence has accumulated that placental strands of DNA are abundantly found in maternal circulation in the form of cell-free circulating DNAs. The discovery promised that this cell-free DNA can be used to identify fetal chromosomal defects in lieu of the more invasive chorionic villous sampling or amniocentesis [20,21]. Empirical studies have successfully detected aneuploidy of chromosomes 21 (Down syndrome), 18 (Edwards syndrome), 13 (Patau syndrome), X and Y by measuring the amounts of specific fragments of the fetal cell-free DNAs in maternal bloodstream [22–24].

Malignant cells in cancer patients release another form of cell-free DNA into the bloodstream. The malignant process entails the transfer of cancer-promoting cellular contents not only to neighboring cells within the tumor microenvironment but also into the circulation, thereby enabling cancer progression [25]. It is hypothesized that cell-free DNA, along with exosomes, micro RNAs, and cytokines are actively excreted by malignant cells into circulation to promote angiogenesis and metastasis [26,27].

The first report on identifying cell-free DNA in the blood-stream of cancer patients was published by Leon et al. in 1977, in which authors developed a radioimmunoassay for nanogram quantities of DNA in the bloodstream [28]. Later, Stroun et al. also showed that

small fragments of tumor DNA are frequently released into the bloodstream. These cell-free circulating tumor DNAs (ctDNAs) were found to range between 0.5 to 21 kb in length, with a plasma concentration ranging from 0.15 to 12 mg/mL. Concentration of ctDNA in the blood was subsequently investigated as a diagnostic tool to detect different forms of cancer including hematological, colorectal and thoracic neoplasms [29]. More recently, it was shown that ctDNA levels may also assist with determining the prognosis and recurrence of cancer. Holmgren et al. showed that cancer patients who are in remission have asymptomatic dormant micrometastases that may proliferate and eventually lead to recurrence [30]. This finding was followed by studies that focused on identifying these micrometastases in form of CTCs [31,32]. Bettegowda et al. showed that these micrometastases signal can also be identified by measuring ctDNAs [33].

ctDNA in breast cancer

The earliest report on using ctDNA in the diagnosis of breast cancer was published less than a decade ago [17]. Since then, numerous studies have been published discussing different techniques to measure and analyze ctDNA in breast cancer, as well as evaluating the diagnostic and prognostic characteristics of this biomarker. These studies are summarized in the next sections.

Methods for measuring ctDNA

Leon et al. first measured the concentration of cell-free DNA in the bloodstream through radioimmunoassay using the serum of a patient with systemic lupus erythematosus as the source of antibody. The concentration of circulating cell-free DNA among healthy individuals and those with various types of cancer was measured at 13 ± 3 ng/mL and 180 ± 38 ng/mL, respectively. Using a reference range of 0–50 ng/mL would yield a specificity of 93% but a sensitivity of only 50% in identifying cancer patients, therefore authors concluded that ctDNA was of low diagnostic value when detected by radioimmunoassay [28].

Newer measurement techniques revealed that naturally occurring circulating DNA has two sources: one is the DNA originating from apoptotic cells and another from living tumor cells. Studies measuring the length of DNA fragments using polymerase chain reaction (PCR) showed that apoptotic DNA fragments are uniformly truncated and are less than 200 base pairs long, while living tumor cells produce DNA fragments that are more variable in length and can reach several kilo base pairs [34–37]. Using an “integrity index”—the ratio between longer and shorter fragments—was suggested as a diagnostic metric for cancer. The integrity index is reported to be two times higher in breast cancer patients compared to the healthy population [38]. This finding is also supported by studies on other types of cancer [39–41]. However, in one study lower integrity index was associated with metastatic type of breast cancer [42].

Once ctDNA is identified in the bloodstream, methods for identifying genetic and epigenetic profiles of these fragments are available and may assist in detecting specific cancer driving mutations that tumors have released in the circulation. A recent systematic review of

literature has shown that ctDNA is a highly specific and effective biomarker for detecting specific mutations in lung cancer patients [43]. Numerous studies have used similar methods to identify genetic and epigenetic alterations in breast cancer patients. Quantitative real-time PCR has been widely used to measure the concentration of specific ctDNA fragments. Agostini et al. designed primers that would allow distinguishing shorter and longer DNA fragments in breast cancer patients using ALU115 and ALU247 [38]. They reported a sensitivity and specificity > 99% in discriminating healthy individuals from cancer patients using a cutoff of 2.0 ng/mL for ALU247 and comparable results with a cutoff of 9.3 ng/mL for ALU115.

Other PCR-based methods have been used to quantify and analyze ctDNA in breast cancer. BEAMing (Beads, Emulsion, Amplification and Magnetics) uses emulsion PCR technology to separately amplify individual molecules and then quantify specific variants using flow cytometry. It detects somatic mutations found in ctDNA with high sensitivity (1:5000 to 1:10,000 mutants per wild type molecule) [44,45]. Using BEAMing, Higgins et al. have identified mutations in the PIK3CA gene from ctDNA which were 100% concordant with tumor biopsies [46]. A related technique called droplet digital PCR (ddPCR) can be used to identify specific mutations in the ctDNA. This method was shown to have a sensitivity and specificity of 93% and > 99%, respectively, in identifying known mutations in a case of breast cancer [47]. The ddPCR technique augments mutation detection, requires much smaller amount of input DNA, and its high sensitivity allows detection of ctDNA in patients with low tumor burden.

Next generation sequencing techniques provide a high number of DNA sequences which can be used for detecting genetic alterations in breast cancer patients and can help to develop a routine laboratory base test for screening and monitoring for breast cancer [48].

Technological advances such as high throughput genomics including massively parallel sequencing (MPS) have enabled in-depth characterizations of somatic alterations in breast cancer. Such advances allow for comprehensive individual genomic profiling and more accurate prognostication, precision targeted therapies and monitoring of treatment response [49,50].

Most of the methods enumerated above require advanced and expensive genetic assays, which limit their utility in clinical practice. Goldshtein et al. tried to address this limitation by developing an inexpensive method for measuring ctDNA using a fluorescent assay. To perform the assay, a diluted fluorochrome stain is added to the plasma samples and the fluorescence in the sample is measured [51]. This technique does not require prior processing of the samples (e.g. DNA amplification), however, its diagnostic value is suboptimal. In one study, its sensitivity and specificity in detecting early stage breast cancer were measured at 72% and 75%, respectively; this was described as higher than other common biomarkers but lower than the more expensive sequencing methods [52]. In a more recent study, sensitivity and specificity of array-based genome-wide DNA methylation testing in ctDNA samples obtained in early stage breast cancer was 86.2% and 82.7%, respectively.

Beck et al. argue that it is best to restrict the scope of ctDNA sequencing in breast cancer patients to known mutations [48]. This would allow for using more targeted techniques, such as plasma-seq, for identifying and measuring ctDNA, instead of massively parallel sequencing the whole genome [53].

Epigenetic characteristics of breast cancer can also be determined by ctDNA analysis. For instance, Agostini et al. used the MethyLight® method, a fluorescent-based real-time PCR assay, to identify the epigenetic characteristics of ctDNA in breast cancer patients and reported that this method can identify ALU247 methylations with a sensitivity and specificity of > 99% and 69%, respectively [38,54].

A detailed comparison of the aforementioned method was the subject of a recent systematic review and meta-analysis by Lin et al. in which they pooled the various methods used for ctDNA measurement in breast cancer and concluded that the overall diagnostic sensitivity and specificity of these methods is 0.7 and 0.87, respectively, with the qPCR method having better accuracy than other quantitative methods, and NGS showing the highest promise as a high sensitivity method [55].

Diagnostic threshold of ctDNA concentration

Multiple studies have documented an elevated level of ctDNA in patients with breast tumors and its direct correlation with tumor burden [17,33,52,56–61]. Levels of ctDNA drop after the resection of primary tumor [52,59]. Table 1 provides a summary of these studies. However, establishing a threshold level of ctDNA concentration as diagnostic is still a problem.

Cell-free DNAs circulates in the bloodstream of healthy individuals at a concentration of approximately 10 ng/mL [62], and various cytotoxic processes (including cancers, lupus and pulmonary embolism) can significantly increase the levels of cell-free DNA in the blood [63–65]. Other processes such as clotting of the sample, apoptosis, cell lysis by the necrotic pathway or breakdown of any pathogens such as bacteria may also falsely increase the cell-free DNA level in the blood [38,66], although it may be possible to distinguish these DNA fragments from the tumor-related type using the molecular weight of the fragments [67].

As a result, elevated plasma concentration of cell-free DNA may not be a cancer-specific biomarker. A more reliable measure is the fraction of cell-free DNA that has the characteristics of tumor DNA and can be used to accurately diagnose carcinogenesis. Leary et al. showed that tumor DNA concentration 0.75% has a sensitivity of 90% and specificity of 99% in diagnosis of breast cancer [57].

Another issue in defining a threshold value for ctDNA concentration is with respect to its ability to discriminate benign versus malignant tumors. While some studies have shown that ctDNA levels may not be significantly different in benign and malignant disease [56,68], others have shown a significant difference in the levels of ctDNA in patients with benign disease (e.g. fibroadenoma, adenosis and lobular hyperplasia) versus those with malignant disease [17,58,69].

Genetic and epigenetic profiles

Specific cancer related mutations identified in ctDNA may be used to more accurately distinguish benign breast disease from malignancy. Detecting specific genetic or epigenetic changes associated with breast cancer in ctDNA may point to those driving the disease. Once chromosomal alterations such as copy number changes or rearrangements of malignant disease (e.g. amplification of cancer driver genes HER-2neu or CDK6) are found, targeted therapy can be utilized [57,70].

Genetic analysis of ctDNA fragments may focus on specific mutations in genes known to be associated with breast cancer, such as BRCA1/2 [71], and different kinases and phosphatases [72]. Epigenetic analysis, on the other hand, focuses on DNA methylation, which can provide significant diagnostic information especially in asymptomatic patients with non-suspicious mammograms and normal breast examinations [73].

Overall, genes in which mutations have been studied in ctDNA from breast cancer patients include *ESR1* [74–76], *PCDH20*, *OR4X1*, *ALK*, *DNPEP*, *SH3TC2*, *DDR2*, *MLL3* [53], *GAPDH* [68], *MET*, *CD1A*, *IQCA1*, *ZFYVA21*, *KIAA0406* [60], *NOTCH1*, *AKT1*, *FBXL4*, *EIF4B* [33], *MDM4*, *NFASC*, *CAMK1G*, *STK24*, *ERBB2*, *CDK6* [57], *TP53* [33,60], and *PIK3CA* [33,46,47,53,60,77]. Methylation changes in *MAL*, *SFRP1* [38], *RASSF1A* [38,78], *SFN*, *P16*, *PCDHGB7*, *HOXD13*, *hMLH1* [78], *ERβ* and *RARβ2* [79] have also been identified in ctDNA fragments of breast cancer patients.

Monitoring disease progression

Both the plasma level of ctDNA as well as its genomic profile can be valuable in monitoring disease progression. Several studies have shown that plasma levels of ctDNA are significantly higher in those who had higher stages of breast cancer, nodal involvement or larger tumors [38,52], and a recent study showed an association with overall survival as well [80]. However, more evidence is needed to establish the prognostic value of ctDNA concentration in breast cancer patients.

Schwarzenbach et al. showed that increased microsatellite instability (MSI) and loss of heterozygosity (LOH) in ctDNA fragments strongly correlates with tumor stage and disease progression [81]. Leary et al. have proposed that rearrangements identified using ctDNA, including deletions and inversions in *MDM4*, *NFASC*, *CDK6* and *STK24* genes can be used to develop less expensive, more specific biomarkers that could be used for quantitative monitoring of disease progression, for instance using polymerase chain reaction (PCR) tests [57]. Hypermethylation of the promoter region of *BRCA1* in patients with invasive ductal breast cancer, as detected by ctDNA, has also been associated with poor survival [82]. More recently, Janku et al. studied the utility of identifying mutations through ctDNA in determining the survival of patients with various types of cancer, including breast cancer [83]. They demonstrated that higher percentage of mutant ctDNA (> 1%) was associated with shorter overall survival, irrespective of the type of mutation. These findings were in agreement with a subsequent analysis of data from the BOELRO-2 trial in which patients

with *ESR1* mutations (Y537S and D538G) in ctDNA were shown to have shorter survival compared to those without such mutations [74].

Predicting response to therapy

It has been shown in mouse models that the ctDNA level increases immediately after chemotherapy in breast cancer [84]. However, long-term changes in ctDNA level are more important in assessing response to therapy, because many patients develop recurrent or metastatic disease several years after adjuvant therapy [5,6]. Shaw et al. studied 65 cases of breast cancer and 8 healthy female controls to identify copy number variations (CNVs) or single nucleotide polymorphism (SNP) calls using Nexus Copy Number 5.1 Discovery Edition and SNP 6.0 Arrays [85]. They showed that 6–9 years after primary tumor, patients in the cancer group had higher levels of ctDNA containing CNVs and SNP calls compared to the healthy group. While authors noted that these CNVs and SNP calls were concordant with those found in the primary tumor, they also observed that in those patients who eventually had a recurrence, higher levels of changes in CNVs were detected between subsequent ctDNA samples. The majority of these changes were amplifications of known oncogenes including *DUB3*, *BRF1*, *MTA1* and *JAG2*. Authors proposed that this information could be used to design targeted chemotherapy regimens for these patients [85].

Fiegl et al. analyzed the *RASSF1A* methylation identified in ctDNA of 148 patients with breast cancer before and 1 year after surgery and treatment with Tamoxifen as adjuvant therapy and they showed that *RASSF1A* methylation was an independent predictor of poor outcome (relative risk for relapse and death was reported as 5.1 and 6.9, respectively). They concluded that measurement of *RASSF1A* methylation in plasma DNA allows monitoring for the efficacy of systemic therapy [86]. Later studies confirmed that *RASSF1A* methylation modulates the effect of docetaxel in breast cancer therapy [87], and that its levels positively correlate with estrogen receptor expression in breast cancer [88].

More recently, Schiavon et al. showed that *ESR1* mutations can be detected using ctDNA of breast cancer patients [76]. *ESR1* mutations are known to cause resistance to aromatase inhibitors (AIs), and *ESR1* mutation analysis after prior AI therapy can direct the choice of endocrine therapy in patients with hormone-receptor-positive patients [89]. This study was conducted on 171 women with advanced breast cancer, showed that ctDNA assays for *ESR1* are as accurate as tumor biopsies and women with *ESR1* mutation found in their ctDNA had a shorter progression-free survival period upon AI-based therapy (hazard ratio = 3.1). A more recent study also showed that NGS can be used to accurately monitor the response to therapy, through mutation or gene amplification; they demonstrated this in patients with various mutations including *ESR1*, *HER2*, etc [90]. On the other hand, Riva et al. showed that in tripple-negative breast cancer patients, neoadjuvant therapy almost always resulted in a decline in ctDNA levels [91].

Lastly, ctDNA mutation analysis can be used as a novel indicator of resistance to therapy, as demonstrated by a recent study of 18 patients in which serial gene-panel ctDNA sequencing showed that in patients with resistance to *HER2* - blockade, a concurrent aplimification in

HER2 levels in the ctDNA can be detected with a sensitivity of 85.7% and a concordance of 82.1% [92].

Capturing intra-tumor genetic heterogeneity

A single case of breast cancer may be associated with more than one somatic mutation, and the repertoire of mutations may change during the course of therapy. In a proof-of-principle study, De Mattos-Arruda et al. reported that it is possible to use MPS to capture all primary and metastatic mutations from the ctDNA [49]. This study was conducted on a case of synchronous estrogen receptor-positive/*HER-2*neu-negative, highly proliferative, grade 2, mixed invasive ductal-lobular carcinoma with bone and liver metastases. Somatic non-synonymous mutations were detected in the primary tumor (*CDKN2A*, *AKT1*, *TP53*, *JAK3*, *TSC1*, *NF1*, *CDH1*, *MLL3*, *CTNNB1*, *PIK3C2G*, *GATA1*, *EPHB1*, *ESR1* and *PAK7*), all of which were also detected in the liver metastasis; mutations affecting *FLT4* and *MAP2K2* were present in the liver metastasis, but could not be reliably detected in the primary tumor. All of these mutations were detected in ctDNA. Authors concluded that analysis of ctDNA mirrored the pharmacodynamic response to the targeted therapy as assessed by positron emission tomography-computed tomography in cases where the response was different for different metastatic sites [49].

In contrast, Heidary et al. obtained samples from four tumor foci in a single patient with breast cancer as well as involved lymph nodes and successfully identified all of the primary and metastatic mutations in CTC samples acquired from the bloodstream of the same patient [53]. However, authors observed an unexpectedly low mutant allele fraction in ctDNA samples from the same patient, and concluded that the mechanism of release of ctDNAs may vary across different patients and diseases.

It has also been shown that the genetic profile of breast cancer may change over time, and Gevensleben et al. showed that disease phenotype change at progression can be identified using digital PCR of ctDNA [93].

Comparison with other biomarkers

Several authors have compared the utility of ctDNA in breast cancer with other available biomarkers, to justify or challenge its diagnostic or prognostic value. Previous biomarkers proposed for early diagnosis of breast cancer [94], included circulating caspase and protease activity [56], circulating mRNA [95], exosome [96], circulating nucleosome [56], and circulating tumor cells [53].

The evidence on the comparative effectiveness of these biomarkers is scarce. Roth et al. discovered that the tumor DNA predominantly circulates in the form of nucleosomes, and not as cell-free DNA [56]. They showed that while nucleosome concentrations and ctDNA levels are strongly associated with each other, nucleosome concentration has a better diagnostic value for discriminating healthy patients from those with breast cancer (area under receiver operator curve = 0.934 versus 0.769). They also compared the diagnostic value of ctDNA with that of circulating caspase and protease activity and concluded that

circulating caspase and protease activity has superior diagnostic value, especially for detection of malignant breast cancer.

CTCs have also been used as a biomarker in breast cancer [97]. While Heidary et al. have showed that levels of CTC do not strongly correlate with the burden and dynamics of breast cancer [53], Madic et al. have demonstrated that CTC counts were correlated with overall survival but a similar association was not observed with ctDNA levels [98]. It should be noted, however, that the utility of CTC in diagnosis of breast cancer is limited by the fact that CTCs are frequently apoptotic [99], and high CTC levels are only observed in metastatic disease [100], (CTCs are produced as a result of micrometastases into the bone marrow [16]), while ctDNA can help with identifying breast cancer in earlier stages. Bettegowda et al. studied different types of localized and metastatic cancer and showed that all cases that had detectable CTC in the blood also had detectable ctDNA levels, but the converse was not always true [33].

Exosomes, which are cell-derived vesicles containing DNA, miRNA, mRNA, proteins and enzymes, provide a mechanism for intercellular communication including among tumor cells [101]. It is believed that dysregulation in this communication is involved in pathogenesis of cancer. It has been shown that exosomes play an important role in cancer cell invasion and metastasis, stem cell stimulation, angiogenesis, apoptosis, interaction with immune system cells, and drug resistance [102,103]. Hannafon et al. reported selective encapsulation or elevated enrichment of certain microRNAs in exosomes release in the bloodstream of patients with breast cancer [104], and other studies have reported elevated CEA and CA 15-3 in the exosomes of such patients [103]. Additionally, the presence of ceratin microRNAs in exosomes has been shown to have a strong association with specific subtypes of breast cancer, e.g. miR-373 with triple negative and ER and PR negative cancer [105,106]. However, a head-to-head comparison of ctDNA and exosomes as diagnostic or prognostic modalities in breast cancer has not been performed to date.

Another biomarker frequently used for breast cancer is cancer antigen 15-3 (CA 15-3). While this biomarker has high sensitivity for diagnosis, its levels do not change in association with progression or remission [107]. In contrast, ctDNA levels have been repeatedly shown to correlate with the progression of the disease (see above).

Dawson et al. have compared ctDNA, CA 15-3 and CTC levels in patients with stages I and II breast cancer and showed that all these biomarkers are positively correlated with each other and with disease progression but ctDNA showed superior sensitivity and has a greater dynamic range that correlates with changes in tumor burden [60]. Similarly, Shaw et al. showed that ctDNA shows greater correlation with changes in tumor burden, compared to CA 15-3 and CTC levels [108].

Conclusions

Current evidence suggests that ctDNA levels increase in breast cancer patients, a finding that might be of significant value in identification of micrometastases and monitoring the progression of the disease (see Box 1). It is worth noting that diagnostic and prognostic

value of ctDNA has been discussed for different subtypes of breast cancer, including triple-negative breast cancer (TNBC) [105], luminal breast cancer, [78] and HER2-positive breast cancer [109].

Methods used to measure and analyze ctDNA in the bloodstream span from PCR and next generation sequencing methods to fluorescent assays. Measuring ctDNA can also provide a noninvasive approach for identifying the carcinogenic mutations or epigenetic variations, thereby leading to initiation of gene-specific therapy at earlier stages of the disease. This could potentially allow for genetic and epigenetic evaluation of cancer through a liquid biopsy, i.e. without the need for direct biopsy.

Nevertheless, the prognostic value of ctDNA as well as its role in monitoring the progression of the disease is still open to question.

Technical issues surrounding obtaining, using and analyzing ctDNA samples and the lack of standards in its measurement and interpretation are two of the most important factors limiting the use of ctDNA in breast cancer diagnosis and therapy at this time [110–112]. Additionally, while several studies show that ctDNA levels rise in breast cancer patients, and decline in response to therapy, the actual mutations identified in the ctDNA may often be discordant with what is found through direct biopsy [113], and tumor heterogeneity may hinder the value of ctDNA quantification as a measure of tumor burden [114]. Large-scale prospective studies are needed to evaluate the effectiveness of ctDNA as a diagnostic and prognostic measure for breast cancer, e.g. in reducing mortality or reducing morbidities and cost of care.

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Box 1**High level summary of evidence in the utilization of ctDNA for breast cancer diagnosis and treatment****Methods of identification**

- Radioimmunoassay
- Polymerase chain reaction (PCR), including quantitative PCR (qPCR), droplet digital PCR (ddPCR) and Beads, Emulsion, Amplification and Magnetics (BEAMing)
- Next generation sequencing (NGS)
- Massively parallel sequencing (MPS)
- Methylation assay
- Fluorescent assay

Diagnostic characteristics

- Plasma-level of ctDNA
- Fragment size (“integrity index”)
- Genetic and epigenetic profile of the ctDNA

Utility for monitoring disease progression and response to therapy

- Changes in the ctDNA level in plasma
- Changes in the mutations identified through ctDNA
- Identification of treatment resistance through ctDNA sequencing
- Capturing intra-tumor genetic heterogeneity
- Potential association with survival

Key limitations

- Lack of standardized in measurement and interpretation
- Discordance of ctDNA genetic content with primary tumor biopsy results
- Scarcity of longitudinal trials with long follow-up periods

Table 1

Summary of studies on the use of ctDNA for identification of breast cancer.

Study	Disease stage	ctDNA concentration or fraction	ctDNA quantification or evaluation method	Genetic markers identified
Leary et al. [57]	Stage IV	1.4–47.9%	Digital PCR with PARE biomarker-specific primers	MDM4, NFASC, CAMK1G, STK24 and amplification in ERBB2 and CDK6
Huang et al. [17]	Stages I–IV	65 ng/mL (9–566 ng/mL)	Real-time quantitative PCR	Not done
Bettegowda et al. [33]	Stages I–IV	1000–10,000 mutant fragments/5 mL	Digital PCR, WGS	NOTCH1, TP53, AKT1, FBXL4, PIK3CA, EIF4B
Zanetti-Dallenbach et al. [58,68]	Stages I–IV	41,149 (3644–192,482) GE/mL	Real-time quantitative PCR	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene
Roth et al. [56]	Stages I–IV	Only reported for subgroups	PicoGreen assay	Not done
Agassi et al. [52]	Stages I–III	1010 ± 642 ng/mL	Direct Fluorescence assay	Not done
Catarino et al. [59]	Stages I–IV	105.2 ng/mL (average)	Real-time quantitative PCR	Not done
Dawson et al. [60]	Stages I–III	5.18–119746.38 copy/mL	Digital PCR, tagged amplification deep sequencing	TP53, PIK3CA, CD1A, IQCA1, MET, ZFYVE21, KIAA0406
Gong et al. [61]	Stages I–III	471 ng/mL (cut off value)	Real time PCR	GAPDH

PARE: personalized analysis of rearranged ends; PCR: polymerase chain reaction; WGS: whole genome sequencing; GE: genome equivalent.