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REVIEW ARTICLE

Advances in liquid biopsy using circulating tumor cells and circulating cell-free tumor DNA for detection and monitoring of breast cancer

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Abstract

Overview the progress of liquid biopsy using circulating tumor cells (CTCs) and circulating cell-free tumor DNA (cfDNA) to detect and monitor breast cancer. Based on numerous research efforts, the potential value of CTCs and cfDNA in the clinical aspects of cancer has become clear. With the development of next-generation sequencing analysis and newly developed technologies, many technical issues have been resolved, making liquid biopsy widely used in clinical practice. They can be powerful tools for dynamic monitoring of tumor progression and therapeutic efficacy. In the field of breast cancer, liquid biopsy is a research hot spot in recent years, playing a key role in monitoring breast cancer metastasis, predicting disease recurrence and assessing clinical drug resistance. Liquid biopsy has the advantages of noninvasive, high sensitivity, high specificity and real-time dynamic monitoring. Still application is far from reality, but the research and application prospects of CTCs and cfDNA in breast cancer are still worth exploring and discovering. This article reviews the main techniques and applications of CTCs and cfDNA in breast cancer.

Keywords Circulating tumor cells (CTCs) · Circulating cell-free tumor DNA (ctDNA) · Breast cancer · Liquid biopsy

Introduction

Breast cancer is the most common malignancy in women worldwide and represents the leading cause of cancer-related death next to lung cancer in women younger than 45 years [1]. With the development of molecular biology technology, the concept of diagnosis and treatment of breast cancer has gradually changed from being classified based on pathological morphology to molecular typing such as the expression of human epidermal growth factor receptor 2 (HER2). Acceptable anti-HER2-targeted therapies further demonstrate the importance of molecular typing in individualized treatment. The molecular and phenotypic characteristics of CTCs are more important than simply counting these cells. Many currently available methods for cancer evaluation also suffer from technical limitations in that they often lead to false-negative results; for traditional tumor biopsies often fail to reflect the complete cancer gene expression profile, making it difficult to monitor the true tumor status dynamically [2, 3]. Therefore, 'liquid biopsy' has attracted substantial interest in recent years. Besides CTCs, ctDNA is the second most investigated analysis of liquid biopsies [4]. Liquid biopsy provides a real-time assessment of breast cancer (MBC) and holds the promise to help select treatment regimens and monitor the treatment efficacy. From 2015 to 2018, many improvements in liquid biopsy have been carried out and some novel testing tools have been introduced in the field of liquid biopsy, which itself deserves an updated review.

In this review, we highlight the latest advances in CTCs and cfDNA in breast cancer. For the purposes of discussion, the development of detection techniques and the clinical application of breast cancer are discussed separately according to the category of liquid biopsy, and some recent examples of related research in the literature are used. Our goal is to provide a comprehensive overview covering existing challenges and the latest developments.

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Biological characteristics of liquid biopsy

Circulating tumor cells

In 1869, Ashworth, an Australian scholar, found similar tumor cells in the peripheral blood of one patient with metastatic disease and first proposed the concept of CTCs [5]. CTCs are present in the blood of many patients with solid tumors. Most of these cells, which are thought to be involved in metastasis, die in the circulation, presumably due to the loss of matrix-derived survival signals or circulatory shear stress [6]. One important process involved in tumor metastasis, contentiously known as EpCAM-based CTCs capture, is the epithelial–mesenchymal transition (EMT). However, there are currently very few data that can directly demonstrate the existence of the EMT process in CTCs [7]. Other than EMT, the existence of cancer stem cell (CSC) subpopulation in CTCs is another potential phenomenon worthy of exploration. Recent evidence suggests that EMT plays a role in expanding the pool of tumor cells with CSC features. In a recent study of detecting the expression level and heterogeneity of CSC markers in breast cancer [8], Markiewicz et al. found that CSC markers were enriched in primary tumors, metastatic lymph nodes and CTCs with mesenchymal features, but their heterogeneity was decreased in metastatic lymph nodes. In addition, the mesenchymal CTCs phenotype was correlated with poor prognosis of the patients. Boulding et al. [9] explored the role of LSD1 in global epigenetic regulation of EMT, CSCs, tumor microenvironments and therapeutic resistance in breast cancer and found that LSD1 activation promoted inducible EMT programs and modulated the tumor microenvironment in breast cancer. Some other studies [10] reported that PTBP3-mediated regulation of ZEB1 mRNA stability promoted EMT in breast cancer. Another interesting observation is the CTC-clusters, also known as circulating tumor microemboli (CTM). CTC-clusters may represent one of the key mechanisms initiating the metastasis process [11]. However, a series of pathophysiological events by which CTC-clusters originate enter the circulation and reach the distant sites remain to be identified. Giuliano et al. [12] discussed the potential mechanism underlying the formation and spread of CTC-clusters and the role of CTC-clusters in these processes. Data suggested that CTC-clusters may have 100 times more metastatic potential than single CTCs. King et al. [13] systematically studied the physical characteristics of CTC-clusters of 2–5 tumor cells composed of CTCs and provided evidence that CTC-clusters could enhance the metastatic potential.

Enrichment and analysis of CTCs

In recent years, there has been great interest in innovating technologies to detect CTCs, but the isolation of rare CTCs in a large number of blood samples has become a technical challenge due to the scarcity of CTCs. Therefore, it is imminent to develop a highly efficient and highly sensitive method to capture highly active CTCs. Most currently available techniques used to enrich and detect CTCs are based on one or more characteristics of CTCs to distinguish them from surrounding normal blood cells such as biological characteristics (surface protein expression, the presence of mutations, the expression of specific genes, viability and invasiveness) and/or physical properties (size, density, pH, charge and deformability). It is noteworthy that most prior arts for isolation of CTCs are based on the expression of epithelial cell adhesion factor (EpCAM), a protein marker that is expressed on the surface of epithelial origin cells. CellSearch® (Veridex, LLC, Raritan, NJ, USA) is the only system currently approved by the Food and Drug Administration (FDA) for clinical use in a number of systems [14]. The CellSearch TM system is an EpCAM-based immunocapture system used to monitor patients with metastatic cancer [15–17]. In the process of metastasis, EMT occurs in CTCs [18], reducing the adhesion between epithelial cells to migrate and invade and become mesenchymal cells [19]. The antigenic expression of CTCs is down-regulated or lost during EMT, and some CTCs cannot be detected by classical detection methods such as CellSearch TM. In addition, the system is expensive and limited by the expression of membrane surface markers. Therefore, the negative enrichment technology of CTC-iChip came into being. This technology first uses the magnetic beads covering the surface proteins (CD45 and CD15) that can identify immune cells to mark leukocytes so that CTCs can be obtained from the blood sample when they pass through the microfluidic chip by washing out red blood cells, platelets, plasma proteins and the remaining beads [20]. Additionally, a variety of technologies can distinguish CTCs from nonspecifically captured cells, such as cytomorphological characterization of CTCs [21], immunohistochemical/immunofluorescent detection of tumor-specific antigens [22–24] or various real-time polymerase chain reaction approaches. CTCs technological innovation is an important direction in the field of CTCs research. Ates et al. [25] used two covalent and two bioaffinity antibody immobilization methods to assess their CTCs capture efficiency and selectivity, and an anti-epithelial cell adhesion molecule (EpCAM) as the capture antibody. They found that both methods could provide high selectivity for CTCs with EpCAM expression and also demonstrated that antibody immobilization via EDC-NHS reaction in a microfluidic channel could achieve high capture efficiency and selectivity.

Jan et al. [26] reviewed the advantages and disadvantages of the fourth-generation technology and used new cell substrate concept to improve the capture efficiency of CTCs. The University of Wisconsin team used the CapioCyte technology to capture CTCs using EpCAM, HER2 and EGFR

antibodies and identified CTCs using immunofluorescent staining (CK/CD45/DAPI) [27]. So far many techniques for separating CTCs have been developed [28–31].

Compared with single CTCs, CTC-clusters are even rare in the circulation, but they have a value of predicting metastasis 23–50-fold as high as that of CTCs [18]. At present, there are some devices that can separate CTC-clusters [32–35]. Wang et al. [36] conducted a novel time-dependent analysis of longitudinally collected CTCs and CTC-clusters and found that CTC-clusters added additional prognostic values to CTCs enumeration alone, and a larger-size CTCs cluster conferred a higher risk of death in MBC patients. Recently, a Korean team succeeded in prolonging the survival rate in a mouse model of breast cancer by dissociating CTC-clusters using urokinase [35], which is the first step in CTCs cluster-related therapy. In addition, CTCs in clusters with elevated CTCs have also been shown to have poor prognosis in both prospective cohorts of advanced or metastatic breast cancer [37, 38]. Sofia Gkountela et al. found that treatment with FDA-approved Na+/K+-ATPase could dissociate CTC-clusters and suppress metastasis [39]. These studies demonstrated the metastatic potential and oncogenic profile of CTCs and CTC-clusters by combining the genetic features of tumor metastasis and CTCs with primary tumors. The CTCs counting is critical to address prognostic interpretation and treatment response. Comparison of methods for CTCs detection and analysis is shown in Table 1.

Ex vivo culture of CTCs

In vitro culture of CTCs is a challenge due to their low numbers in the peripheral blood of most cancer patients. Epithelial tumor cells are difficult to culture, even when starting with millions of tumor cells. The reason may lie in the pretreatment of blood samples, irreversible cell injury during enrichment and sorting and inadequate culture conditions [47]. In the past few years, several groups have achieved important advances in vitro cultures of circulating tumor cells [31, 48–51]. Wu et al. [52] described a one-step microfluidics-based immunomagnetic isolation method to isolate CTCs directly from the whole blood of lung adenocarcinoma patients. This method avoids harsh sample preparation and enrichment steps and therefore preserves the viability (> 90%) of CTCs during the in vitro isolation. Sobral-Filho et al. [53] reported a method for direct visualization of nanoparticle-coated CTCs under dark field illumination, and their work validates the use of metallic nanoshells as labels for direct visualization of CTCs. Targeted molecular and functional analysis in vitro cultured CTCs has received tremendous attention. In vitro and vivo culture of CTCs provides both functional and important clinical information about the use of anti-metastatic drugs in personalized therapies for cancer patients.

HER2 diagnostics in CTCs

Anti-HER2 therapies are prescribed according to the HER2 status of the primary tumor. Increasing evidence indicates that the HER2 status may undergo changes over time during relapse or progression of breast cancer [54, 55]. In such a case, re-evaluation of the HER2 status by the molecular characteristics of CTCs is a potential clinical application. An optimal individualized treatment regimen can be selected by comparing the HER2 status in CTCs with that in primary tumor tissues [56–58]. In a recent randomized study, CTCs from the peripheral blood were enriched by size-based

Table 1 Different technologies to isolate CTCs

Methods	Platforms	Advantages	Disadvantages	References
Technologies based on density, size, others	Microfluidic chip	High throughput, high sensitivity and high specificity	no uniform standard	[24, 34]
	Ficoll, OncoQuick	Fast processing time	General preprocessing steps as other enrichment methods	[21, 40]
	ISET, CTC-biopsy	High recovery	Lower specificity	[41, 42]
	DEP array	High specificity can maintain cell activity better	The range of isolated cells is small	[43, 44]
Selection for tumor-specific markers	CellSearch, microfluidic CTC-chip, Adna test, MACS, RARE	FDA certification; wide range of applications; good recovery	Rely on EpCAM; CTCs experiencing EMT process are missed	[22–24, 45]
Negative depletion of leukocytes	CTC-iChip, immunomagnetic separation	Does not rely on EpCAM; applies to all types of cancer detection CTCs	CD45/CD15-positive CTCs were missed; low specificity	[20]
Cluster isolation	Cluster-chip	label-free, physical capture of circulating tumor cell clusters	Not commercially available	[18, 32–36]
Nucleic acid analysis	PCR, RT-PCR, ICC, FISH, single cell sequencing et al.	High sensitivity	Not quantifiable, biased CTCs detection	[28, 46]

separation and then cultivated in vitro; they then observed the CTCs character by qPCR by focusing special attention on the HER2 and ESR status. Interestingly, the HER2 and ESR status of CTCs differed from that of the primary tumor. The HER2 status in CTCs changed not only from HER2 + to HER2 in 50% patients but from HER2 to HER2 + in 33% patients [59]. Given the efficacy and high cost of anti-HER2-targeting drugs, real-time monitoring of HER2 status is particularly important for guiding the use of targeted drugs, knowing that there is an inconsistency in the expression of HER2 in traditional tissue biopsies and CTCs.

Circulating tumor DNA (ctDNA)

Circulating free DNA is a DNA double-stranded fragment, usually in the form of a protein complex, and the size of ctDNA fragments varies from 70 to 200 bp and is usually larger than that of non-tumor cfDNA [60]. The majority of cfDNA is derived from apoptotic and necrotic cells that release their fragmented DNA into the circulation. cfDNA detection is a new method of detecting tumors at the molecular level and therefore has a broad application prospect [61]. But, we still need to know more about the biology behind the release of cfDNA into the circulation. Moreover, the clearance of cfDNA is also not fully understood. cfDNA has a short half-life of less than 2 h and is cleared through the liver and kidneys [62]. Renal dysfunction affects cfDNA clearance [63], which might be a confounding factor modulating the concentration of cfDNA in some patients with cancer. ctDNA can be taken up by host cells, and this uptake affects the biology of these cells.

Under physiological conditions, the release of cfDNA is not only due to necrosis and apoptosis, but also due to inflammation, trauma or exhaustive exercise. For cancer patients, cfDNA is a DNA fragment derived from tumor cell apoptosis, necrosis or secretion released into the blood circulation, also called ctDNA [64, 65]. Intriguingly, ctDNA retains the genetic variant and epigenetic features of tumor tumors, such as mutations, insertions, deletions, rearrangements and DNA methylation of oncogenes or tumor suppressor genes, making it a potential biomarker [66]. PIK3CA and TP53 are the two most commonly mutated genes in breast cancer. In particular, ctDNA contains the same mutations and genomic rearrangements as the primary solid tumors. These mutations and rearrangements drive the development and progression of cancer, so ctDNA can serve as a potential tumor marker [67].

ctDNA detection systems

The cfDNA in the peripheral blood has a low content and is easily degraded. Therefore, an efficient and accurate cfDNA detection system is very important. ctDNA analysis is highly influenced by pre-analytics. Clinical laboratories can detect cfDNA in a variety of different body fluid samples. Current studies have shown that the best type of specimen is plasma, and it is best to use an anticoagulation tube containing cell stabilizer or an EDTA anticoagulation tube to collect blood. There is already a technical document reading specifications for pre-analytical procedures for cfDNA isolation: CEN/TS 16835-3:2015 Molecular in vitro diagnostic examinations - Specifications for pre-examination processes for venous whole blood - Part 3: Isolated circulating cell free DNA From plasma. The pretreatment of specimens should follow the principle of fast and stable and adopt different schemes for different specimens to ensure the concentration and stability of the substances to be tested as much as possible to ensure the quality of the test.

Nucleic acids in single cells do not meet the mg-level sample requirement for sequencing. In order to obtain more information from a small number of samples, such as whole transcriptome amplification (WTA) and whole genome amplification (WGA) came into being [68].

Direct ALU-qPCR is a simple, robust, highly sensitive and high-throughput method for measuring the integrity of free circulating DNA in serum. Iqbal et al. found that the mean serum DNA integrity, ALU 247 level and ALU 115 level were significantly higher in patients than those in healthy females [69–71]. The detection of cfDNA content and integrity has a certain clinical value in the diagnosis and treatment of breast cancer. However, the lack of standardized testing procedures has hindered its widespread clinical application.

Detection of tumor-specific mutations in cfDNA of breast cancer patients, including somatic point mutations (such as PIK3CA, TP53 and ESR1), DNA methylation (such as APC, BRCA and RASSF1A) and DNA loss, is recognized as the presence of ctDNA [72]. The current methods of detecting ctDNA mainly include digital polymerase chain reactions (dPCR) and next-generation sequencing (NGS), RNAseq [73–76]. Thress et al. [77] used two non-digital platforms (cobas® EGFR Mutation Test and therascreenTM EGFR amplification refractory mutation system assay) and two digital platforms (Droplet DigitalTM PCR and BEAMing digital PCR [dPCR]). It was found that these methods have advantages and disadvantages, and different detection methods are suitable for the corresponding mutation type. In addition, dPCR can only analyze a limited number of single base-pair changes, and the flux is relatively low. Therefore, dPCR can currently only be used for hot spot mutation detection and cannot be applied to the discovery of new mutations. It is worth noting that the ARMS method is currently approved by the China Food and Drug Administration (CFDA) for clinical ctDNA testing and is relatively popular in

clinical practice. While dPCR and mass spectrometry-based nucleic acid detection methods are limited to laboratories, it is still difficult to enter clinical practice.

Next-generation sequencing (NGS) provides a reliable means for qualitative analysis of ctDNA. Second-generation sequencing can identify both known and unknown mutation sites and perform individualized monitoring through comparison with existing cancer gene databases after whole exon or genome sequencing. A recent study [72] reported CAPP-Seq for cancer searches for recurrent mutation-associated exons in non-small cell lung cancer (NSCLC) by searching the catalogue of somatic mutations in cancer (COSMIC) and then the cancer gene database (the cancer Genome atlas, TCGA) whole genome sequencing result screening. This study has shown that a ctDNA ratio even as low as 0.02% can be detected by this method.

The ctDNA detection and analysis technologies based on PCR or NGS have their own advantages: The former is higher than the latter in detection sensitivity and specificity; on the contrary, the latter can detect new mutations. Therefore, we should make a reasonable choice or a match between the two based on the actual situation. Despite the limitations of ctDNA analysis, ctDNA detection can provide very important reference information. It is worth noting that the current detection of ctDNA for non-small cell lung cancer EGFR-targeted therapy documented by NCCN. In the future, with more advanced technology and more clinical evidence, more ctDNA will be detected for the concomitant diagnosis of targeted drugs. The assessment of ctDNA levels may also be an important indicator of prognosis. Prospective studies in larger cohorts of patients are required to validate the role of ctDNA as a prognostic biomarker.

Current research shows that in addition to CTCs and cfDNA, exosomes and miRNAs are also the popular research object in the field of liquid biopsy [78, 79]. Detection of exosomes and miRNAs is important in the diagnosis and treatment of tumors. Exosomes are vesicle-like bodies that are actively secreted extracellularly by a variety of cells, including cancer cells and normal cells, with a diameter of 30–100 nm. It carries important information such as proteins, lipids, DNA and RNA to mediate intercellular communication [80]. Wang et al. found that exosomal protein CD82 expression may be used as a new biomarker for breast cancer diagnosis [81]. MicroRNA (miRNA) is a short noncoding RNA of a posttranscriptional regulatory gene, which plays an important role in normal physiological activities. It acts as a carcinogenic or tumor suppressor regulator. Son et al. reported that miR-374a-5p promoted tumor progression in triple negative breast cancer by directly targeting arrestin beta 1 [82]. It can be seen that exosomes and miRNAs are of great significance and broad application prospects in clinical treatment.

Potential clinical applications of liquid biopsy in breast cancer

More studies have shown that the emergence of CTCs is closely related to cancer metastasis. CTCs can be used as a biomarker for evaluating the development of cancer, detecting the existence of distant tumors and predicting the prognosis. In recent years, cfDNA has been considered as another substance besides CTCs for the diagnosis of noninvasive molecules in cancer. In breast cancer, relevant studies have focused on the cfDNA concentration, DII, methylation changes, gene changes and exosomes [83]. Tuomela et al. [84] also found that DNA derived from apoptotic cells could increase the invasiveness of breast cancer cells. For the first time in the New England Journal of Medicine, Dawson et al. [54] reported the use of circulating tumor DNA in monitoring the prognosis of breast cancer patients.

Prognosis assessment

Technology for detecting CTCs has continued to develop over the years. With the development of technology, the role of CTCs has been further clarified. CTCs can be as a prognostic factor in metastatic breast cancer patients. Using both the CellSearch® and the newly developed filtration-based platform, Huebner et al. [85] analyzed the correlation of CTC-positivity between both methods and their ability to predict prognosis in a prospective clinical study. They found that overall survival (OS) of patients without detectable CTCs was significantly higher than that of patients with one or more CTCs, regardless of the method used ($p < 0.001$), confirming that the presence of CTCs was a strong predictor of poor prognosis in patients with metastatic breast cancer.

Multiple prospective and retrospective trials have supported the adverse prognostic significance of ≥ 5 CTCs for progression-free survival (PFS), and OS. Bidard et al. [86] summarized 1944 patients with metastatic breast cancer in a recent pooled analysis and created a clinic pathological prognostic model to determine the added impact of CTCs for PFS and OS. Their multivariate analysis showed that the CTCs count was the strongest prognosticator for PFS, and OS. Bidard et al. [87] conducted a meta-analysis in nonmetastatic breast cancer patients treated by neoadjuvant chemotherapy and found that the CTCs count was an independent and quantitative prognostic factor in early breast cancer patients treated by neoadjuvant chemotherapy. This is a supplement to the current prognostic models based on tumor characteristics and response to therapy. On January 1, 2018, the clinical value of CTCs testing was further clarified in addition to retaining the CM0 (I +) staging system in the globally activated AJCC Eighth Cancer Staging System [88]. The latest guidelines list CTCs as another breast cancer prognostic assessment tool in addition to the four biological

markers (ER/PR, HER2, Ki67 and histological grading), suggesting that the presence of CTCs in the peripheral blood of breast cancer patients may predict a poor prognosis [87].

In addition, a small number of studies showed that ctDNA detection was earlier than clinical detection of distant metastases. Olsson et al. [46] used ddPCR to continuously monitor ctDNA in 20 patients with early stage breast cancer and found that the accuracy of predicting tumor recurrence by monitoring ctDNA was as high as 93% and was 11 months earlier than the clinically found recurrence and metastasis. Undoubtedly, this is of great value for the prediction of recurrence of early breast cancer.

A research team from Cambridge, England [89], conducted long-term follow-up observations on 30 patients with metastatic breast cancer and compared the biological indexes of ctDNA, carbohydrate antigen 15-3 and CTCs with prognosis. They found that patients with high levels of ctDNA and CTCs were at high risk for death, and that ctDNA had higher specificity and sensitivity than CTCs. These findings suggested that ctDNA might be superior to CTCs in assessing patient outcomes, though more multicenter clinical data are needed to verify the conclusion. Rossi et al. [90] suggested that comprehensive liquid biopsy analysis may represent a tool to assess both tumor burden and molecular features and believed that liquid biopsy may become an effective tool for predicting the prognosis of patients with metastatic breast cancer.

Klaus Pantel, who is universally recognized as the father of CTCs, published an important review in 2018, in which they summarized the progress of liquid biopsy in screening early cancer, predicting relapse and assessing resistance and discussed the role of ctDNA and CTCs in the diagnosis and treatment of early cancer [91]. Thus, we believe that combining CTCs and ctDNA with traditional tumor markers can increase the sensitivity and specificity of cancer detection, which may be more helpful for clinical applications.

Drug resistance test

Drug resistance is a core challenge in cancer research, but the degree of heterogeneity in resistance mechanisms in cancer is unclear. Paoletti et al. [92] conducted NGS of CTCs from patients who experienced disease progression after targeted therapy and found 85% concordance in at least one or more prioritized somatic mutations and CNA between paired CTCs and tissue metastases. Khoo et al. [51] presented a reliable label-free approach to efficiently monitor drug response of patients via serial sampling of patient-derived CTCs cultures in an integrated microfluidic device. Yu et al. [49] maintained that drug sensitivity testing of CTCs lines with multiple mutations could help detect potential new therapeutic targets.

Individualized treatment

Endocrine therapy and anti-HER2-targeted drug therapy are important means for individualized breast cancer treatment. Many studies reported that with disease progression, estrogen receptor (ER), progesterone receptor (PR) and the HER2 status underwent different changes in primary tumor sites and metastatic lesions. At the same time, compared with the molecular phenotypes of CTCs, inconsistency has also been found [58, 93]. The molecular phenotype of CTCs can feed back the molecular biological information of tumors in real time, providing a basis for the rational use of drug therapy. The introduction of cfDNA-guided personalized therapy into clinical practice has already begun. ctDNA detection can also be used to predict the risk of recurrent breast cancer patients receiving neoadjuvant chemotherapy and provide a basis for the development of individualized treatment programs [94, 95]. With increasing application of targeted therapies and the development of acquired resistance, more attention has been focused on developing in vitro and in vivo patient-specific tumor models for individualized therapeutic evaluation of cancers.

Conclusion

In the context of precision medicine, liquid biopsy technology quickly penetrates into the basics of oncology and clinical research with its unique advantages. CTCs and cfDNA have potential application value in screening, diagnosis, prognosis and treatment of patients with breast cancer. The prospects for CTCs and cfDNA are very broad, driving the pace of individualized treatment and precision medicine. However, there are still many challenges and problems to be solved in the development process. A large sample of prospective clinical studies needs to further confirm the clinical value of different types and stages of CTCs and cfDNA in breast cancer patients.

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Compliance with ethical standards

Conflict of interest We have declared that no competing interests exist. The authors alone are responsible for the content and writing of the paper.

Ethical approval The study complies with the Declaration of Helsinki and was approved by the Ethics Committee of Affiliated Hospital of Nantong University, and all patients gave written informed consent.

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