



# Advances in the Detection Technologies and Clinical Applications of Circulating Tumor DNA in Metastatic Breast Cancer

This article was published in the following Dove Press journal:  
*Cancer Management and Research*

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**Abstract:** Breast cancer (BC) represents the most commonly diagnosed cancer among females worldwide. Although targeted therapy has greatly improved the efficacy of treating BC, a large proportion of BC patients eventually develop recurrence or metastasis. Traditional invasive tumor tissue biopsy is short of comprehensiveness in tumor assessment due to heterogeneity. Liquid biopsy, an attractive non-invasive approach mainly including circulating tumor cell and circulating tumor DNA (ctDNA), has been widely utilized in a variety of cancers with the advances of sequencing technologies in recent years. The ctDNA that is found circulating in body fluids refers to DNA released from tumor cells and has shown clinical utility in metastatic breast cancer (MBC). With the results of genomic variants detection, ctDNA could be used to predict clinical outcomes, monitor disease progression, and guide treatment for patients with MBC. Moreover, the drug resistance problem may be addressed by ctDNA detection. In this review, we summarized the technological developments and clinical applications of ctDNA in MBC.

**Keywords:** circulating tumor DNA, metastatic breast cancer, genomic variants, drug resistance

## Introduction

Breast cancer (BC) represents the most commonly diagnosed cancer and the leading cause of cancer death among females worldwide.<sup>1</sup> In China, 272.4 thousand women were newly diagnosed as BC each year, bringing heavy medical and financial burden to the whole society.<sup>2</sup> The 2011 St. Gallen conference categorized BC into four subtypes based on the molecular landscape of the tumor, including luminal A, luminal B, human epidermal growth factor receptor-positive (HER2+), and triple-negative (ductal) disease.<sup>3</sup> This surrogate classification for BC subtypes has been shown to successfully predict tumor presenting features, nodal involvement, recurrence patterns, and disease-free survival.<sup>4</sup> Even though the 5-year mortality rate of BC has dropped by 34% since 1990, a number of patients eventually developed resistance to the therapy.<sup>5</sup> For instance, the drug resistance rate of estrogen receptor-positive (ER+)/progesterone receptor-positive BC patients could reach 70%.<sup>6</sup> Meanwhile, the treatment of advanced BC (ABC) remains a challenge due to the common drug resistance.

Tumor tissue biopsy is the gold standard specimen for tumor genotyping. However, tumor tissue biopsy is invasive, painful, and potentially risky, and performing biopsy in some tumor sites may not be convenient.<sup>7</sup> Moreover, in

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metastatic settings it may be short of comprehensiveness due to tumor heterogeneity.<sup>8</sup> Therefore, alternative less-invasive detection methods are desperately required to capture the entire genetic map of the tumor and consequently improve the current treatment options. Liquid biopsy is an attractive non-invasive approach that utilizes readily available body fluids to provide a real-time comprehensive assessment of the tumor, through the analysis of genomic variants in circulating tumor DNA (ctDNA) and circulating tumor cell (CTC).<sup>9</sup> In cancer patients, ctDNA is a small proportion of circulating cell-free DNA (cfDNA) in the bloodstream, which was first described by Mandel et al in 1948.<sup>10</sup> With the rapid developments of sequencing technologies in recent years, the clinical applications of ctDNA in various tumors such as lung cancer, BC, and gastric cancer have been extensively explored.<sup>9,11,12</sup> For BC, ctDNA was shown to provide supplementary information for tumor tissue biopsy in terms of prognosis assessment, drug resistance test, and individualized treatment.<sup>13</sup> Importantly, additional mutations detected by ctDNA could reflect the utility of liquid biopsy to capture heterogeneity of metastatic sites in ABC.<sup>14</sup>

To offer an update of current literature, this review will discuss the recent developments of ctDNA in metastatic BC (MBC) from these aspects: main detection technologies, concordance between ctDNA and tumor tissue biopsy, pre-analytical factors, tumor burden, genomic variants, specific gene mutations, drug resistance, and tumor heterogeneity and sub-clone.

## Main Detection Technologies for ctDNA in MBC

It has been demonstrated that cfDNA can be released from apoptosis, necrosis, and macrophage phagocytosis of the cells, whereas the concentration of cfDNA in the bloodstream of healthy individuals is low (approximately 10 ng/mL).<sup>15,16</sup> The concentration of cfDNA can be increased by certain events like exercise, cancer, and autoimmune disease.<sup>17–19</sup> In patients with BC, the concentration of cfDNA may increase to 0.5, 235, 422, and 1280 ng/mL classified by tumor stage I, II, III, and IV, respectively.<sup>20</sup> However, the concentration of ctDNA or the concentration ratio of ctDNA: cfDNA can be very low given that the majority of cfDNA consists of background germline DNA.<sup>21</sup> Therefore, highly sensitive approaches capable of detecting the genomic profile of limited available

amounts of ctDNA are demanded. So far, diverse detection methods mainly based on Polymerase Chain Reaction (PCR) and Next-Generation Sequencing (NGS) have been applied, while each has its own strength and weakness.<sup>22</sup>

## PCR-Based Technologies

The development of traditional simple PCR has shown some limitations in liquid biopsy such as its low detection sensitivity.<sup>23</sup> Currently, digital PCR (dPCR) is frequently used for the detection of ctDNA because of its high sensitivity and ease of use. Two common dPCR methods, the droplet digital PCR (ddPCR) and BEAMing [beads, emulsification, amplification, and magnetics] assay, can accurately detect point mutations present at frequencies  $\leq 0.1\%$ , nevertheless restricted to the analysis of a small number of genomic loci and the need of “a priori” knowledge of the investigated genomic aberrations.<sup>24</sup> A workflow by means of uniplex or multiplex pre-amplification combined with dPCR was successfully used to investigate the estrogen receptor 1 (ESR1) mutation status of the samples of MBC patients who progressed on endocrine treatment.<sup>25</sup> The combination of allele-specific, asymmetric rapid PCR and melting analysis assay was used to detect the phosphoinositide 3-kinase catalytic subunit alpha (PIK3CA) hotspot mutations (E545K, H1047R) and showed a high concordance (81.1%,  $P = 0.001$ ) with ddPCR.<sup>26</sup> Gyanchandani et al utilized a novel whole-genome amplification of cfDNA via rolling-circle amplification before ddPCR and provided physiological evidence that ctDNA was accessible by fingerstick and sustained presence/absence of mutation detection after whole-genome amplification.<sup>27</sup>

## NGS-Based Technologies

NGS, compared with PCR, can interrogate larger regions such as gene panels without prior knowledge of mutations.<sup>28</sup> The main methods of NGS consist of targeted sequencing, whole-exome sequencing, and whole-genome sequencing.<sup>8</sup> Li et al performed targeted deep sequencing on 17 blood samples from 17 Chinese MBC patients and identified 60 somatic mutations in the blood samples of all patients.<sup>7</sup> MammaSeq, a breast cancer-specific NGS panel, was shown to detect 43 protein-coding mutations in 14 ctDNA samples from 7 patients with ABC, targeting 79 genes and 1369 mutations.<sup>29</sup> Despite of the comprehensive sequencing ability, the error rate of NGS complicates the calling of mutations with variant allele frequencies (VAFs)  $< 5\%$ .<sup>28</sup>

Error correction through random molecular barcodes has been incorporated into NGS ctDNA assays to reduce this error rate, enabling mutation calling with VAFs < 0.1%.<sup>30</sup> Masunaga et al analyzed the cfDNA samples from 34 MBC patients and revealed a higher sensitivity (0.1%) of the molecular barcodes-NGS than NGS without barcode (1%) by reducing background errors.<sup>31</sup> Nevertheless, increasing proportions of false positives and negatives have been reported at low mutant allele frequencies, which brings the issue of detection reliability.<sup>32</sup> Importantly, reproducibility is one of the main caveats of high throughput technologies. The study by Stetson et al suggested that most NGS assay discordance is a result of technical variations.<sup>33</sup> Gerratana et al confirmed the detection performance of a novel NGS platform, PredicinePLUS<sup>TM</sup>, when compared with Guardant360<sup>TM</sup> and that different NGS platforms could be potentially compared provided that certain sample management and analytical requirements are met.<sup>32</sup>

## Hybrid Techniques

Hybrid techniques such as the combination of PCR and NGS allow to circumvent both the sensitivity limits of NGS (1–2%) and the inherent need for “a priori” knowledge of the target of PCR.<sup>22</sup> Studies using hybrid techniques such as synchronous coefficient of drag alteration, tagged-amplicon deep sequencing, and safe sequencing system have been summarized in a previous review.<sup>9</sup> Recently, Keup et al analyzed 17 genes in the cfDNA of 44 MBC patients by a targeted PCR-based NGS approach. This method constructed a library using a customized targeted DNA panel with integrated unique molecular indices before sequencing. The comprehensive analysis of cfDNA variants not only confirmed the benefit of unique molecular indices in the variant verification, but also identified new promising variants with clinical relevance.<sup>34</sup>

## The Concordance Between ctDNA and Tumor Tissue Biopsy

The ctDNA cannot be used to replace the need for metastatic tissue biopsy of patients until it is proven to represent the genomic information detected in solid tissue.<sup>35</sup> A number of studies have investigated the difference between ctDNA and tumor tissue biopsy in MBC and some of them have revealed high concordances (77–89%).<sup>36–40</sup> The remarkable concordance suggests that ctDNA assays can be confidently used to molecularly stratify patients for prognostic and predictive purposes. Furthermore, compared to tumor tissue

biopsy, ctDNA may have the superiority of obtaining more comprehensive mutation information.<sup>41</sup> For example, Maxwell et al found that the proportion of genomic alteration in MBC patients was lower in tumor than in blood (69% vs 91%;  $P = 0.06$ ).<sup>42</sup> Even so, several studies reported significant differences between the genomic results of tumor tissue biopsy and cfDNA in MBC patients.<sup>43–46</sup> Diverse analysis tools, temporal spacing between ctDNA analysis and tumor biopsy, and tumor heterogeneity due to the substantial selection pressure from therapies perhaps contribute to this inconsistency.<sup>43,46,47</sup>

## Pre-Analytical Factors

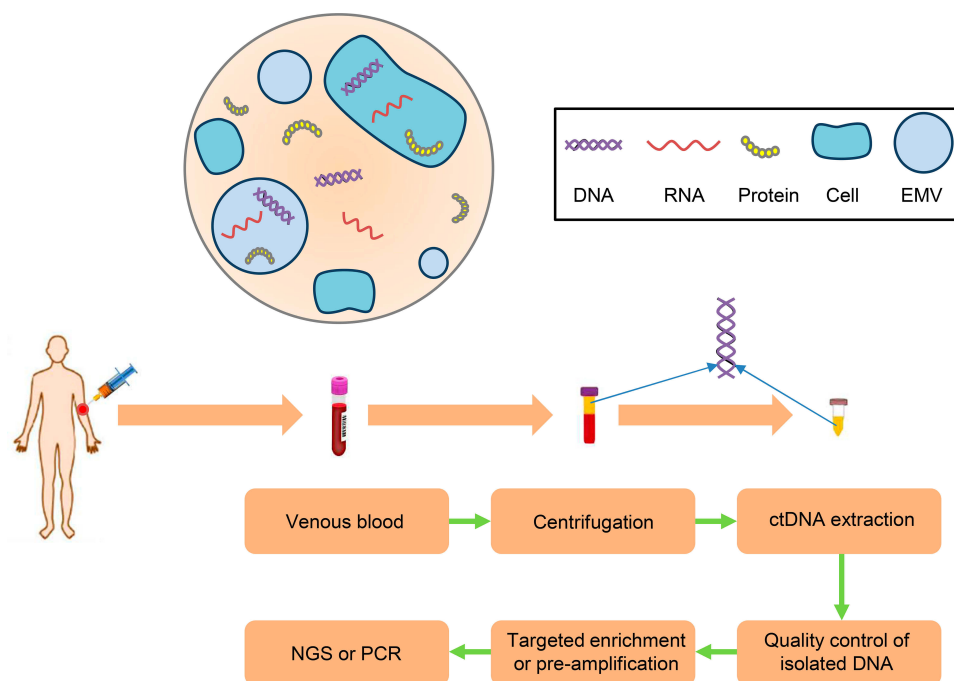
The quality of ctDNA analysis was affected by a lot of pre-analytical factors.<sup>9</sup> Hakan et al reported a significant increase in isolation efficiency with very fresh blood samples of at least 15 milliliters which were processed in minutes.<sup>48</sup> Risberg et al investigated the potential pre-analytical influential factors of ctDNA levels. The results revealed that using cell-stabilization tubes, storing at 4 °C after collection for a short period, and performing the centrifugation in different types of centrifuges greatly improved the feasibility of collecting high-quality specimens.<sup>49</sup> What's more, higher frequencies of ESR1 and PIK3CA mutations in the plasma than in the serum were revealed, indicating that plasma samples might be the preferred source of cfDNA.<sup>50–52</sup> Possible influential factors in each step of the ctDNA detection from sample collection to sequencing are shown in Figure 1.

## Assessing Tumor Burden According to the Quantity and Quality of ctDNA/cfDNA

Tumor burden provides key information about objective response and disease progression.<sup>53</sup> MBC patients with high panel-specific mutational load had a significantly shorter time to progression than those with a low mutational load ( $P = 0.0112$ ).<sup>42</sup> The molecular tumor burden index, a measure of the percentage of ctDNA in samples, was positively correlated with tumor size as evaluated by computed tomography ( $P < 0.0001$ ) and detected disease progression 8–16 weeks earlier.<sup>54</sup>

## The ctDNA Concentration

Dynamic changes in ctDNA levels closely reflect changes in tumor burden and increases in ctDNA levels may predict the progressive disease (PD) several months before



**Figure 1** The whole process of ctDNA analysis and possible influencing factors.

**Notes:** The concentration of ctDNA and mutation frequency are significantly higher in ABC than early BC. Using cell-stabilization tubes, storing at 4°C after collection for a short period, and performing the centrifugation in different types of centrifuges greatly improved the feasibility of collecting high-quality specimens. Plasma samples may be the preferred source of ctDNA than serum. The type of detection technology also affects the results of ctDNA analysis. EMV, extracellular micro vesicle; ctDNA, circulating tumor DNA; ABC, advanced breast cancer; NGS, next-generation sequencing; PCR, polymerase chain reaction.

the standard imaging.<sup>55</sup> In recent years, several methods were used to determine the ctDNA concentration in MBC and investigate its relationship with clinical outcomes. Cheng et al evaluated the ctDNA concentration of MBC patients by measuring the short and long fragments of two repetitive DNA elements, ALU and LINE1, and found that ctDNA concentration could serve as an independent prognostic marker.<sup>56</sup> Li et al used the VAF of mutations from the major mutated clones to access the ctDNA levels of MBC patients. The results showed that a higher ctDNA level in plasma was associated with a shorter progression-free survival (PFS).<sup>7</sup>

## The Cutoff of ctDNA Concentration

A number of studies have demonstrated the prognostic value of a CTC enumeration > 5 at baseline and follow-up in patients with MBC.<sup>55,57,58</sup> Recently, Cristofanilli et al used a CTC threshold of 5 cells per 7.5 mL for the stratification of 2346 MBC patients in a retrospective and pooled analysis of individual patient data from 18 cohorts and confirmed the identification of two subgroups of MBC (Stage IV<sub>indolent</sub> and Stage IV<sub>aggressive</sub>), independent of clinical and molecular variables.<sup>59</sup> Nevertheless, a cutoff of ctDNA concentration that correlates with a worse

prognosis has not been identified yet. A recent retrospective study revealed a statistically significant difference in PFS and overall survival (OS) for baseline %ctDNA (the maximum mutant allele fraction in each sample) < 0.5 vs. ≥ 0.5 ( $P = 0.003$  and  $P = 0.012$ ).<sup>55</sup> Stover et al presented the genomic characterization of a large metastatic triple-negative BC (MTNBC) cohort. A cfDNA tumor fraction threshold of ≥ 10% was associated with significantly worse metastatic survival.<sup>60</sup> In one study by Shatsky et al, ABC patients with at least one alteration with 5% ctDNA had poorer median OS (6.7 months) vs. patients with less than 5% ctDNA (17.9 months) ( $P = 0.01$ ).<sup>43</sup>

## The DNA Integrity

The cfDNA concentration directly reflects the quantity of cfDNA, while the cfDNA integrity (cfDI) represents the quality of cfDNA, which has been investigated as a diagnostic or prognostic marker in multiple cancers.<sup>61–63</sup> In general, cfDI is calculated as a ratio of longer DNA to shorter DNA for ALU repeats (247 and 115 bp). Plasma ALU-247, ALU-115, and DNA integrity may be proven to have clinical utility in BC diagnosis.<sup>64</sup> In metastatic settings, Cheng et al showed a significantly increased cfDI ( $P = 1.21\text{E-}7$  for ALU and  $P = 1.87\text{E-}3$  for LINE1) in both

repetitive DNA elements after one cycle of therapy. In addition, the multiple cox regression model indicated an independent prognostic role of cfDI.<sup>56</sup>

## Detecting Genomic Variants

Besides the ctDNA concentration, the mutation frequency of ctDNA in ABC is significantly higher than that in early BC.<sup>20,23</sup> Analyzing the genomic variants including somatic mutations, somatic copy number alterations (SCNAs), and DNA methylation (DName) may have important prognostic significance.<sup>65</sup>

### Somatic Mutations

Somatic mutations identified in ctDNA are widely representative of the tumor genome as they can provide the spectrum of mutations not limited to a single region of the tumor.<sup>66</sup> In recent years, several studies identified the somatic mutation profiles of ABC.<sup>40,67-69</sup> Chung et al sequenced 62 genes in ctDNA from patients with ER+ MBC and found that the most frequently altered genes were TP53 (38%), ESR1 (31%), and PIK3CA (31%).<sup>40</sup> Ding et al identified HER2 mutations in both the tyrosine kinase (n = 14) and extracellular (n = 4) domains in MBC patients.<sup>67</sup> In a retrospective study by Yi et al, authors determined the frequency and spectrum of common cancer-related gene mutations in ctDNA among different ABC subtypes. 96.0% (96/100) female ABC patients had somatic genomic alterations including copy number variants (CNVs) and point mutations and different subtypes of ABC had distinct features in terms of genetic alterations.<sup>68</sup> In one study by Zill et al, the somatic mutation landscape of 70 cancer genes from cfDNA deep sequencing analysis of 21,807 patients with advanced cancers was depicted. The results revealed the most commonly amplified genes (MYC and FGFR1) and the most common resistance alterations (ESR1 L536/Y537/D538) in ABC.<sup>69</sup>

### Somatic Copy Number Alterations

A study by Stover et al presented the largest genomic characterization of MTNBC to date. They found that certain SCNAs were more frequent in MTNBC patients relative to primary TNBC including copy number gains in drivers NOTCH2, AKT2, and AKT3.<sup>60</sup> Theoretically, all HER2+ patients should show a copy number gain of erb-b2 receptor tyrosine kinase 2 (ERBB2). However, in a study by Yi et al, only 8.8% patients in the HER2+ group showed an ERBB2 copy number gain. The reason

might be that the ctDNA assay was not sufficiently sensitive to accurately detect CNVs.<sup>68</sup> This viewpoint was supported by a recent meta-analysis of Yang et al. The pooled results showed that single nucleotide variants detection through cfDNA from MBC patients had higher sensitivity (0.78 [0.71–0.84]) and specificity (0.92 [0.87–0.95]), when compared to tissue DNA detection. However, the cfDNA-based detection of CNV was shown to have no diagnostic value due to the low sensitivity (0.42 [0.24–0.62]) in the cases of CNV.<sup>70</sup>

### DNA Methylation

DName has been considered as a hallmark of cancer over the last decade.<sup>71</sup> DName means the covalent addition of a methyl group to cytosine within the context of CpG dinucleotide and is chemically and biologically stable.<sup>72,73</sup> Le et al examined the promoter methylation status of a panel of markers in a range of BC cell lines spanning the epithelial-mesenchymal spectrum with methylation-sensitive high-resolution melting. A distinction in profiling DNA methylation was revealed between epithelial and mesenchymal phenotypes.<sup>74</sup> This study provided important insight into selecting an optimal panel of methylation markers to assess minimal residual disease, which is a major determinant of the initiation of metastatic tumors.<sup>75</sup> Panagopoulou et al assessed the methylation status of a panel of cancer-related genes in BC. The methylation of WNT5A was statistically significantly correlated to greater tumor size and poorer prognosis characteristics and in advanced stage disease with shorter OS. In the metastatic group, SOX17 methylation was significantly correlated to shorter PFS and OS.<sup>76</sup> These studies highlighted that DNA methylation should be further evaluated as a potential liquid biopsy-based biomarker in metastatic settings.

## The Significance of Detecting Specific Gene Mutations

The identification of specific point mutations such as PIK3CA and ESR1 in BC may provide key information for the selection of treatment regimens.<sup>77</sup> In MBC, the mutation profile of specific genes could be utilized to predict clinical outcomes. Importantly, crucial adjustments of treatment regimens could be made corresponding to the changes of genetic information.<sup>78</sup> The detection sites and clinical relevance of detecting specific gene mutations in the ctDNA of ABC patients from studies in recent years are summarized in Table 1.



**Table I** Selected Studies Detecting Specific Gene Mutations in Recent Years

Mutant Gene	Detection Sites	Researcher	Study Type	Patients Classification	Clinical Relevance
PIK3CA	Not specific	Ma et al <sup>45</sup>	Phase I study	HER2+ MBC	Predicting the efficacy of pyrotinib
	R88Q, R93Q/W, K111E/N, G118D, E365K, C420R, E542K, E545G/K, Q546K, and H1047L/R/Y	Baselga et al <sup>36</sup>	Phase III study	HR+HER2-ABC	Associated with longer PFS in patients with PIK3CA mutation than patients without PIK3CA mutation
	E542K, E545K, and H1047L/R	O'Leary et al <sup>82</sup>	Phase III study	HR+HER2-ABC	Predicting PFS of ABC patients on palbociclib and fulvestrant.
	E542K, E545K, and H1047R	Park et al <sup>81</sup>	Phase II study	MTNBC	Revealing PIK3CA mutational status in cfDNA from blood samples of MTNBC
	E542K, E545K, and H1047R	Takano et al <sup>65</sup>	Phase II study	HER2+ MBC	Exploring the predictive value of the differential benefit of anti-HER2 drugs
	E542K, E545G/K, Q546K, M1043I, and H1047 L/R/Y	Di Leo et al <sup>37</sup>	Phase III study	HR+HER2-ABC	PFS was in favour of buparlisib plus fulvestrant versus fulvestrant alone in patients with PIK3CA mutations
	E545K, and H1047R	Li et al <sup>84</sup>	Formal-prospective /retrospective study	ABC	Associated with shorter median PFS and worse OS
	E542K, E545K, and H1047L/R	Kodahl et al <sup>38</sup>	Observational study	ABC	Changes in PIK3CA ctDNA level correlated with treatment response
	E545K, and H1047R	Tzanikou et al <sup>26</sup>	Observational study	MBC	Concordance of PIK3CA hotspot mutations between ctDNA and CTCs
ESR1	E542K, E545K, and H1047R	Jacot et al <sup>83</sup>	Observational study	ER+/HER2-ABC	Four-week persistence of cfDNA PIK3CA mutation appears highly correlated with PFS
	E380Q, D538G, and Y537S/N/C	Takeshita et al <sup>94</sup>	Retrospective study	ER+ MBC	Prevalence of ESR1 mutation in Japanese MBC patients
	E380Q, D538G, I298N, V560E, and Y537S/N	Yi et al <sup>68</sup>	Retrospective study	ABC	Positively related to the line of endocrine therapy
	D538G, and Y537S/N/C	Beije et al <sup>93</sup>	Observational study	MBC	Conferring endocrine resistance
	D538G, and Y537S/N	Takeshita et al <sup>46</sup>	Retrospective study	MBC	Independent distribution of ESR1 mutations between plasma and tumor tissue
	E380Q, D538G, S463P, L536R, and Y537S/N/C	O'Leary et al <sup>82</sup>	Phase III study	HR+HER2-ABC	ESR1 ctDNA dynamics offering limited prediction of clinical outcome.
HER2	D538G, and Y537S/N/C	Allouchery et al <sup>86</sup>	Retrospective study	HR+ MBC	ESR1 mutation frequency increased with the development of relapse and progression
	E380Q, D538G, Y537S/N/C, V534E, L536Q/R	Paoletti et al <sup>88</sup>	Phase I study	ER+HER2-MBC	Elevated ESR1 mutation associated with worse PFS
	Not specific	Ma et al <sup>95</sup>	Single-arm phase II study	HER2 <sup>mut</sup> , non-amplified MBC	As a screening tool to identify future trial patients
	Not specific	Sakai et al <sup>101</sup>	Retrospective study	HER2+ MBC	Predicting primary resistance to T-DMI
	Not specific	Li et al <sup>102</sup>	Phase I study	HER2+ MBC	Multiple genetic alterations significantly associated with shorter PFS compared to none or one genetic alteration

(Continued)

Table 1 (Continued).

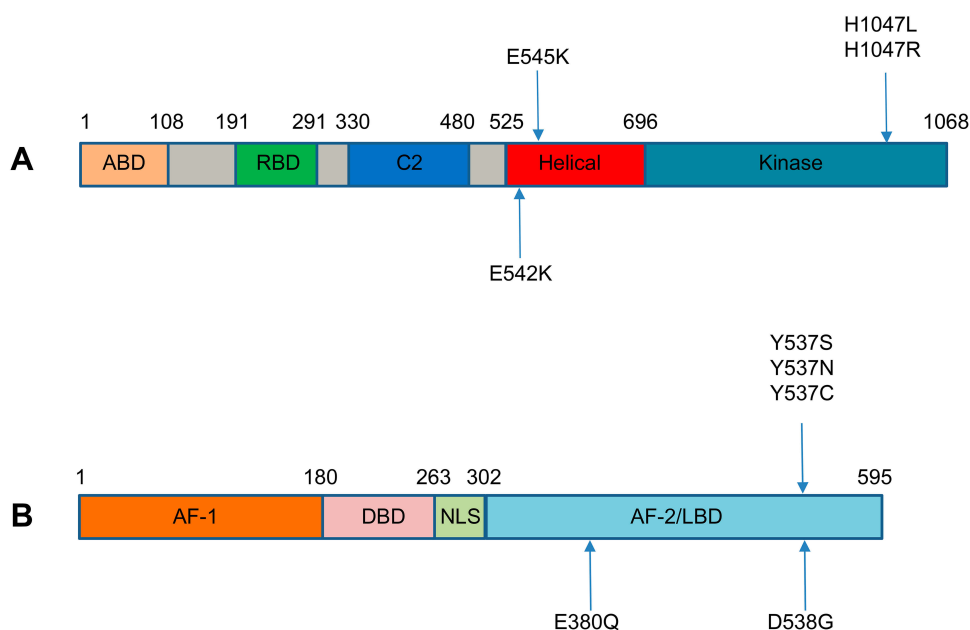
Mutant Gene	Detection Sites	Researcher	Study Type	Patients Classification	Clinical Relevance
RB1	Not specific	Condorelli et al <sup>96</sup>	Observational study	HER2+ MBC	Conferring therapeutic resistance of CDK4/6 inhibitor
BCL2	Not specific	Lok et al <sup>97</sup>	Phase Ib study	BCL2+ MBC	Combination therapy for patients with BCL2-positive tumors

**Notes:** PIK3CA, phosphoinositide 3-kinase catalytic subunit alpha; ESR1, estrogen receptor 1; HER2, human epidermal growth factor receptor 2; RB1, retinoblastoma 1; BCL2, B-cell lymphoma 2; MBC, metastatic breast cancer; HR, hormone receptor; ABC, advanced breast cancer; MTNBC, metastatic triple-negative breast cancer; ER, estrogen receptor; PFS, progression-free survival; OS, overall survival; CTC, circulating tumor cell; TDM-I, trastuzumab emtansine; CDK4/6, cyclin-dependent kinase 4 and 6; +, positive; -, negative.

## PIK3CA

PIK3CA, an oncogene encoding the p110 $\alpha$  component of the phosphoinositide 3-kinase (PI3K), represents one of the most frequent driver mutations observed in BC.<sup>38</sup> As shown in Figure 2, the four most frequent hotspot mutations of PIK3CA are located within two exons (exon 9: E545K and E542K; exon 20: H1047R and H1047L), accounting for 80–90% of all PIK3CA mutations in human malignancies.<sup>79,80</sup> High-frequency (35/73 (47.9%)) PIK3CA hotspot mutations were present in the plasma-ctDNA of MBC patients.<sup>26</sup> Park et al reported that more than 70% of the cfDNA samples of patients with MTNBC had PIK3CA hotspot mutations and the mutation frequency increased after the treatment.<sup>81</sup>

In recent years, a number of studies supporting the prognostic role of PIK3CA in ABC have been carried out.<sup>36–38,45,65,82–84</sup> O’Leary et al found that PIK3CA ctDNA level after 15 days’ treatment strongly predicted the PFS of ABC patients on palbociclib and fulvestrant (hazard ratio 3.94, log-rank  $P = 0.0013$ ).<sup>82</sup> In a study by Jacot et al, a detectable circulating PIK3CA mutation after 4 weeks’ hormone therapy was highly correlated with a worse PFS.<sup>83</sup> A retrospective study by Li et al revealed that PIK3CA p.H1047R mutation was more frequent in HER2+ disease and associated with worse OS. Moreover, this was the only mutation associated with shorter PFS on multivariate analysis in HER2+ patients who were treated with trastuzumab, suggesting



**Figure 2** Major mutation sites of (A) PIK3CA and (B) ESR1.

**Notes:** Most of the reported mutations in PIK3CA cluster in conserved regions within the region coding for the helical and kinase domains of p110 $\alpha$ , including E542K, E545K, H1047L, and H1047R. ESR1 point mutations mainly localized in the ligand-binding domain, including 3 hotspot mutations in codons 380, 537, and 538. ABD, adaptor binding domain; RBD, ras binding domain; AF, activation function; DBD, DNA binding domain; NLS, nuclear localizing signal; LBD, ligand binding domain.

that trastuzumab had lower activity in these patients.<sup>84</sup> Takano et al found that in patients without PIK3CA mutations, lapatinib plus capecitabine (LX) yielded relatively longer PFS ( $P = 0.035$ ) and OS ( $P = 0.025$ ) than trastuzumab plus capecitabine (HX), indicating that these patients might benefit from LX rather than HX.<sup>65</sup> In a Phase I study by Ma et al, all patients with PIK3CA or TP53 mutations were relatively insensitive to pyrotinib, whereas a significantly higher response rate was revealed in patients with wild-type PIK3CA and TP53 ( $P = 0.013$ ).<sup>45</sup> The BELLE-2 study was the first randomized Phase 3 clinical trial to include a prospective analysis of PFS in patients with ctDNA PIK3CA mutations. A significant difference in PFS in the buparlisib group vs. the placebo group was recorded in patients with ctDNA PIK3CA mutations, but not in patients with non-mutant ctDNA.<sup>36</sup> On this basis, Di Leo et al demonstrated that PFS was in favour of combination therapy (buparlisib plus fulvestrant) vs. fulvestrant alone in patients with PIK3CA mutations in the BELLE-3 study.<sup>37</sup> Overall, the ctDNA was analyzed in 1698 ABC patients of these studies and 552 (31.5%) of them had PIK3CA mutations. Of note, 1429 of these patients had HR+ ABC and 31.1% (445/1429) had PIK3CA mutations. Available data indicates that H1047R (118/256) and E545K (94/256) are the two most frequent mutation sites in PIK3CA (Table 2). These studies suggest that PIK3CA may be a powerful target to predict the clinical outcomes and guide treatment decisions especially for HR+ or HER2+ MBC.

### ESR1

The activation of ESR1 is a key feature of 70–80% BC patients in whom ER expression is detected.<sup>85</sup> As shown in Figure 2, ESR1 point mutations mainly localized in the ligand-binding domain (LBD), including 3 hotspot mutations in codons 380, 537, and 538. Allouchery et al found

that the mutation frequency of ESR1 increased with the development of relapse and progression (5.3% and 33%, respectively), suggesting that detecting circulating ESR1 mutations should be focused on the metastatic setting.<sup>86</sup> A secondary analysis of the BOLERO-2 clinical trial by Chandarlapaty et al demonstrated that each of the ESR1 mutations was associated with shorter OS.<sup>87</sup> Paoletti et al found that patients with persistently elevated ESR1LBDm + ctDNA had worse PFS than patients who did not ( $P = 0.0007$ ).<sup>88</sup> However, an analysis of the data from PALOMA-3 study indicated that ESR1 mutations selected by prior hormone therapy were frequently sub clonal, with ESR1 ctDNA dynamics offering limited prediction of clinical outcomes.<sup>82</sup>

Endocrine therapy is the basis of treatment for ER+ MBC patients. However, 40% of these patients obtain no clinical benefit from first-line endocrine therapy, and virtually all of the patients in whom the tumor initially responds eventually develop resistance.<sup>89</sup> ESR1 mutation emergence has been recently highlighted as a frequent mechanism of acquired aromatase inhibitor (AI) resistance in the metastatic setting.<sup>90,91</sup> It was found that the mutation frequency of ESR1 was significantly higher in HR+ patients than in HR- patients, especially in the HR+/HER2- group. In addition, ESR1 mutations were positively related to the line of endocrine therapy. These results support the viewpoint that ESR1 mutations are acquired from endocrine therapy.<sup>68,92</sup> Beije et al reported that the ESR1 mutations of cfDNA were more prevalent in the progressing cohort (42%) than in the baseline cohort (11%) ( $P = 0.04$ ), which strongly suggested a role in conferring endocrine resistance in MBC.<sup>93</sup> Takeshita et al showed that there were distinct populations of ESR1 mutations in metastatic tissue and plasma.<sup>94</sup> Each ESR1 mutation might have different clinical significance, and it would be necessary to investigate them all. This viewpoint is consistent with Chandarlapaty et al In their analysis, the ESR1 D538G mutation group derived a large benefit from treatment with a mechanistic target of rapamycin kinase (mTOR) inhibitor, whereas the ESR1 Y537S mutation group did not.<sup>87</sup> Altogether, the ctDNA was analyzed in 685 ABC patients and 169 (24.7%) of them had ESR1 mutations in these studies. Notably, 524 of these patients had HR+ ABC and 26.9% (141/524) had ESR1 mutations. D538G (23/56) and Y537S (18/56) are the two most frequent mutation sites in ESR1 (Table 3). Selected by the treatment pressure, ESR1 mutations in ABC often evolve into polyclone, which may weaken its prognostic

**Table 2** The Distribution of PIK3CA Mutation Sites

Researcher	E542K	E545K	H1047L	H1047R	Total
Park et al <sup>81</sup>	3	2	0	15	20
Takano et al <sup>65</sup>	0	0	0	8	8
Di Leo et al <sup>37</sup>	25	50	7	54	136
Kodahl et al <sup>38</sup>	2	5	3	11	21
Tzanikou et al <sup>26</sup>	0	30	0	15	45
Jacot et al <sup>83</sup>	4	2	0	4	10
Li et al <sup>84</sup>	0	5	0	11	16
	34	94	10	118	256



**Table 3** The Distribution of ESR1 Mutation Sites

Researcher	E380Q	D538G	Y537S	Y537N	Y537C	Total
Yi et al <sup>68</sup>	2	7	7	2	0	18
Beije et al <sup>93</sup>	0	8	2	1	1	12
Takeshita et al <sup>46</sup>	0	2	4	4	0	10
Paoletti et al <sup>88</sup>	0	6	5	5	0	16
	2	23	18	12	1	56

value. Still, detecting the mutation profile of ESR1 may assist in knowing the resistance mechanism of endocrine therapy and guiding clinical treatment (for example, the use of fulvestrant in ABC patients after AI treatment).

## Other Gene Mutations

Besides ESR1 and PIK3CA, the clinical impacts of other gene mutations such as HER2, retinoblastoma 1 (RB1), and B-cell lymphoma 2 (BCL2) have been investigated in ABC.<sup>95–97</sup> HER2 is a well-established therapeutic target in BC.<sup>98–100</sup> HER2 gene amplification in ctDNA and ER/PR status may predict primary resistance to T-DM1 (trastuzumab emtansine), which is an antibody-drug conjugate with high efficacy approved for the treatment of patients with HER2+ ABC.<sup>101</sup> In a phase I study by Li et al, HER2 bypass signaling pathway along with PI3K/AKT/mTOR pathway and TP53 was shown to be significantly associated with poorer PFS in patients with HER2+ MBC treated with the combination of pyrotinib and capecitabine (median, 16.8 vs. 29.9 months,  $P = 0.006$ ).<sup>102</sup> Theoretically, HER2- (non-amplified) BC patients do not benefit from HER2-directed drugs.<sup>103</sup> However, recent identification of recurrent HER2 mutations (HER2<sup>mut</sup>) in a subset of HER2 gene non-amplified BC suggested an additional HER2 targeting opportunity.<sup>95,104–106</sup> RB1 is a tumor suppressor protein that is dysfunctional in several major cancers.<sup>107</sup> Deregulation of the cyclin D1-CDK4/6-RB pathway is implicated in endocrine treatment resistance.<sup>108</sup> Condorelli et al described the acquisition of de novo RB1 mutations in three patients with MBC treated with CDK4/6 inhibitors. None of the RB1 mutations was present in the pre-CDK4/6 specimen, highlighting that these molecular alterations likely emerged under selective pressure from the CDK4/6 inhibitor potentially conferring therapeutic resistance.<sup>96</sup> Further research is needed to identify the frequencies of these mutations and how these mutations temporally emerge under the selective pressure of CDK4/6 treatment. BCL2, a key member of the BCL2 pro-survival family, is often expressed at high levels in poorer-prognosis luminal B tumors and in good-prognosis

luminal A tumors.<sup>109,110</sup> Lok et al demonstrated that combining venetoclax with endocrine therapy had a tolerable safety profile and elicited notable activity in ER+BCL2+ MBC. Their findings warrant further investigation of combination therapy for patients with BCL2+ tumors.<sup>97</sup>

## Drug Resistance

Targeted therapies have remarkably changed the treatment of cancer over the last decade. However, almost all tumors acquire resistance to systemic treatment as a result of tumor heterogeneity and clonal evolution.<sup>111</sup> Multiple mechanisms involved in signal transduction molecules have been demonstrated to be associated with the primary or acquired resistance in ABC, including activation of various intracellular pathways such as the PI3K/AKT/mTOR and CDK4/6/RB pathways.<sup>83,102</sup>

## Resistance to Endocrine Therapy in ER + BC

ESR1 mutation is not only associated with AI treatment failure in ER+ BC patients, but also confers ligand independent cell growth and relative resistance to tamoxifen and fulvestrant.<sup>112,113</sup> Moreover, the crosstalk between PI3K and ER pathway and between PI3K/ERBB2 pathway also cause patients' resistance to endocrine therapy.<sup>114</sup> Zhou et al evaluated the genetic response of ctDNA to chemotherapy in ER + MBC patients. The results showed that more patients with PD exhibited increased PIK3CA mutation frequency than non-PD patients (56.25% vs 0%,  $P = 0.002$ ). Furthermore, ESR1 and GATA3 mutations were shown to correlate with PIK3CA mutation, suggesting that the accumulation of PIK3CA, ESR1 and GATA3 mutations might significantly increase the risk of endocrine therapy resistance.<sup>115</sup>

## Resistance to CDK4/6 Inhibitors

Selective CDK4/6 inhibitors have become the standard of care for ER+HER2- MBC patients.<sup>116</sup> These agents prevent cell cycle progression from G0/G1-phase to S-phase by directly blocking the activity of the cyclin D-CDK4/6

holoenzyme, leading to the proliferation inhibition of sensitive tumor cells.<sup>117</sup> The potent anticancer activity of CDK4/6 inhibitors has been demonstrated in pre-clinical and clinical studies.<sup>118,119</sup> However, little is known about the mechanisms of resistance to these agents. Therefore, there is a pressing need to develop biomarker approaches to identify which ER+ BC patients will derive long-term benefit from CDK4/6 inhibition. O' Leary et al conducted ctDNA sequencing to 195 patients from the PALOMA-3 study. The evolution of ESR1 mutations was observed, with selection of ESR1 Y537S as the variant most likely promoting resistance to fulvestrant in the combination.<sup>120</sup> Formisano et al identified FGFR1/2 amplification or activating mutations in 14/34 (41%) post-progression specimens by sequencing the ctDNA in 34 patients after progression on CDK4/6 inhibitors. In addition, the presence of FGFR1 alterations in baseline plasma tumor DNA was associated with a shorter PFS in the MONALEESA-2 study, suggesting that aberrant FGFR signaling was a potential mechanism of escape from endocrine therapy plus CDK4/6 inhibitors.<sup>121</sup>

## Tumor Heterogeneity and Sub-Clone

Intra-tumoral genetic heterogeneity presents a challenge to targeted therapy response, as sub-clones may have divergent response to therapy.<sup>82</sup> A study by Ma et al provided evidence that tumor heterogeneity present in ctDNA could predict therapeutic outcome. The results indicated that patients with higher tumor heterogeneity had significantly worse PFS outcomes, with a median PFS of 30.0 weeks vs. 60.0 weeks for patients with low tumor heterogeneity ( $P = 0.02$ ).<sup>54</sup> Sub-clonal mutations are reported most commonly in ESR1. Multiple concurrent ESR1 genomic alterations including mutation, rearrangement, and amplification were observed in 40% of ESR1-altered cases, suggesting polyclonal origin.<sup>40</sup> Paoletti et al proposed that integrating multiple biomarkers (ctDNA ESR1LBDm, CTC enumeration, and CTC-ER status) could improve outcome prediction and endocrine treatment resistance mechanisms' identification over a single biomarker.<sup>88</sup> Li et al explored potential predictive biomarkers of HER2+ MBC patients and found that compared to none or one genetic alteration, multiple genetic alterations in HER2 bypass signaling pathway, PI3K/AKT/mTOR pathway, and TP53 were significantly associated with poorer PFS (median, 16.8 vs 29.9 months,  $P = 0.006$ ).<sup>102</sup>

## Summary and Conclusions

Compared to tumor tissue biopsy, ctDNA can non-invasively detect more comprehensive genomic information from limited amount of plasma. In recent years, great progress has been made in the detection technologies of liquid biopsy from PCR to NGS, providing strong technical support for ctDNA analysis. A lot of efforts were made to increase the sensitivity and reduce the error rate of ctDNA sequencing, leading to an improvement of the quality control of ctDNA analysis. Recent studies have revealed high concordances (77–89%) between ctDNA and tumor tissue biopsy, which confirmed the detection capability of ctDNA and its role in replacing tumor tissue biopsy. In addition, a series of pre-analytical factors such as sample freshness, storage tube and temperature, and centrifugation speed could affect the analysis results of ctDNA and should be controlled appropriately. Tumor burden, providing key information about objective response and disease progression, could be reflected through the evaluation of ctDNA concentration and integrity. The prognostic value of specific gene mutations such as PIK3CA in MBC has been confirmed in numerous studies. Hopefully, by longitudinally monitoring the treatment response and ctDNA variants of MBC patients, the drug resistance may be addressed, however, which needs to be further validated in large-scale prospective trials. Moreover, given that the previous ctDNA tests are mainly for patients with advanced cancers, the applications of ctDNA in early-stage cancers, such as early diagnosis, relapse monitoring, and screening may be well explored. To conclude, ctDNA has promising prospects in cancer research, especially in promoting the realization of personalized medicine, and perhaps it will completely change the existing patterns of cancer treatment in the near future.

## Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

## Disclosure

The authors declare that they have no conflict of interest.

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