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Breast Cancer Biomarkers: Utility in Clinical Practice

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Abstract

Breast cancer is a heterogeneous disease. For the past decades, new technical tools have been developed for biomarkers at the DNA, RNA and protein levels to better understand the biology of breast cancer. This progress is essential to classify the disease into clinically relevant subtypes, which may lead to new therapeutic opportunities. Novel biomarker development is paramount to deliver personalized cancer therapies. Further, tumor evolution, being natural or under treatment pressure, should be monitored and “liquid biopsies” by detecting circulating tumor cells or circulating free tumor DNA in blood samples will become an important option. This paper reviews the new generation of biomarkers and the current evidence to demonstrate their analytical validity, clinical validity and clinical utility.

Keywords

Biomarkers; Breast cancer; Prognosis; Prediction; Clinical utility

Introduction

Breast cancer is the most frequent female neoplasm. For early breast cancer, standard treatment remains complete excision with or without radiotherapy. Indication of an adjuvant systemic treatment is based on standard prognostic characteristics as age, menopausal status, tumor size, lymph node status, histological grade, estrogen receptor (ER) and HER2 status. To help clinicians, *Adjuvant!Online* (AOI), an easy computer-based nomogram, combines some of these markers to evaluate recurrence and death risks with or without adjuvant systemic therapy. However, this clinical tool widely used in practice presents some drawbacks: the prognostic prediction is only based on stage of disease (tumor size, node involvement), tumor grade and ER positivity; treatment effectiveness is adjusted only for age and ER positivity in postmenopausal patients; and the validation was obtained on

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Compliance with Ethics Guidelines

Conflict of Interest

Fanny Le Du declares that she has no conflict of interest.

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Human and Animal Rights and Informed Consent

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patients without major co-morbidities and <70 years of age [1]. Therefore, patients with same risk and same treatment have different outcomes, an indication of breast cancer heterogeneity. During the last few years, research has focused on identification of potential markers (specified DNA sequence, RNA levels or expressed protein) to improve sub-group classification and correlate it with clinical outcome and therapy response. We will review some of the most promising biomarkers focusing on their reproducibility and robustness (analytical validity), their ability to identify accurately relevant breast cancer survival (clinical validity) and how these biomarkers could favor a better approach of the treatments (clinical utility) [2]. In addition we will also review the role of "liquid biopsies" in detecting circulating tumor cells (CTCs) or circulating free tumor DNA (cfDNA) in blood samples as a biomarker option.

Molecular testing for early breast cancer

Nowadays, many new tools in the field of molecular profiling have been developed for early-breast cancer to accurately predict outcomes and to estimate the benefit of adjuvant treatment. We will first discuss of tumor tissue markers from gene expression assays (summarized in Table 1 [3]) to proteomics assays, and then, we briefly analyze the germline markers.

Tumor tissue markers

Gene expression assays—OncotypeDX® measures 21 genes by quantitative reverse transcriptase-PCR (qRT-PCR), using formalin-fixed paraffin-embedded (FFPE) tissues to determine a Recurrence Score (RS). This score estimates the likelihood of distant metastasis at 10 years from the date of diagnosis, and stratifies patients in to three risk groups: low, intermediate and high for RS values <18, 18–30, >30, respectively [4]. Scientific societies such as ASCO® [5], NCCN® [6] and ESMO [7] have recently included the OncotypeDX assay in their guidelines. The analytical value of this biomarker was assessed by a high reproducibility (Pearson's r=0.86) [8]. It was firstly validated as an independent prognosis marker [4] then as predictive of tamoxifen response[9] for ER–positive, lymph-node negative early stage breast cancer in the NSABP-B14 population. In NSABP-B20 cohort of ER-positive, node-negative patients tamoxifen-treated with or without chemotherapy, RS assay was assessed as predictor of chemotherapy response [10]. In the most recent TransATAC study, RS prognostic value was highlighted in post-menopausal both node negative and positive patients, treated either by tamoxifen or anastrozole [11]. The prognostic value and predictive response to chemotherapy was also validated in the node positive SWOG8814 cohort. No benefit of CAF-regimen chemotherapy was proved for low-RS (p=0.97) but an increased disease-free survival (DFS) was highlighted for high-RS group (p=0.03) [12]. Others studies revealed that the 21-gene signature was better than standard clinicopathological variables at predicting recurrence [13]. But even with these new classifiers, results remain intermediate for 22 % to 40% of the population for whom prognosis are still heterogeneous and treatment decisions still difficult [4,14]. Studies have shown that in approximately 30% of cases, knowledge of RS results impacts the oncologist's recommendation. Most changes were from combined chemo-endocrine therapy to endocrine therapy alone [15,16], but impact on outcomes was not studied. Phase III trials are ongoing to prospectively validate clinical utility. The TAILORx and the RXPONDER trials will validate the clinical utility of Oncotype DX® to assign ER-positive to adjuvant systemic treatment. They both investigate whether hormone therapy alone or hormone therapy together with combination chemotherapy is better for women who have an RS of 11–25, in node-negative for TAILORx and node-positive cohort for RXPONDER [17,18].

Mammaprint® evaluates the expression level of 70 genes on fresh-frozen or FFPE tissue sample to define low or high risk of relapse. This DNA-microarray has been FDA approved

as a prognosis marker of distant metastasis free survival for T1-2 early breast cancer [19–21], after a 100% inter-laboratory concordance was obtained (failure rate of 19% because of insufficient samples) [21]. Similar to the 21-gene RS, the 70-gene signature outperforms the clinicopathological risk indexes habitually used (e.g. AOI) with a discordance rate of 37% and a larger low risk population [21–23]. On ER-positive population, *Knauer et al.* [24] studied in a pooled analysis the benefit of adding chemotherapy to endocrine treatment among high risk patients compared to low risk patients. However, the test for interaction was not significant and the predictive value of the signature was not validated. Straver et al. [25] showed that a pathological complete response (pCR) after neoadjuvant chemotherapy was unlikely in the good prognosis-signature group versus the poor one. The MINDACT European project prospectively will validate the clinical utility of Mammaprint® as a predictor of chemotherapy benefit [26,27].

The **PAM50 assay**, a qRT-PCR of 50 genes, identifies five “intrinsic” subgroups of breast cancers: luminal A, luminal B, HER2-enriched, normal-like and basal-like [28]. This assay provides a risk of relapse (ROR) score, predicting the likelihood of recurrence over 10 years. These subtypes combined with usual clinical variables improve prediction of relapse at 5 years for node negative or positive breast cancer [29]. PAM50 results are concordant with Oncotype DX®: 83% of the low RS are luminal A [14]. Moreover, ROR is predictive of pCR after neoadjuvant chemotherapy with a sensitivity of 97% and a negative predictive value (NPV) of 97% [29] with a pCR rate for luminal A and HER2-enriched of respectively 3% and 50% [30]. The utility of PAM50 could be into a more precise classification of breast cancer with similar molecular aberrations, response treatments and outcomes, which could be attractive for therapy selection. However, currently no data are available to change our treatment recommendations according to the ‘intrinsic’ subtypes.

The **EndoPredict** (EP) score is based on the quantification of mRNA levels of 11 genes by qRT-PCR issued from FFPE samples. Its high inter-laboratory reproducibility (Pearson’s r=0.994) attests of its analytical validity [26]. As regards of its clinical validity, continuous EP-score was validated as an independent predictor of distant relapse in ER-positive, HER2-negative breast cancer treated with endocrine therapy (p=0.010). Its combination with two clinicopathologic risk factors (tumor size and nodal status) outperformed others prognosis tools like AOI or Ki67 (c-score 0.732 to 0.788). This combined marker, called EPclin, identifies risk groups with obvious differences in the 10-year distant relapse rate from 4% for low to 28% for high risk [32]. *Muller et al.* [33] studied the impact of this results on daily practice. Almost 38% of the patients were proposed to change treatment between pre- and post- EPclin test: 12% changed from endocrine to chemo-endocrine therapy while 25% change from combined treatment to endocrine treatment alone.

The **Breast Cancer Index** (BCI) combines two independent biomarkers, HOXB13:IL17BR and the 5-gene molecular grade index. It reflects the risk of distant metastasis at 10 years post-diagnosis. Among ER-positive lymph node negative disease, BCI provides new independent prognosis information for both distant-recurrence free survival (DRFS) (p=0.0002) and BCSS (p<0.0001) [34,35]. In the neoadjuvant setting, *Mathieu et al.* [36] demonstrate the ability of BCI to predict chemosensitivity with a NPV of 98.4% to achieve pCR and 86% for breast conservation surgery, for the low risk group. So far, this biomarker didn’t show added value to others gene expression assays which limited its clinical utility [37].

The **Genomic Grade Index** (GGI), a 97 genes micro-array, created by *Sotiriou et al.* [38], reclassifies histological grade 2 (HG) patients into two groups: HG1-like or HG2-like. This approach improved the accuracy and prognostic value of HG in ER-positive invasive breast carcinoma, on a heterogeneous cohort [38–40]. High GGI tumors had a significantly higher

pCR rate after neoadjuvant chemotherapy for both ER positive [39] and negative [40] but very limited information validating the robustness of GGI is available.

Proteomic assays—The **Mammostrat®** test is an immunohistochemical (IHC) five-protein assay. Increased Mammostrat scores correlates with lower DRFS (p=0.005) and OS (p=0.0023) for ER-positive early breast cancer despite adjuvant treatment [41]. The impact of Mammostrat on breast cancer outcomes also has been shown in lymph node-positive and ER-negative disease [41,42]. The recurrence-free-survival (RFS) was improved by 21% (from 64% to 85%) for Mammostrat® high risk patients with the addition of chemotherapy to endocrine treatment [43].

The **immunohistochemical (IHC) 4** prognostic score is based on four widely measured IHC markers (ER, progesterone receptor (PgR), HER2, and Ki-67) using FFPE tumor samples. It was created and validated as a prognosis factor using the ATAC trial cohort of ER-positive early breast cancer. This inexpensive biomarker, for which three of four measures are routinely performed, could be as informative as OncotypeDx®. However, these results can only by obtained by standardized procedures because of the lack of reproductibility of IHC [44]. On this point, the International Ki67 in Breast Cancer Working Group recently proposed guidelines to reduce interlaboratory variability and improve interstudy comparability of **Ki67**, mostly using IHC with monoclonal antibody MIB1. Strengthening the analytical validity of Ki67 may enable using it for prognosis, prediction of responsiveness/resistance to endocrine therapy or chemotherapy and monitoring neoadjuvant treatment [45].

Genomic and/or proteomic approach to predict specific drugs response— Some biomarkers could be more relevant in predicting response to specific drugs. The predictive role of **TP53**, regarding preclinical studies, remains a matter of debate concerning sensitivity to taxanes. To answer the question, *Bonnefoi et al.* [46] led a randomized phase III trial on non-metastatic chemonaïve breast cancer. TP53 status, using yeast assay was not predictive of preferential sensitivity to taxanes. Such results were also supported by a retrospective analyze of the BIG 02-98 III trial [47].

Likewise, retrospective studies suggested an impact of the **topoisomerase 2α** amplification/overexpression (TOP2A) on responsiveness to anthracycline-based regimens [48]. Moreover, the BCIRG-006 trial highlighted that HER2-positive breast cancers without TOP2A amplification had more benefits from trastuzumab than the all cohort, in contrast, tumors coamplified for both TOP2A and HER2 showed identic benefit in the anthracycline non trastuzumab arm and the trastuzumab arms [49]. However, standardization of measurement is mandatory because, so far, different methodological approaches (qRT-PCR, DNA microarrays, IHC or mostly fluorescence in situ hybridization (FISH)) have been used with inconsistent results and poor agreement [50]. To avoid reproducibility problem, *Desmedt et al.* [51] proposed an anthracycline-based score (A-Score) combining three signatures: *TOP2A* gene, tumor invasion and immune signatures. The A-Score was significantly associated with pCR in the anthracycline-based arm and was characterized by a high NPV of 98% either HER2-status. This score could become, if further validated, a useful clinical tool to identify patients who could avoid high risk toxicity anthracycline therapy.

The validation of new biomarkers, from in-vitro cell lines identification through prospective randomize trials, is mandatory before clinical use. The best example of such process is the **uPA/PAI-1** marker for whom the final result of the first prospective biomarker-based therapy trial ‘Chemo-N0’ just published. These 10-year results validate the benefit from adjuvant chemotherapy in the high-uPA/PAI-1 group. Thus, the American Society of

Clinical Oncology (ASCO) recognized uPA/PAI-1 as risk-group-classification markers for routine clinical decision-making in node-negative breast cancer [52].

The use of germline markers for therapy selection

Another approach in dealing with biomarkers is to study molecular specificity of the patient rather than of the tumor. Thus, Cytochrome P450 2D6 (CYP2D6) was evaluated for its implication in metabolizing tamoxifen into a clinically active metabolite. Currently results of various studies looking at this question are discordant. The largest retrospective analysis of the the BIG1-98 trial samples, didn't demonstrate an interaction between CYP2D6 phenotypes and BCSS in a tamoxifen-treated population [53]. Prospective studies are ongoing to clarify impact of CYP2D6 metabolizer on tamoxifen treatment (ECOG3108 [54] in metastatic setting). So far, this test should not be used to select adjuvant endocrine therapy.

The predictive role of BRCA1 and BRCA 2 mutations to therapy benefit have been controversial. Retrospective results suggested an enhanced response to platinum salts and reduced to taxanes in the neoadjuvant setting. [55] However, *Arun et al.* [56] demonstrated high response rates to anthracycline-taxane-based regimens in BCRA mutation carriers undergoing neoadjuvant chemotherapy. Lately, BRCA mutation status have been used as a selection biomarker for clinical trials with PARP inhibitors and platinum salts in patients with early and metastatic breast cancer [57].

Molecular testing for metastatic breast cancer

In this second part, we will review how we could monitor metastatic breast cancer evolution in order to provide an accurate and personalized treatment. We will discuss tumor tissue markers, then "liquid biopsies".

Tumor tissue markers

Over the last few years biomarkers discordance between primary tumor and disease recurrence has been a topic in the field of breast cancers. The largest retrospective study led by *Linstrom et al.* [58] revealed a discordance in ER status of 32.4% ($p<0.001$). This discordance, confirmed in other studies, varies from 13.4% [59] to 40% [60] for ER, and correlates with worse overall survival (OS) ($p=0.002$) [59,61]. As per HER2 status, *Niikura et al.* [62] retrospectively highlighted a 24% rate of change (ranged from 0% to 64% dependents on the study), also associated with a shorter OS ($p=0.003$) [61,62]. ER and HER2 changes were confirmed in recent prospective ConvertHER [63], DESTINY [64] and BRIT studies [65]. New markers as PTEN levels or *PI3KCA* mutational status have also shown to be discordant (26% and 18% respectively) between primary tumors and metastasis [66].

As new targeted therapies are brought into clinical trials, using primary tumor signatures for patient selection in metastatic setting might not be relevant. All these data highly suggest biological changes due to treatment exposure or natural evolution of neoplasm. New molecular signature seems mandatory to provide an accurate and relevant targeted treatment at relapse.

Pusztai et al. [67] recently presented results of the BEAT-IT study. Molecular analysis of metastatic breast cancer biopsies was successfully performed in approximately 75% for IHC (PTEN, androgen receptor, MET), FISH (*EML4-ALK*, *MET*) and mutation analysis (CMS46), without adverse events. Data were used to select clinical trials of targeted therapies, the study continues patient accrual and data on outcomes should follow. Similarly, *Andre et al.* [68] led the prospective SAFIR-001 trial. Whole-genome arrays (e.g. array

Comparative Genomic Hybridization and DNA sequencing) detected targetable genomic alteration in 70% of DNA breast cancer metastatic biopsies. The most frequent genomic alterations were *PI3KCA* mutations, *CCND1*, *FGF4* and *FGFR1* amplifications. Updated results regarding the percentage of patients who benefit from selected targeted therapy according to the molecular screening are expected. Genomic analyzes to determine a molecular target at relapse seems feasible and could notably modify our treatment options in the future.

Molecular testing utilizing blood samples

Repeat **tumor biopsy** is one of the solutions proposed by scientists. But even repeats, single snap-shots of the tumor could be insufficient to obtain an accurate genomic landscape of the breast cancer due to high intratumoral heterogeneity [69]. Moreover, tumor seeding risk [70] and adverse events of invasive techniques mostly for patients who undergone anti-angiogenic treatment could contraindicate traditional biopsy [71]. Finding more accessible biomarkers would be helpful to monitor metastatic disease and anticipated treatment response.

Circulating tumor cells—During the last decades, scientists have developed many **circulating tumor cells** (CTCs) platforms. Various technologies are employed: 1) using specific antibodies (e.g. EpCAM (epithelial cell adhesion molecule) alone or combination) in immunomagnetic (e.g. CellSearch®) or microfluidics assay; 2) using size-bazed filtration; 3) using electrical properties [72]. Currently, only CellSearch® is FDA approved for prognosis in metastatic breast cancer based on the prospective study of *Cristofallini et al.* [73]. Detection of ≥ 5 CTC significantly predicts worse progression free survival (PFS) and OS [74]. These results were strengthened by a recent meta-analysis, led by *Zhang et al*. [75], where CTCs detection was predictive of poor outcome irrespective of the time point of detection and the stage of breast cancer (early stage or metastatic one).

Therefore, more than a snapshot evaluation, CTCs could provide a dynamic evaluation of prognostic gradually the treatment regimens. In fact, among patients with ≥ 5 CTCs at baseline, half will have < 5 CTCs after chemotherapy courses, correlated with both increased PFS and OS ($p<0.0001$) [73–75], thereby indicating potential chemosensitivity. Moreover, changing levels of CTCs has been correlated with radiologic response, evaluated by RECIST ($p<0.001$) [76]. CTCs appeared to be an earlier and accurate biomarker for anticipating chemotherapy outcomes. For metastatic population treated with endocrine therapy, *Giuliano et al.* [77] couldn't show a real impact of endocrine therapy alone on CTC count which remained a strong prognostic factor at baseline. This enhances the question of more aggressive therapies for high CTC counts patients. For example anti-HER2 therapy highly targets CTCs with a decreased rate after treatment of 94 to 100% in metastatic disease [74,77]. However, for HER2-positive breast cancer treated by anti-HER2, the prognostic value of CTC is not longer sustainable because of the significant survival benefit from therapy [77,78].

The latest published studies on CTCs evaluated their HER2 expression. In fact, most of them reported a low concordance rate between HER2 status on primary or secondary tumor and CTCs from 23% to 50% [79,80]. This could define a new cohort, with poor PFS ($p=0.036$) [81], potentially eligible for anti-HER2 treatment. Thus, a phase II study has been conducted on a small cohort of HER2-negative metastatic disease and HER2-positive CTCs, who underwent lapatinib. This clinical trial was negative for objective response rate but even so show how we could integrate CTCs analyze in future trial design [82].

The major issue regarding these "liquid biopsies" remains their analytical validity. In fact, Cellsearch®, the only FDA approve tool had low sensitivity and high false negative rate.

CTCs are currently detects in 44% to 65% of the metastatic population [74,83,84]. *Sieuwert et al.* [85] revealed that normal-like breast cancer cells were undetectable by this tool because of low or no EpCAM expression. Since then, the proposed solution was to combine anti-EpCAM with other anti-epithelial antibodies - like anti-CD46 [86] or anticytokeratine [87]- or with size-based filtration. However, these solutions could be insufficient, mostly because normal-like subtype not only loose epithelial markers but also express both vimentin and TWIST1, markers of epithelial–mesenchymal transition (EMT) [85]. This discovery could potentially explain why patients considered as cured by adjuvant treatment could relapse. Furthermore, by using EpCAM+/Cytokeratine+/CD45- as definition of CTCs, 66% of patients - regardless to their metastatic status – were positive for CTCs. However, by adding more markers (e.g. ERα, HER2, ALDH1) especially EMT ones (e.g. fibronectine and vimentin), to the detection process, 34% of negative results became positive for CTCs [84]. This result strengthens the limitation of the approved standard CTC isolation test. An accurate method of detection is needed especially because CTCs gaining mesenchymal markers are associated with poorest PFS than those who don't (6.6 vs 15.3 months, p=0.0000), in metastatic breast cancer patients [88]. As a matter of fact, the high incidence of EMT-CTCs in metastatic versus early stage breast cancer supports the notion of EMT-CTC implication in metastatic process [84,89].

Circulating free tumor DNA—More recently, another type of "liquid biopsy" has been developed to evaluate cancer genetics: circulating free tumor DNA (cfDNA). A recent prospective study, led by *Dawson et al*. [90], on 30 metastatic breast cancers with genetic alterations (*PI3KCA*, *TP53* and structural variation) detected cfDNA in 97%. Presence of circulating DNA fragment carrying tumor sequence alterations, like mutations, promoter-methylation, could impact outcome, as summarized in Table 2.

Moreover, cfDNA appears in several studies like a prognosis marker of survival. *Dawson et al.* [90] demonstrated that an increased rate of cfDNA reduced OS (p=0.001). In fact, in this population, 89% of relapses were associated with an increased cfDNA level by a factor of 505 from the nadir. Furthermore, for 53% of recurrence, levels of cfDNA increased, on average 5 months before the establishment of progressive disease by imaging. The authors conclude that cfDNA often provides the earliest measure of tumor response so far. *Sorensen et al.* [91] used cfDNA to monitor treatment on 28 metastatic breast cancer treated with trastuzumab and chemotherapy. A decreased rate of cfDNA after trastuzumab treatment was observed in 40% and associated with an improved response rate (p= 0.02) and OS (p= 0.05). *Murtaza et al*. [92] tracked genomic evolution of metastatic disease in response to treatment by analyzing cfDNA, in few cases. For example, an activated mutation in *PIK3CA* following paclitaxel and a mutation in *MED1* following tamoxifen and trastuzumab were reported. Monitor genetic markers of treatment resistance might lead to prevent progression by changing treatment before imaging detection of relapse or increased cfDNA levels.

Lack of standardization in cfDNA detection is the main issue to access an accurate analytical validity. In fact, DNA can be extracted from plasma or serum and genetic alterations can be analyzed by several technical tools with limitations for each of them: a prior tumor biopsy is needed to select the mutation to monitor for some (e.g. digital PCR [90], BEAMing [93]); high quality DNA is needed for large analyze of genetic landscape for others expansive ones (New generation of Sequencing (NGS) [90]).

Up to now, high detection rates are not reproducible in early stage cancer probably because of insufficient DNA quantity of low tumor burden [94,95]. Moreover, discordant results concerning the specificity of cfDNA detection at baseline, is raising an issue because of high false positives in benign lesions in some cohorts [96,97].

Disseminated tumor cells in bone marrow

Disseminated tumor cells in bone marrow (DTC-BM) were found to be prognostic in metastatic disease at the beginning of the 2000's. BM aspiration has a limited ability for repeated measures because it is an invasive and painful procedure. As a consequence, CTCs detection currently surpasses this tool thanks to more convenient patient procedure and significant congruence between DTC-BM and CTC [98]. However one needs to remember that DTC may have a different biological significance compared to CTCs and further studies are granted.

Conclusion

Up to date, evidence suggests that some women with ER-positive disease might be cured by endocrine therapy alone whereas others require chemotherapy. The main issue with the majority of the available tests that claim to be predictive is the clear lack of clinical validity. Results of ongoing prospective randomized clinical trials will strengthen this weakness.

Using primary tumor signatures for patient selection in metastatic setting is not standard practice, but a good screening tool for clinical trials of targeted therapies as they can have a initial validation on the go. Circulating biomarkers will allow better monitoring of metastatic disease.

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Table 1

Summary of gene expression assays in early stage breast cancer [2,37]

Assay	Tissue sample	Method	Number of Genes/Proteins	Analytical validity	Clinical validity	Ongoing clinical prospective studies	Patients
OncotypeDX®	FFPE	qRT-PCR	21	YES[8]	YES[4,8,11]	TAILORx trial[18]	N±ER+
Mammaprint®	Fresh frozen/FFPE	DNA microarray	70	YES[21]	YES[19,20,24,25]	MINDACT trial[27]	N±ER±
PAM50	FFPE	qRT-PCR	50	NO	YES[29,30]	NO	N±ER+
EndoPredict	FFPE	qRT-PCR	11	YES[31]	YES[32,33]	NO	N±ER+
Breast Cancer Index	FFPE	qRT-PCR	7	NO	YES[34–36]	NO	N−ER+
GGI	Fresh frozen	DNA microarray	97	NO	NO	NO	N±ER±

Abbreviations: FFPE using formalin-fixed paraffin-embedded, *qRT-PCR* quantitative reverse transcriptase-PCR

Table 2

Clinical impact of cfDNA detection in metastatic setting

Study (year)	Patients (n)	Source	Technique	Genetic alterations monitored	Clinical impact
Müller et al. (2003)[99]	10	Serum	MethyLight	RASSF1A and/or APC methylation	na
Board et al. (2010)[94]	72	Plasma Serum	ARMS-Scorpion PCR	PI3KCA	na
Higgins et al.(2012)[93]	85	Plasma	BEAMing	PI3KCA	na
Sorensen et al(2010)[91]	28	Plasme	Real-time PCR	HER2	Decreased cfDNA increased OS (p=0.05)
Yamamoto et al (2012)[100]	58	Serum	OS-MSP	RASSF1A, GSTP1 and RARβ methylation	na
Dawson et al. (2013)[90]	30	Plasma	Digital PCR, Tam-Seq	PI3KCA, TP53, structural variation	Increased cfDNA reduced OS (p<0.001)

Abbreviations: OS-MSP one-step methylation-specific polymerase chain reaction PCR Polymerase Chain Reaction