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REVIEW

The potential for liquid biopsies in the precision medical treatment of breast cancer

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ABSTRACT Currently the clinical management of breast cancer relies on relatively few prognostic/predictive clinical markers (estrogen receptor, progesterone receptor, HER2), based on primary tumor biology. Circulating biomarkers, such as circulating tumor DNA (ctDNA) or circulating tumor cells (CTCs) may enhance our treatment options by focusing on the very cells that are the direct precursors of distant metastatic disease, and probably inherently different than the primary tumor's biology. To shift the current clinical paradigm, assessing tumor biology in real time by molecularly profiling CTCs or ctDNA may serve to discover therapeutic targets, detect minimal residual disease and predict response to treatment. This review serves to elucidate the detection, characterization, and clinical application of CTCs and ctDNA with the goal of precision treatment of breast cancer.

KEYWORDS Circulating tumor cells (CTCs); circulating tumor DNA (ctDNA); cell free DNA (cfDNA); biomarker; cancer

Introduction

The concept of personalized medicine in oncology, or customizing cancer treatment based on specific biomarkers predictive of drug response, is rapidly progressing based on innovative translational research strategies and the implementation of biomarkers in clinical trial design. The key to personalizing medicine is to identify biomarkers which are easily accessible and could be repeatedly accessed to inform physicians about an individual's tumor biology, predict which therapeutic interventions may be of greatest benefit, monitor treatment responses to therapy, identify mechanisms of therapeutic resistance to guide subsequent therapies, and assay for microscopic relapse[1].

Primary tumor gene expression profiling is prognostic in terms of probability of recurrence; however, breast cancer patients would benefit greatly from a blood test that could provide a real time assessment of the cancer cells that are most likely to produce distant metastatic disease. Circulating biomarkers may provide insights into how to better treat breast cancer patients by focusing on the very cells that are the direct precursors of distant metastatic disease, and probably very different than the primary tumor's biology. Two circulating biomarkers through which personalized medicine can be achieved are the study of circulating tumor cells (CTCs) and that of circulating tumor DNA (ctDNA). These have been promoted as potential real time "liquid biopsies", minimally invasive biomarkers capable of providing a global picture of the tumor burden and vital genetic information about the targeted tumor from a blood draw[2-4]. However, challenges still remain in the identification, isolation, and ultimate harnessing of these methodologies in order to effectively guide treatment. This review serves to identify the detection, characterization, and application of ctDNA and CTCs with the goal of precision treatment of breast cancer.

CTCs

CTCs are recognized to be rare cells that are shed from the primary or metastatic tumor and can be isolated from the peripheral blood of patients with solid and liquid tumors[5]. CTCs were first reported in 1869 in the blood of a man with metastatic cancer by scientist Thomas Ashworth, who performed a thorough comparison of the morphology of the

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circulating cells to tumor cells leading him to conclude that "one thing is certain, that if they (CTCs) came from an existing cancer structure, they must have passed through the greater part of the circulatory system"[6,7]. CTCs are an independent prognostic factor in all stages of breast cancer[8-12]. CTC's rarity and short half-life, probably measured in hours, renders isolation a challenge; therefore a successful assay should ideally be able to process a large amount of cells in a relatively short amount of time while being able to specifically enumerate and capture malignant cells from a vast blood background - requiring the ability to detect one CTC per 10^5-10^7 mononuclear cells[13]. Such assays include a large panel of technologies based on their multiple physical and biological properties such as size, deformability, surface protein expression, viability, and invasion capacity properties distinguishing them from the surrounding hematopoietic cell lineage[5,14]. Our group has previously reviewed the state of this scientific field and speculated that CTCs would gain considerable interest as molecular techniques that allowed for their characterization developed and could permit translation to the clinic[15].

CTCs have been molecularly profiled via several approaches, including using next-generation sequencing (NGS)[16,17]. These advances have revealed some mechanisms of drug resistance including epithelial to mesenchymal transition (EMT)[18-20]. In addition, these breakthroughs can help us characterize those cancer cells capable of metastasis and optimize targeted therapies based on each patient's unique tumor biology, not simply rely on the standard four therapeutic groups of breast cancer: (1) hormone receptor (HR) positive patients who have estrogen and/or progesterone-dependent tumors but are HER2 (human epidermal growth factor type 2) negative (ER/PR+HER2−); (2) HR positive and HER2 positive (ER/PR+HER2+) patients who have amplification or overexpression of HER2, in addition to estrogen and/or progesterone-dependent tumors; (3) HR negative group with HER2 overexpression/amplification (ER/PR−HER2+), and (4) triple-negative breast cancers (TNBCs), which are defined by a lack of expression of ER, PR, and HER2[21,22]. Optimization of detection techniques is required in order to molecularly characterize CTCs and incorporate this information into the current clinical landscape.

CTC detection techniques

Over the last two decades there have been several methods developed for isolating and analyzing CTCs. Overall they may be separated into affinity based, physical properties based, and functional assays. Many affinity-based techniques isolate cells based on expression of cell-surface markers, such as the epithelial cell adhesion molecule (EpCAM), and include the CellSearch assay (the only FDA approved method of detecting CTCs in breast cancer), the Herringbone-CTC chip, and flow cytometry-based approaches. Many of these selection technologies are criticized for their reliance on cell-surface expression of EpCAM to capture (and define) CTCs because some tumors down-regulate expression of this marker during EMT. In addition, preanalytic variables, such as time to assay are significant factors in CTC detection and may be more important than EpCAM high status, with the exception of claudin low tumors[23]. To address this limitation, physical property and functional based technologies have been developed that isolate CTCs on the basis of physico-chemical properties-such as size, density, surface charges, unique functional characteristics-that distinguish them from other blood cells[24]. Please see **Table 1** for a summary of the various isolation techniques being utilized[25-93].

Additionally, there are various pre-analytical conditions to take into consideration such as the time interval between blood draw and assay, type of tube utilized, use of fixatives or preservatives, and temperature. Our group has focused on pre-analytic variables pertaining to the amplification of picogram quantities of RNA as well as time to CTC assay influencing the number of cells recovered[23,94].

EMT

According to recent findings, more invasive CTCs may lose their epithelial antigens by the EMT process, rendering detection via EpCAM based technologies challenging. Through the EMT process, epithelial cells lose cell-cell contacts and cell polarity, downregulate epithelial-associated genes, acquire mesenchymal gene expression, and undergo major changes in their cytoskeleton. This cellular process culminates in a mesenchymal appearance and increased motility and invasiveness[95,96].

In the actuation of the EMT program, epithelial markers such as E-cadherin and cytokeratins are downregulated, whereas mesenchymal markers, such as vimentin and fibronectin are frequently overexpressed. Furthermore, intermediate phenotypes between epithelial and mesenchymal differentiation are described to co-exist in human cancer[97]. Cancer cells can be induced to undergo EMT by several signaling pathways, most notably those involving the cooperation between TGF-β 1 signaling and oncogenic RAS or other receptor tyrosine kinases, as well as Wnt, Notch, and the signaling activated by Hedgehog[98],

Table 1 A summary of the various isolation techniques being utilized

Technique	Description	Reference
Physical properties based assays		
Dean Flow Fractionation (DFF)	The device maintains to process 3 mL of whole blood in an hour using centrifugal forces with >90% CTC recovery. The continuous collection of sorted CTCs and short residence time in the device significantly shortens the CTCs exposure time to constant shear in the channel, thus minimizing any undesirable shear induced changes to the CTCs' phenotype.	25
Cell density-based enrichment	Density gradient separation of CTCs from other cells in the blood may be performed using commercially available density gradient liquid separation kits. This process generates a layered separation of cell types based on their density. Limitations include a possible loss of CTCs due to an unwanted migration into the plasma fraction, as well as the formation of nonspecific aggregates containing CTCs at the bottom of the gradient.	26-28
Size-based cell enrichment by filtration	Direct enrichment of epithelial cells by filtration is based on the observation that the vast majority of peripheral blood cells are among the smallest cells in the human body. They can be eliminated by blood filtration using polycarbonate membrane calibrated pore filters. This method is quite simple, involving one single step.	29-34
Selective size amplification (SSA)	It offers advantages not only in resolving the trade-off between recovery rate and purity—optimizing both—but also in reducing the mechanical stress exerted upon the CTCs during filter transit. The major reasons for this enhanced performance include distinctive size discrimination between WBCs and CTCs as well as the benefits of the solid microbeads mitigating cell deformation within the MOA filter gap.	35
3D microfiltration	This device consists of two parylene membrane layers with pores and a gap precisely defined by photolithography. The positions of the pores are shifted between the top and bottom membranes. The bottom membrane supports captured cells and minimizes stress, which is concentrated on the cell membrane and sustains cell viability during filtration under very low pressure.	36
ISET (isolation by size of epithelial tumor cells)	Size-based enrichments of CTCs have been described by membrane filter devices such as ISET.	29,30,37
NanoVelcro CTC Chip	By switching device temperature in a physiologically endurable range (i.e. 4-37°C), thermoresponsive conformational changes of nanosubstrate-grafted polymer brushes alter the accessibility of capture agent to specifically capture (37°C) and release (4°C) CTCs to give viable CTCs in desired purity.	38
Telomescan	A novel cancer detection platform that measures telomerase activity from viable CTCs captured on a parylene-C slot microfilter. Using a constant low pressure delivery system, the new microfilter platform is capable of cell capture from 1 mL of whole blood in less than 5 min, achieving 90% capture efficiency. Addition of an adenovirus-containing GFP to peripheral blood assay, incubation with cancer cells allows precise enumeration and visualization of CTCs.	39,40
Affinity based assays		
CellSearch	The only FDA-approved technology for CTC detection is based on immunomagnetic enrichment. It employs an immunomagnetic enrichment step to isolate cells that express the epithelial cells' adhesion molecule (EpCAM). Additionally, to be identified as a CTC, the cell must contain a nucleus, express cytoplasmic cytokeratin, and have a diameter larger than 5 μm. This technology has demonstrated the prognostic utility of enumerating and monitoring CTC counts in patients with metastatic breast, prostate, and colorectal cancers. Semiautomated analyzer enriches CTCs with ferrofluid nanoparticles coated with anti-EpCAM antibodies, then CD45-, CK8+, CK18+ and CK19+ cells are counted by a four-color semiautomated fluorescence microscope.	41-47

Table 1 (*continued*)

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Technique	Description	Reference
CTC-chip	Capture of CTCs by EpCAM-coated microposts under strict manipulation of velocity and shear force. It enables a high yield of capture (median, 50 CTCs per milliliter) and purity (ranging from 10% to 50%), most likely caused by the gentle one-step microfluidic processing. Captured cells remain viable after capture, although the absence of cell fixation currently limits the time allowed between blood collection and microfluidic analysis to a few hours. Captured CTCs are visualized by staining with antibodies against cytokeratin or tissue-specific markers. For CTC enumeration, the entire device is imaged at multiple planes using a semiautomated imaging system while on-chip lysis allows for DNA and RNA extraction and molecular analyses. Nuclear fluorescence and CK stain for positive selection and CD45 stain for negative selection; CTCs captured are directly recognized by cameras, based on morphology, viability and expression of tumor markers. It has a total of 98% cell viability and high detection rate, making further analysis possible.	48-51
Herringbone-chip	Its chambers were made of transparent materials, allowing imaging of the captured CTCs, using traditional histopathological stains, transmitted light microscopy and immunofluorescence-conjugated antibodies. The Herringbone-chip has been tested in metastatic prostate and lung cancer patients, verifying results with those obtained with the CTC-chip method of analysis: Herringbone-chip shows higher flow rates and higher CTC capture efficiency and purity.	52,53
AdnaTest	Immunomagnetic separation with EpCAMs and MUC1 coupled antibodies; further analysis by isolation, direct lysis, mRNA extraction and application of multiplexed RT-PCR for HER2, EpCAM and MUC-1. Possibility to characterize CTCs for stem cells and epithelial mesenchymal transition. It lacks flexibility and automation. Cannot enumerate cells due to lysis. False-positive results due to the expression of the same antigens on nontumor cells; false-negative results due to loss of antigens on CTCs.	54-58
EPISPOT (Epithelial ImmunoSPOT)	Detects only viable cells after the depletion of CD45- positive cells, and was introduced for CTC analyses. Avoiding direct contact with the target cells, this technique assesses the presence of CTCs based on secreted or released proteins during 48h of short-term culture.	59,60
Collagen Adhesion Matrix (CAM) assay	It has been reported in breast, prostate and ovarian cancer: CAM ingestion and epithelial immunostaining identifies CTCs based on their invasive properties *in vitro*.	61
MAINTRAC	A specialized laser scanning cytometer provides another EpCAM-based approach.	62
Biocept	Utilizes proprietary antibody based enrichment technique to detect rare CTCs found in a patient's blood sample (1 in 1 million).	63
Photoacoustic flowmetry	Making use of the broadband absorption spectrum of melanin, it has been tested to detect melanoma cells and has been combined with nanoparticles targeting cell surface antigens to broaden its applicability in CTC detection.	64,65
MagSweeper	A magnetic stir bar coated with an antibody to EpCAM. The device can process 9 mL of blood per hour and purified cells of interest can be individually selected for subsequent molecular analysis, since the MagSweeper technology preserves cell function and does not perturb gene expression.	66-70
DEPArray (Silicon Biosystems)	An automated system with fluorescence imaging that captures cells in a chip based upon electric movement. DEPArray achieved 100% purity, eliminating all white blood cells (WBC), in the isolation of a mixed population of tumor cell lines downstream of CellSearch enrichment. This enabled molecular profiling of pure tumor cells from whole blood spiked tumor cell lines.	71
CTC-iChip	Whole blood is now processed through a microscale system at speeds of 8 mL/hour while preserving the high sensitivity afforded by microfluidic isolation techniques. Furthermore, rapid and gentle isolation of CTCs, as well as their collection in suspension, increases the integrity of these cells and their RNA quality. Moreover, the system can be run in either a positive selection or a negative depletion mode. The robustness of this platform was demonstrated by staining CTCs per clinical pathology protocols, which yielded high-quality diagnostic images. The negCTC-iChip allowed for isolation of CTCs from a nonepithelial cancer (melanoma) and from cancer that has undergone EMT and lost virtually all detectable EpCAM expression (TNBC). Limitations: low CTC purity to facilitate routine molecular analyses of CTCs and total blood volume needed to enable early cancer detection.	72

Table 1 (*continued*)

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Technique	Description	Reference
Negative depletion CTC enrichment strategy	Relies on the removal of normal cells using immunomagnetic separation in the blood of cancer patients. This method is based on the combination of magnetic and fluid forces in an axial, laminar flow in long cylinders placed in quadrapole magnets.	73
Millennium Sciences IsoFlux	The blood is centrifuged. Immunomagnetic particles are added to the PMBC layer that target the cells of interest. It is then transferred into a microfluidic cartridge. A permanent magnet is placed on the roof of the channel to attract the labeled target cells.	74
Cynvenio Liquid Biopsy platform	This platform uses high throughput sheath flow microfluidics for the positive selection of CTC populations. Furthermore the platform quantitatively isolates cells useful for molecular methods such as detection of mutations in 50 oncogenes.	75
Photoacoustic flowmetry	Making use of the broadband absorption spectrum of melanin, it has been tested to detect melanoma cells and has been combined with nanoparticles targeting cell surface antigens to broaden its applicability in CTC detection.	64,65
Cytometric assays		
FACS (Fluorescence-activated cell sorting)	It enables simultaneous analysis of multiparameters, such as size, viability, DNA content and expression of different markers for CTCs detection. It has high specificity, but low sensitivity.	76-79
Slide-based automated scanning microscopes (Ikoniscope and Ariol)	Maximizes scanner utilization with brightfield-multi-channel fluorescent and FISH capture capabilities. Introduced for detecting CTCs; still need to be validated.	80,81
Fiber-optic array– scanning technology (FAST)	It involves deposition of nucleated cells on the surface of a large glass slide, with scanning of cells positive for epithelial or tumor-specific antigens. Ultra-high-speed automated digital microscopy using fiber-optic array scanning technology has been developed to detect CTCs mounted directly on a slide that are labeled by antibodies with fluorescent conjugates.	82,83
Multiphoton intravital flow cytometry	It detects CTCs tagged *in vivo* using injected fluorescent ligands as they flow through the vasculature.	48
Functional based assays		
Folate-conjugated nanotubes and magnetic uPA-conjugated nanoparticles + photoacoustic flow cytometry assay	This assay has been validated in a mouse model. Most cancer cells express folate receptors and high levels of the urokinase plasminogen activator (uPA) receptors. Thus, CTCs can be dually targeted *in vivo* (in the bloodstream) with folate-conjugated nanotubes and magnetic uPA-conjugated nanoparticles and subsequently detected with two-color photoacoustic flow cytometry. Future studies on humans will inform whether this new platform can diagnose tumor cell dissemination.	84
Molecular detection		
RT-PCR	It allows the analysis of expression of candidate genes specific to epithelial tumor cells by mRNA evaluation, often combined with other enrichment techniques. It has high sensitivity. Disadvantages include RNA degradation, false-positive results due to nonspecific amplification, contaminations and pseudogenes; false negative results due to low expression levels.	85-91
Enzyme-linked immunosorbent spot technology	Immunological assay based on the ELISA (identification and count of cells able to secrete proteins like MUC1 and CK19 in short-term culture), after immunomagnetic depletion of CD45+ cells. Disadvantages include: CTC isolation not possible, further analysis not available, need of active protein secretion and technically challenging.	55-57
QuantiGene ViewRNA CTC Platform	CTC is isolated by size; sample is prepared (fixed, baked, permeabilized and protease digested) to enable RNA accessibility. Target RNA Probe Sets are hybridized followed by a sequential hybridization of signal amplification and detection components. Once processed, filters are transferred to a microscope slide for image processing and analysis.	92
CK19 mRNA Assay	Assays targeting specific mRNAs are the most widely used alternative to immunological assays to identify CTCs. In breast cancer, the CK19 mRNA has been most frequently used in clinical studies. Many transcripts (e.g. encoding CK18, CK19, CK20, Mucin-1, prostate-specific antigen and carcinoembryonic antigen), however, are also expressed at low levels in normal blood and BM cells 93, so quantitative RT-PCR assays with validated cutoff values are required to overcome this problem.	93

which may be potential drug targets.

In addition, certain transcription factors (TF), including TWIST1, SNAIL1, SLUG, ZEB1, and FOXC2 can induce EMT in mammary epithelial cells and/or breast cancer cells[99]. Moreover, blocking the expression of TWIST1 in the highly metastatic 4T1 murine mammary cell line reduced both metastatic burden and the number of CTCs in mice bearing xenograft mammary tumors, thus linking EMT, metastasis, and the presence of CTCs[99]. These findings suggest that the expression of epithelial-cell surface markers, such as EpCAM, may not be optimal for detecting a heterogeneous population of CTCs including those with a mesenchymal phenotype. Evidence exists that EpCAM-negative CTCs might have undergone EMT[54].

Raimondi et al.[95] investigated the expression of EMT and stem cell markers in CTCs from 92 metastatic breast cancer patients. CTCs were isolated by CELLection Dynabeads coated with the monoclonal antibody toward EpCAM. Samples positive for CTCs presence (CD45-/CK+) were evaluated for the expression of ER alpha, HER2, ALDH1, vimentin, and fibronectin. Samples negative for CTCs presence (CD45-/CK-) were also evaluated for the expression of vimentin and fibronectin, used as markers of EMT. In 34% of patients, they detected cells with negative CK/CD45 expression but positive expression of vimentin and fibronectin[95]. This mesenchymal phenotype is more common for the basal-like molecular subtype of breast cancer[100]. However, further analysis is needed to classify CTC expression with known subtypes of breast cancer for both validation and clinical relevance.

One study on mRNA analysis shows that the low abundance of EpCAM expression is associated with increased vimentin in basal-like breast cancer[101]. Another study further demonstrated dynamic changes in epithelial and mesenchymal composition in circulating breast cancer cells with mixed probes using RNA *in situ* hybridization, particularly in CTC clusters. Although rare in the circulation compared with single CTCs, CTC-clusters have 23 to 50-fold increased metastatic potential. CTC clusters are oligoclonal in origin, derived from groupings of primary tumor cells that together enter the circulation[102]. They can be captured via several techiniques such as microfluidic devices, including a specifically designed cluster chip which operates at sub-physiological flow rates, preventing these highly deformable cell groupings from squeezing through small pores under higher flow pressures[103], direct precipitation of all blood cells onto specially prepared slides, followed by high-speed microscopic scanning[104], as well as enrichment based techniques[105,106].

Claudin-low tumor cells

The most commonly used markers to identify carcinoma cells are cytokeratins, which have become a standard detection marker for CTCs[56,57,93,107]. Since hematopoietic cells rarely express cytokeratin proteins detectable by immunostaining[108,109], specific detection can be achieved for cells from epithelial tumors, such as breast carcinomas from blood cells. However, if tumor cells undergo EMT to migrate and invade the body, the cytoskeleton is rearranged and epithelial markers such as E-cadherin, claudins, and cytokeratins are downregulation[110]. Many detection methods using cytokeratin antibodies are not able to detect all CTCs secondary to this downregulation[111]. If indeed not all CTCs are detected by the common detection methods, false-negative results would hamper the clinical implementation of CTCs as a diagnostic marker. To improve CTC detection of claudin low tumors, the use of CD146 and CD49f as selection markers has been shown to improve detection of those cell lines showing EMT-features[112]. Our group has recently demonstrated that EpCAM based capture of CTC mimics using a panel of ten cell lines recovered all intrinsic subtypes of breast cancer except the claudin-low group[23]. Furthermore, detection techniques not dependent on epithelial markers may be used for detection of these cells, see **Table 1**.

Apoptotic CTCs

As tumors increases in volume, so too does the cellular turnover and hence the number of apoptotic and necrotic cells. Under normal physiologic circumstances, apoptotic and necrotic cellular remains are cleared by infiltrating phagocytes. In theory this does not happen efficiently within the tumoral mass, leading to CTC escape as well as the accumulation of cellular debris and its inevitable release into the circulation(ctDNA)[113,114].

Although many CTCs migrate early from the primary tumor into the circulation, many may be cleared within a few days[115]. According to the seed and soil hypothesis, the survival of these cells depends on their distinctive biologic characteristics as well as on the microenvironment at the secondary site[116]. Only rare subsets of cells finally succeed in establishing a cross-talk with stromal cells in secondary organs that promotes tumor cell survival, angiogenesis, and metastatic outgrowth. This could be related to the induction of senescence in CTCs, to a low proliferative potential, or to the presence of apoptotic cells. Mehes et al.[117] first noted apoptotic cells significantly contribute to the circulating

tumor cell fraction in breast cancer patients after he performed a detailed microscopic analysis. An "inclusion type" cytokeratin staining pattern and nuclear condensation indicated apoptosis in the CTCs isolated. Furthermore, apoptosis-related DNA strand breaks could be demonstrated by applying the TdT-uridine nick end labeling assay in these cells[117]. The monoclonal antibody targeting the neoepitope M30, revealed by caspase cleavage of CK18 in early apoptosis, has also been shown to be a marker for apoptotic CTCs[118]. Kallergi et al.[119] noted the presence of exclusively apoptotic CTCs in a patient may represent a favorable prognostic factor, whereas the preponderance of proliferating cells (determined by Ki-67) could be related to poor patient outcome in breast cancer. The median percentage of apoptotic CTCs per patient in his study was lower in patients with advanced disease compared with those with early disease. Adjuvant chemotherapy reduced both the number of CTCs per patient and the number of proliferating CTCs. Kallergi et al.[119] concluded that the detection of CTCs that survive despite adjuvant therapy implies that CTC elimination should be attempted using agents targeting their distinctive molecular characteristics.

CTC enumeration clinical trials

Numerous studies including Bidard et al.[12] pooled analysis reveal that CTC count is independently prognostic of progression-free survival and overall survival in metastatic breast cancer (MBC) patients[120]. Other trials by Lucci et al.[9] and a meta analysis by Zhang et al.[10] have further demonstrated CTC detection is prognostic in non-metastatic breast cancer as well[11].

Additionally, The Southwest Oncology group (SWOG) completed a prospective clinical trial (SO500) to evaluate the utility in changing therapy versus maintaining therapy in MBC patients who have elevated CTCs after one cycle of a new first-line chemotherapy[121]. Although CTC status was prognostic, simple enumeration did not predict for a benefit in switching to an alternate cytotoxic therapy, likely because simple enumeration has a low predictive value and cannot predict a specific course of treatment. Other limitations to this trial include chemo/cross resistance and statistical limitations.

The early DETECT trials revealed that serial CTC measurements before and after chemotherapy are prognostic and can be used to monitor treatment benefit[122,123]. Subsequent trials being conducted by the DETECT study group are evaluating targeted agents based on phenotypes of CTC in patients with MBC[124]. To fully realize the potential of CTCs as a useful biomarker, simple enumeration is not sufficient. Detailed molecular profiling of CTCs must be performed to inform the discovery of therapeutic predictors and actionable targets, important steps forward in personalizing medicine.

CTC molecular profiling

Several mechanisms exist to profile CTCs including protein expression, genomic, gene expression and functional characterization[125,126]. They have been cultured *in vitro*, used in xenotransplantation *in vivo*[16,127], and assessed using next-generation sequencing (NGS)[16,17]. Several studies have shown that recurrent/metastatic breast tumors and CTCs show discordance in hormone receptors (ER and PR) and HER2 expression from the primary tumor[112,128-130]. Furthermore, patients with TNBC do not have identified targeted therapies and are currently treated by chemotherapy alone and have very limited treatment options upon distant metastasis. Therefore, comparing genomic alterations in cancer-related genes between primary, metastatic breast tumors and CTCs and may provide insights into mechanisms of tumor metastases and drug resistance that may help guide targeted therapeutics[131]. Recent technical advancements in massively parallel or high-throughput NGS offer multigene mutational profiling that provides comprehensive genetic information on breast cancer molecular pathology, paving the road for newer and more effective therapeutic targets[131]. NGS has changed the perspective on genome profiling, since DNA sequencing has the potential to identify structural changes, including gene fusions and even point mutations, in addition to copy number. Furthermore, NGS using RNA Seq would permit full genomic and transcriptomic profiling, maximizing the ability to study individual genes, pathway perturbations, and chromosomal losses/gains. Additionally, NGS panels for inherited cancer has increased the potential to identify pathogenic variants in genes that would not typically have been tested (in breast cancer, genes other than BRCA1/2) and to offer more tailored preventative management to patients and family members[132]. Each sequencing technique has specific advantages and disadvantages (**Table 2**)[133-144].

In addition there are a variety of NGS platforms available including second generation, sequencing of an ensemble of DNA molecules with wash-and-scan techniques and third generation, sequencing single DNA molecules without the need to halt between read steps, whether enzymatic or otherwise[145] (**Table 3**)[136,146-160].

The advantages of sequencing CTCs include: (1) the ability

Table 2 Advantages and disadvantages of DNA sequencing

Method	Basic technique	Advantages	Disadvantages	Reference
Sanger-chain termination method (first generation sequencing)	Fluorescent dye-labeled bases; DNA fragments separated by capillary electrophoresis	High sensitivity, gold standard complete sequence	Very time consuming; cannot detect deletions, translocations or copy number changes	133-136
Pyrosequencing-sequencing by synthesis method	Chemiluminescent detection; DNA polymerase synthesizes cDNA to a target template; pyrophosphate release is detected at each base addition	More sensitive than Sanger; provides % of mutated *vs*. wild-type DNA; works well with fragmented DNA	Short read length limits technique to hot spots. Limited accuracy at detecting changes in homopolymer runs. Scalability is limited compared with other NGS methods	136-140
Allele-specific RT-PCR	Primers span DNA sites of interest and probes detect specific mutations	Very high sensitivity widely used for clinical testing for oncogene mutations in CRC and NSCLC	Scalability constraints limit application to hot spots	136,141-143
RT-PCR melting curve analysis	Heterogeneous DNA PCR products melt at different temperatures than homogenous DNA/PCR products	High sensitivity provides percentage of mutated versus wild-type DNA	Often difficult to resolve differences in melt curves. Difficult to standardize. Multiplex capability is limited	136,144

to fully sequence large numbers of genes (hundreds to thousands) in a single blood test and simultaneously detect deletions, insertions, copy number variations, translocations, fusion events and exome-wide base substitutions (including known "hot-spot mutations") in all known cancer-related genes in a cell(s) most likely to cause metastases, which may be important for predicting response to select therapies; (2) results from sequencing are presented as either positive or negative and do not appear as gradations as with immunofluorescence; (3) analysis can be automated to reduce interpreter bias; and (4) allows us to evaluate tumor heterogeneity[161].

Molecular profiling of CTCs has been shown to predict response and or resistance to therapy. RNA sequencing via CTC-iChip revealed activating mutations in PIK3CA, FGFR2, and ESR-1 in breast cancer patients. Drug sensitivity testing revealed that the selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene, and the selective ER degrader (SERD) fulvestrant, were ineffective in ESR-1 mutant cells, either alone or in the clinically approved combination with inhibitors of the phosphatidylinositol 3-kinase-mammalian target of rapamycin (PI3K-mTOR) pathway (everolimus). Cultured CTCs were highly sensitive to the PIK3CA inhibitor BYL719 and the FGFR2 inhibitor AZD4547[127].

A trial conducted by Gradilone et al.[162] revealed that in MBC patients the presence of CTCs expressing multidrug-resistance-related proteins, and ALDH1, is predictive of response to chemotherapy. Liu et al.[163] reported on a patient affected by chemo-refractory metastatic HER2-positive breast cancer receiving lapatinib. The detection and characterization of CTCs was evaluated and depletion of the EGFR-positive CTC pool in the blood was associated with tumor response, whereas disease progression was related to a recurrence in CTCs, indicating expression of EGFR may predict response to lapatinib-based treatments.

Sequencing techniques also have several disadvantages including: (1) limitations with regards to sensitivity that make single cell analysis difficult[164,165], including amplification bias[166]; and (2) leukocyte contamination which may cause false positive/negative results due to impaired visualization of amplified transcripts. However, several groups have been able to overcome these challenges via various techniques, such as micromanipulation, laser-capture microdissection, and flow cytometry with promising results[167,168]. For instance Magbanua et al.[169] demonstrated that it is feasible to isolate CTCs away from hematopoietic cells with high purity through immunomagnetic enrichment followed by fluorescence activated cell sorting (IE-FACS) and profile them via array comparative genomic hybridization analysis. With newer amplification techniques such as whole genome amplification, in combination with NGS and FACS, some researchers have shown it is possible to profile copy number in a single cell in various cancer types, including in breast cancer[170-173]. Single cell analysis has identified clinically significant genomic disparity between primary tumors and CTCs[3,174-176] therefore analysis of CTCs in this fashion may surmount the plaguing concern of tumor heterogeneity.

Table 3 Next-generation sequencing platforms

Device	Method	Generation	Major uses	Run time	Length of reads	Reference
Illumina Hi Seq 4000	Flow cell-based, reversible dye termination and four-color optical imaging	2nd	WES WGS SNP WTA	<1-3.5 days	2×150 bp	136,146-151
Roche 454 pyro-sequencing GS FLX+	Emulsion PCR with bead-based pyro-sequencing and CCD light imaging	2nd	WGS WTA Targeted Seq SNP	10 h	Up to 1000 bp	136,146,151-153
Life Technologies SOLiD 5500xl	Sequential dinucleotide ligation; flow cell-based four-color optical imaging	2nd	WES WGS SNP	6 days	2×60 bp	136,146,150,151,154
Life Tech-nologies Ion Torrent PGM	Semi-conductor based nonoptical detection; standard dNTP sequencing chemistry	2nd	Targeted panel Demand Sequenc-ing	<1 day	400 bp	136,151,155
Complete Genomics CGA platform	DNA nanoball arrays coupled with combinatori-al probe anchor ligation	2nd	WGS	Slow: weeks to months	~70 bases	136,151,156
Pacific Biosciences PacBioRSII	Zero-mode waveguide, individual polymerase; single molecule sequencing using fluorescent dNTPs	3rd	Long read, full microbial genome	<1-6 h	10-15 kb	136,151,157,158
Seqll Heliscope	True Single Molecule Sequencing, massive parallel sequencing without amplification	3rd	Targeted WGS WES WTA	~8 h	25-60 bp	136,151,156,159
Nanopore Minion	Nanopore exonuclease sequencing, no need for amplification	3rd	Long read, full microbial	1 min-48 h	230-300kb	160
Illumina Hi Seq 4000	Flow cell-based, reversible dye termination and four-color optical imaging	2nd	WES WGS SNP WTA	<1-3.5 days	2×150 bp	136,146-151
Roche 454 pyro-sequencing GS FLX+	Emulsion PCR with bead-based pyro-sequencing and CCD light imaging	2nd	WGS WTA Targeted Seq SNP	10 h	Up to 1000 bp	136,146,151-153
Life Technologies SOLiD 5500xl	Sequential dinucleotide ligation; flow cell-based four-color optical imaging	2nd	WES WGS SNP	6 days	2×60 bp	136,146,150,151,154
Life Tech-nologies Ion Torrent PGM	Semi-conductor based nonoptical detection; standard dNTP sequencing chemistry	2nd	Targeted panel Demand Sequenc-ing	<1 day	400 bp	136,151,155
Complete Genomics CGA platform	DNA nanoball arrays coupled with combinatorial probe anchor ligation	2nd	WGS	Slow: weeks to months	~70 bases	136,151,156
Pacific Biosciences PacBioRSII	Zero-mode waveguide, individual polymerase; single molecule sequencing using fluorescent dNTPs	3rd	Long read, full microbial genome	<1-6 h	10-15 kb	136,151,157,158
Seqll Heliscope	True Single Molecule Sequencing, massive parallel sequencing without amplification	3rd	Targeted WGS WES WTA	~8 h	25-60 bp	136,151,156,159
Nanopore Minion	Nanopore exonuclease sequencing, no need for amplification	3rd	Long read, full microbial	1 min-48 h	230-300kb	160

CCD: charge-coupled device; NA: not applicable; NGS: next-generation sequencing; WES: whole exome sequencing; WGS: whole genome sequencing; SNP: single nucleotide polymorphism; WTA: whole transcriptome analysis.

Tumor heterogeneity

Tumor heterogeneity can exist within the same site of disease or between sites of disease[177,178] and contamination with adjacent normal tissue can lead to false negative results or confound successful targeted therap[179]. As Navin and Hicks[168] pointed out, tumor heterogeneity exists in breast cancer because the malignant cells often arise from ductal tissue and are constrained by the duct structure until they begin to invade surrounding stromal tissue. They exhibit regions of growth, regions of hypoxia and necrosis, and regions of interaction with blood vessels and lymph ducts. Given these variable selection pressures, tumor cells within a single mass are not identical[180]. This heterogeneity in the molecular characterization of single cells, including CTCs could potentially identify rare driver mutations that are diluted out when profiling bulk tumors, including their stroma[168]. Navin et al.[172] revealed that by comparing multiple single-cell copy number profiles, they could provide highly accurate measures of genomic heterogeneity within solid tumors. Furthermore, by comparing multiple single-cell profiles, they showed that it was possible to reconstruct the evolutionary lineage of a tumor and understand its pattern of progression.

In addition, Heitzer et al.[181] has described a CTC sequencing approach for single-cell analysis, suggesting that contamination with normal cells may be reduced. Secondly, although the sequencing of CTCs does not change the fact that the readout is still at the level of the genome, we anticipate that, especially in cases in which metastatic lesions are inaccessible, CTC sequencing will strengthen conclusions regarding mutations that are drivers versus those that are passengers as they may be present not only in the primary/metastatic lesion but also in the cells that were able to escape into the circulation. The conserved nature of these mutations may suggest an important functional contribution to disease progression. Heitzer et al.[181] demonstrated the sequencing of CTCs may identify relevant private mutations, present but not detected in tumor tissue. In contrast to CTCs, single cell analysis is not possible when evaluating ctDNA as a biomarker.

CtDNA

Circulating DNA was first identified by Mandel and Metais[182] in 1948 but no association with disease was hypothesized. Only 30 years later, in 1977, Leon et al.[183] found circulating tumor DNA (ctDNA) in plasma of patients affected by lung cancer. Cell free DNA (cfDNA) was identified in the peripheral blood of healthy individuals however patients with cancerous tumors have higher quantities and detection is associated with poorer prognosis[4,184]. Due to reduced cell turnover and more efficient removal of defective cells from the circulation by phagocytes, the concentration of cfDNA is lower in healthy individuals. CtDNA, or soluble nucleic acids shed into the bloodstream in patients with cancer, are linked to apoptosis and necrosis of cancer cells in the tumor microenvironment. Secretion within exosomes has also been suggested as a potential source of cfDNA. The released tumor cells are usually phagocytosed by macrophages which engulf necrotic cells and release digested DNA into the tissue environment[185]. Additionally, tumors usually harbor a mixture of different cancer cell clones that justify their genomic and epigenomic heterogeneity, together with other normal cell types, such as hematopoietic and stromal cells. Thus, during tumor development and turnover both tumor-derived and wild-type cfDNA can be released into the blood. Consequently, the proportion of cfDNA that originates from tumor cells varies according to the state and size of the tumor. The amount of cfDNA is also affected by physiological factors such as clearance, degradation and filtering events of the blood and lymphatic system. Nucleic acids have a half-life in the circulation ranging from 15 minutes to several hours depending on the rate of clearance from the blood by the spleen, liver and kidney[186,187].

The characteristics of ctDNA suggest they are in part derived from apoptotic cells. For instance, when the lengths of ctDNA strands are measured, they often assume the classic ladder pattern in integer multiples of 180 base pairs, characteristic of the apoptotic process. In fact, most ctDNA fragments measure between 180 and 200 base pairs, suggesting that apoptosis likely produces the majority of ctDNA in circulation[113,114].

Pre-analytic considerations

Cell lysis can occur hours after venipuncture when using standard tubes for blood collection, leading to an increase in contaminating cellular DNA that may hinder analysis of ctDNA. This normal DNA from dying blood cells will contaminate the specimens and dilute ctDNA. This is the reason why plasma is recommended, since its analysis avoids the simultaneous testing of material originally associated with hematopoietic cells. However, centrifugation speed, amount of blood collected, a delay in blood processing and storage temperature (contaminating cells can be removed after storage at -20°C)can influence the amount of cfDNA extracted from plasma[187-189]. Toro et al.[190] conducted a study

comparing the properties of two cell stabilizing reagents in blood samples from MBC patients and measured genome equivalents of plasma DNA by droplet digital PCR. They compared wild type PIK3CA genome equivalents and also assayed for two PIK3CA hotspot mutations, E545K and H1047R. Their results demonstrated that blood stored for 7 days in BCT tubes did not show evidence of cell lysis, whereas PAX gene tubes showed an order of magnitude increase in genome equivalents, indicative of considerable cellular lysis[190]. Several kits exist for DNA extraction with some recent studies comparing the kits. The QIAamp DNA Blood Mini and Circulating Nucleic Acid kits from Qiagen (Hilden, Germany) gave the highest recovery of cfDNA[191,192]. Currently there is no uniform standardization and each study has its own protocol regarding pre-analytical conditions for processing specimens.

Furthermore, levels of cfDNA might reflect physiological and pathological processes that are not tumor-specific. CfDNA yields are higher in patients with malignant lesions than in patients with resected tumors, but increased levels have also been quantified in patients with benign lesions, inflammatory diseases and tissue trauma[193]. Please see **Table 4** for the advantages and limitations of CTCs *vs*. cfDNA[5-20,113,114,183-187].

CfDNA detection and molecular profiling

Several sensitive techniques exist to detect such mutations in cfDNA including droplet digital PCR (ddPCR)[194], CAPP-Seq[195] and Guardant360[196], Sanger sequencing[197], amplification refractory mutation system (ARMS)[198], pyrosequencing[199], pyrophophorolysis-activated polymerization (PAP)[200], tagged-amplicon deep sequencing (TAM-Seq)[201], beads, emulsion, amplification and magnetics (BEAMing)[202], modified semi-nested or nested methylation-specific PCR[203], utilization of microsatellite biomarkers followed by post-PCR product analysis using capillary array electrophoresis[204] among others. It is possible to evaluate nucleic acids for fusion events, copy number variants, indels, and single nucleotide polymorphisms.

NGS has been developed to target the genome at various scales (whole genome[205], whole exome[206], and targeted panels[207-209]) and are a key component toward realizing personalized care in oncology. A genome-wide screen for copy number changes has the advantage that it is an untargeted approach, which does not require any prior knowledge about characteristics of the primary tumor genome or its metastatic deposits. Panel-based targeted sequencing of selected cancer genes or mutational hotspots is a popular approach, and can generally be classified on the basis of the target enrichment method used (e.g. amplicon PCR versus hybridization capture), sequencing chemistry, and scale of the sequencing platform. There is considerable variability across targeted NGS panels implemented in different clinical laboratories in terms of the number and identities of genes tested, and sample throughput. The number of genes targeted varies depending on the technology. Most vendor solutions are generally amplicon PCR-based and target selected mutation hotspots in 1 to 50 cancer genes, and these include the Ion- Torrent AmpliSeq (Life Technologies, Carlsbad, CA)[210], and the Illumina TruSeq Amplicon Cancer Panel (Illumina, San Diego, CA)[207] among others. Targeted enrichment using hybridization capture can incorportate more genes without risk a of problematic primer. Via a customized hybrization capture technique the MSK-IMPACT panel targets 341 genes[208,209]. Foundation Medicine targets 287 genes (however on, formalin-fixed, paraffin-embedded (FFPE)tissue, use of these genomic targets as a ctDNA assay is currently being validated on clinicaltrials.gov protocol NCT02620527)[211,212].

CtDNA clinical trials

Several trials clearly demonstrate that ctDNA is a specific and highly sensitive biomarker in patients with MBC and non-metastatic breast cancer[4,213]. Via molecular profiling of such

Table 4 Advantages and limitations of CTCs *vs*. cfDNA

	Advantages	Limitations
CTC	Allows in depth assessment of viable metastatic tumor cells, DNA, RNA, and protein; may reflect mechanisms of resistance; may reflect treatment efficacy; allows functionally analysis (*in vitro/in vivo* testing)	Few and fragile; requires sophisticated isolation techniques; EMT may cause false negative results; tumor heterogeneity within CTCs; unclear which CTCs cause metastases
Cell free cfDNA	May reflect mechanisms of resistance; may reflect treatment efficacy; sensitive biomarker; available from other sources: urine, plasma, ascitic fluid	Present in inflammatory states and aging (not cancer specific); contamination with DNA from lysed cells; unclear if clinically relevant given being released from dying tumor cells; predetermined somatic alterations need to be identified; does not permit single cell analysis

nucleic acids, researchers have focused on targeting their specific gene mutations and possibly explaining resistance to chemotherapy in metastatic cancer[214].

For instance, Schiavon et al.[215] conducted a study on 171 breast cancer patients with advanced breast disease evaluating ESR1 mutations by ultra high-sensitivity multiplex digital polymerase chain reaction assaysin ctDNA. *ESR1* mutations were found to be a major mechanism of resistance to aromatase inhibitors, as evidenced by shorter progression-free survival (PFS) in those patients with the mutation, and more common in those with metastatic disease.

Additionally in the BOLERO-2 trial, Chandarlapaty et al.[216] demonstrated that patients with the ESR1 mutation D538G in ctDNA derived a 3.1 month PFS benefit from the addition of everolimus (an mTOR inhibitor), whereas those with the Y537S mutation showed stable PFS.

Additionally, it was demonstrated that persistent declines in the levels of the *AKT1 E17K* mutation in ctDNA is associated with durable tumor regression to the AKT inhibitor AZD5363[217].

Another trial assessing mutations in ctDNA as an indicator of therapeutic resistance is the phase 3 BELLE 2 trial. PIK3CA status in cfDNA predicted efficacy of the inhibitor buparlisib in combination with fulvestrant in postmenopausal women with endocrine resistant advanced breast cancer. In patients with PIK3CA mutations identified by ctDNA, median PFS was 7.0 months in patients treated with buparlisib *vs*. 3.2 months in those treated with placebo. In those with non-mutant PIK3CA by liquid biopsy, there was no difference in PFS[218].

Murtaza et al.[206] presented an extensive comparison of biopsy and plasma samples collected from a MBC patient over a 3-year clinical course, in which whole exome sequencing (WES) and deep sequencing of plasma DNA and tumor biopsies revealed resistance to targeted agents such as lapatinib, a tyrosine kinase inhibitor of EGFR (epidermal growth factor receptor) and HER2. Thus deep sequencing of plasma DNA, applied to selected samples with high tumor burden in blood, may help identify the mutations associated with drug resistance[193,219].

The role of ctDNA in evaluating minimal residual disease has also been evaluated by Schiavon et al.[215] in a prospective cohort of 55 early breast cancer patients in which detection of ctDNA in plasma after completion of apparently curative treatment-either at a single postsurgical time point or with serial follow-up plasma samples-predicted metastatic relapse with high accuracy. The concept of tumor dormancy may in part account for minimal residual disease in patients.

Tumor dormancy

Experimental biology studies have documented two types of tumor dormancy: cellular dormancy with solitary cancer cells held in cell-cycle arrest and micrometastatic dormancy with a balanced state of apoptosis and proliferation[220]. Dormant CTCs appear to account for late disease recurrence even after prolonged disease free survival in breast cancer[221]. CTCs are detectable in as many as one-third of patients who are prolonged survivors[222]. Payne et al.[223] confirmed the ability to detect minimal residual disease in breast cancer patients by assessing CTCs, DTCs and ctDNA. Shaw et al.[224] revealed that copy number variations (CNV) detected in ctDNA, mirror the primary tumor up to 12 years after diagnosis in one-fifth of breast cancer patients. SNP/CNV analysis of ctDNA was able to distinguish between patients with breast cancer and healthy controls during routine follow-up. Given these findings, the detection of minimal residual disease in breast cancer via CTCs or ctDNA is possible, and molecular profiling may be able to identify therapeutic targets in the future.

Circulating exosomes

Exosomes are small membrane vesicles (30-100 nm) containing functional biomolecules (proteins, lipids, RNA and DNA) that are released by most cell types upon fusion of multivesicular bodies with the plasma membrane, presumably as a vehicle for cell-free intercellular communication[225]. Circulating tumor derived exosomes were demonstrated to prepare a favorable microenvironment at future metastatic sites and mediate non-random patterns of metastasis via vascular leakiness, stromal cell education at organotropic sites, bone-marrow-derived cell education, and recruitment necessary to complete pre-metastatic niche evolution[226-228]. Furthermore, exosomal DNA was found to reflect the mutational status of parental tumor cells. Therefore future studies on exosomes as a biomarkers may be useful not only to predict metastatic propensity in breast cancer[229], but also to determine organ sites of future metastasis[227] and to monitor treatment response[230].

Circulating microRNAs

MicroRNAs (miRs) are evolutionary conserved, small non-coding RNA molecules consisting of approximately 22 nucleotides. One miR has binding affinity to hundreds of different mRNAs and hence, miRs are involved in the regulation of various cellular processes, such as development,

differentiation, and proliferation[231]. MiRs are released into the blood circulation by apoptotic and necrotic cells or active secretion[232]. They can exist either extracellularly, in association with Argonaut proteins, or in exosomes in the blood circulation[225]. To date, numerous miRs have been identified, and varied circulating miRs concentrations are found in different breast cancer subtypes and some are associated with prognosis and treatment response[233-235]. Given there is already quite a substantial literature in this emerging field, miRs are beyond the scope of this review.

Circulating biomarker characterization and clinical implications

Detailed molecular profiling of tumors and circulating tumor biomarkers such as CTCs and ctDNA has already been changing the therapeutic landscape of cancer across several cancer types. Currently it is standard clinical practice to evaluate genetic alterations in many cancers. For instance, in non-small cell lung cancer (NSCLC), the detection of the somatic activating mutations in EGFR predicts for sensitivity to EGFR inhibitors such as Tarceva[49,236,237]. In melanoma, the presence of BRAF V600E mutation predicts sensitivity to BRAF inhibitors such as vemurafenib[238]. In colon cancer the presence of *KRAS* mutations has been established as a negative predictive marker for treatment with EGF receptor (EGFR) inhibitors[239,240]. However, unlike drug therapy matched somatic mutations in NSCLC, melanoma, and colon cancer, drug therapy matched to the presence of a somatic mutation has yet to be robustly established as a standard approach in breast cancer.

In breast cancer, mutations in the estrogen receptor ESR1 are a potential driver of endocrine resistance[215,216,241]. Furthermore multiple alterations in genes related to the PI3K-mTOR pathway may confer resistance to trastuzumab[218,242]. A recent trial in breast cancer established a CTC PD-L1 assay that can be used for liquid biopsy in future clinical trials for stratification and monitoring of cancer patients undergoing immune checkpoint blockade[243].

As demonstrated in other tumor types and in breast cancer, a better understanding of mechanisms of treatment resistance and biomarkers to predict for response are required to reduce cancer related mortality. The key to standardizing the assessment of somatic mutations in breast cancer would be to integrate tumor, CTCs and ctDNA sequencing data into a systematic and broad-based plan with clinical endpoints. Currently there are several trials investigating anti-HER2 therapies in patients with HER2 negative primary tumors, yet HER2 positive CTCs such as the DETECT 3 trial[244], evaluating lapatinib, CareMore-Trastuzumab[244] evaluating trastuzumab, a monoclonal antibody directed against HER2 in breast cancer and CirCe TDM-1 evaluating TDM-1, trastuzumab linked to the cytotoxic agent DM-1[245]. Incorporating molecular profiling of CTCs/ctDNA in trials such as Molecular Analysis for Therapy Choice (MATCH), a genomic pre-screening study, the National Cancer Institute (NCI) aims to explore the efficacy of existing targeted agents against specific molecular aberrations, and to evaluate if these same therapies have comparable activities across different tumor subtypes[246]. One challenge to this approach is that there are relatively few targeted therapies anticipated to be effective in some solid tumors, such as breast cancer. NGS of circulating biomarkers may help discover new potential rational targeted therapy options.

Conclusions

Profiling primary tumor biology has led to many important insights about the biology of breast cancer, yet relatively few signatures predict treatment sensitivity. A critical barrier to progress may be that MBC and the circulating cells leading to macrometastasis are inherently different than primary breast cancer. In contrast, primary tumors shed CTCs and cfDNA into the systemic circulation where they are subject to metastatic inefficiency and potentially after a period of tumor dormancy may lead to macrometastases[247,248]. The ultimate goal of detecting and molecularly characterizing CTCs and ctDNA would be to shift the current clinical paradigm that incorporates relatively few prognostic/predictive clinical markers (estrogen receptor, progesterone receptor, HER2) to a landscape in which clinicians could measure tumor biology in real time by profiling CTCs or ctDNA in order to discover therapeutic targets, detect minimal residual disease, and predict response to treatment. In order to attain such a goal, CTCs and ctDNA sequencing data must be integrated into systematic and broad-based trials with clinical endpoints. Several trials discussed in this paper strive toward this goal, with the DETECT 3, BOLERO-2, BELLE 2 trials and others focusing on this very concept by evaluating therapeutic response based on phenotypes of CTCs or ctDNA in patients with breast cancer[124,216,218]. In summary, once further established, the molecular characterization of circulating biomarkers has the potential to provide the ideal mechanism of personalizing treatment in breast cancer for the best clinical results by predicting treatment response, evaluating minimal residual disease and allowing for the rational selection of targeted therapies.

Conflict of interest statement

No potential conflicts of interest are disclosed.

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