Role of ctDNA in Breast Cancer

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Breast cancer is a heterogeneous disease, while circulating tumor DNA (ctDNA) is DNA released by the tumor into the bloodstream and can accurately reflect this heterogeneity. In breast cancer, it is used mainly in research or in clinical trials, but it will likely be used in routine clinical practice once certain issues have been worked out and methods of analysis have been improved and standardized. Breast cancer classification and treatment selection are now based on analysis of the tumor but circulating tumor DNA carries many features of the original tumor and can be analyzed from a simple, non-invasive blood extraction.

ctDNA breast cancer liquid biopsy cancer diagnosis

1. Introduction

1.1. The Role of Circulating Tumor DNA (ctDNA) in Breast Cancer

Regardless of the exact origin of malignant cells, breast cancer is a heterogeneous disease. The primary classification of breast cancer is based on immunohistochemistry markers in tumor biopsies: estrogen receptor (ER), progesterone receptor (PR), KI-67, and human epidermal growth factor 2 (HER2). Different subtypes have been proposed with the aim of personalizing treatment and prognosis [1][2], and some groups have suggested using gene expression profiles to characterize five different intrinsic molecular subtypes of breast cancer, with different outcomes (luminal A, luminal B, HER-2 enriched, basal-like, and claudin-low) [3][4][5].

At present, the selection of breast cancer treatment is based on the analysis of tumor biopsy. However, the information obtained from the tumor biopsy is not permanent, and changes and acquired resistance that can occur during cancer treatment cannot be evaluated or analyzed in the original tumor specimen. Studies have found up to 25% of changes in subtypes at or after progression to anticancer therapies [6][7]. Although tumor biopsy is still the gold standard for diagnosis, classification, and treatment decisions, there is a growing interest in improving precision medicine by characterizing and monitoring the tumor genome in blood samples [8], known as liquid biopsy. A liquid biopsy can contain circulating tumor cells (CTCs), ctDNA, and exosomes that can help to understand tumor evolution, resistance, and heterogeneity during treatment [9]. Furthermore, in some cases, a tumor biopsy may not be feasible, and a liquid biopsy would be the only method to obtain a diagnosis or knowledge of the tumor biology.

1.2. ctDNA

In healthy donors, circulating free DNA (cfDNA) is isolated primarily from hematopoietic cells [10]. However, the origin of ctDNA is more complex. Cancer produces not only local infiltration but also malignant cells that are released into the lymphatic or vascular system. CTCs in the blood could be responsible for metastatic progression. The origin of ctDNA is thought to be the cellular breakdown from the tumor through apoptosis, necrosis or phagocytosis, although it could also arise from CTCs and active secretion from cellular structures has also been described [11][12].

Another important consideration is the ability to quantify ctDNA, and the variant allele fraction (VAF) is a crucial parameter. VAF is the percentage of sequence reads detected fitting specific DNA by complete coverage at the locus. Therefore, VAF could be the proportion of DNA carrying the mutant variant [13]. VAF detection in cancer patients can vary; for example, more than 10% could be detected in the metastatic setting, while in early stages of cancer or in minimal residual disease (MRD), less than 0.1% might be detected [14].

2. Methods for ctDNA Detection and Analysis

In cancer patients, ctDNA is found in a variable but usually very low percentage (0.01–1.0%) of the total cfDNA, which is usually less than 1 ng/µL, and varies depending on the stage, location, or vascularization of the tumor [15]. The amount of ctDNA is known to be 2–24 times higher in serum than in plasma [16]. However, this higher amount is associated with contamination by DNA released by blood cells during the coagulation process, so the use of plasma for ctDNA analysis is recommended [17].

qPCR and Sanger sequencing used to be very useful techniques, but due to their low sensitivity, they have been superseded by others. Targeted techniques have been developed, such as droplet digital polymerase chain reaction (ddPCR) and beads, emulsion, amplification and magnetics (BEAMing).

Other targeted DNA sequencing techniques include tagged amplicon deep sequencing (TAM-Seq), cancer personalized profiling by deep sequencing (CAPP-Seq), safe sequencing system (Safe-Seq), and amplicon sequencing (AmpliSeq). These techniques are very useful for analysis of a limited panel of potential mutations in the primary tumor or biopsy specimens [15][18].

Challenges for ctDNA in Breast Cancer

The implementation of ctDNA analysis in routine clinical practice faces several challenges. For example, it is crucial to improve detection of the low fraction of ctDNA in cfDNA and to identify tumor mutations in plasma at VAFs below the background sequencing error threshold. New detection methods have recently been described that can overcome this hurdle [19]. Blood volume is another important issue. To detect a single mutation with a VAF of 0.01% with 95% confidence requires 150–300 mL with 30,000× sequencing coverage, but increasing the numbers of mutations detected would also increase the volume needed [20]. In summary, the sensitivity of ctDNA analysis in localized breast cancer depends on both the blood volume analyzed and the number of mutations screened.

3. ctDNA in Early Breast Cancer

3.1. ctDNA in Breast Cancer Diagnosis

At present, the only available methods for screening and early detection of localized breast cancer, for which there is the option of curative treatment, are self-exploration and imaging tests such as mammography, echography, and magnetic resonance imaging.

Around 80–85% of breast cancers are diagnosed at the early stage but, unfortunately, about 30% of these will relapse with metastatic progression during the follow-up period. Primary risk factors for relapse are well described, including tumor grade, tumor stage, lymph node involvement, and immunohistology characteristics, and are used to define the best therapeutic approach [21]. The gold standard for diagnosis is still tissue biopsy, which provides information on the histology, molecular biology, and genetic profile of the tumor. However, breast cancer is a heterogeneous disease, and several molecular alterations may occur over time and influence treatment response, making it necessary to monitor these modifications and personalize treatment accordingly.

Other potential approaches to the use of cfDNA and ctDNA in early breast cancer are emerging. Interestingly, global cfDNA can be easily quantified and is known to be increased in breast cancer patients compared to healthy subjects [22]. Moreover, high levels of cfDNA correlate with more advanced disease stages [23]. A more complex approach for breast cancer screening uses multiplexed PCR and NGS to identify both clonal and subclonal copy-number variants (CNVs) in the ctDNA of breast cancer patients [24].

3.2. Detection of MRD

The principal neoadjuvant and adjuvant chemotherapy regimens in breast cancer include anthracyclines and taxanes, which are associated with short- and long-term toxicities. Detecting the need to increase or reduce the dose or duration of treatment could decrease these toxicities and also improve overall survival [25]. For this reason, several clinical trials use radiological tests during neoadjuvant treatment to predict a pathological complete response (pCR) and personalize treatment duration and dosage based on these findings [26]. However, ctDNA could be a more sensitive method to evaluate treatment response. In addition, ctDNA analysis could help identify patients unlikely to benefit from adjuvant chemotherapy and could play a crucial role in detecting patients with micro-metastases and a higher risk of future distant metastases, thus improving patient selection for certain treatments and avoiding unnecessary adverse events.

3.3. Epigenetic ctDNA Alterations

Since gene methylation and transcriptional regulation could predict treatment response and patient outcome, epigenetic ctDNA alterations have also been proposed as a promising biomarker in early breast cancer [27][28]. Serial blood samples taken during neoadjuvant chemotherapy were analyzed for the methylation status of BRCA1, MGMT, GSTP1, Stratifin, and MDR1. BRCA1 methylation frequency was different in responders and non-responders [29]. Another study of 336 early breast cancer patients found that patients with methylation of GSP1,

RASSF1a and RARb2 promotors before surgery had a lower overall survival rate at eight years than those without methylation (78% vs. 95%) [30]. Measurement of ctDNA methylation has also been proposed as a method to predict resistance to adjuvant tamoxifen treatment [31], and serum DNA methylation has been proposed as a surrogate marker of tumor DNA methylation for diagnosis and prognosis [32].

4. ctDNA in Metastatic Breast Cancer

The analysis of ctDNA offers a wide range of information in metastatic breast cancer patients. For example, it can provide a prompt diagnosis of disease relapse in previously treated early breast cancer patients. In addition, the assessment of gene mutations in ctDNA can help to select the best therapy for each patient. ctDNA analysis also provides information on the clonal evolution and heterogeneity of the tumor and can be used in the follow up of the disease to detect response or failure to ongoing treatments and determine prognosis. All of this information is crucial for clinical decision-making and patient management [33][34][35][36].

4.1. Tumor Burden Dynamics and Response to Treatment

Fluctuations in ctDNA levels correlate with tumor burden, which makes ctDNA an excellent, non-invasive tool for monitoring tumor evolution, predicting treatment response, and determining prognosis, as shown by Dawson et al. in their prospective study of 30 women with metastatic breast cancer [33][37]. ctDNA is more abundant than CTCs but is also more dynamic and is rapidly cleared from circulation within hours. Furthermore, ctDNA in metastatic breast cancer patients has been shown to accurately represent the mutational profile of individual CTCs. Moreover, an increase in ctDNA levels was able to predict disease progression several months before standard imaging techniques and was able to assess treatment response earlier than any other markers [36][37].

4.2. Prognostic Markers

ctDNA percentage—the number of mutant molecules over the total number of molecules at a given genomic position—is quantitatively associated with outcome, with increasing levels of ctDNA associated with shorter overall survival. This relationship does not hold true for invariable biomarkers, such as T, N, histological grade, ER, PR, HER2, and the Nottingham prognostic index [38][36][39]. Moreover, the study by Dawson et al. demonstrated that while CA 15-3 levels were not a prognostic factor, PIK3CA and TP53 mutations in ctDNA were an early indicator of response to treatment [37].

4.3. Genetic Heterogeneity and Clonal Evolution

ctDNA can also be used to study clonal evolution during treatment and at progression without the need for repeated biopsies, which may not even be feasible if the tumor is in an inaccessible site [33][40]. Due to this difficulty in performing biopsies of metastatic lesions, the phenotype of the primary tumor most often determines treatment decisions in metastatic breast cancer; however, this may lead to inaccurate decisions, since the genetic make-up of the tumor may change over time [41]. Moreover, a biopsy, from either the primary tumor or the metastasis, may not

reflect intratumor heterogeneity, as the biopsy specimen may not be representative of all the tumor cells [33][40]. In contrast, ctDNA can provide insight into the genomic make-up and heterogeneity of inaccessible metastatic lesions [40], which is crucial for detecting the emergence of resistant clones and possible new driver mutations. Furthermore, ctDNA can provide information on the current status of the disease, which can help guide clinical management and the choice of the appropriate targeted therapy during follow up [42][40][41][43][44].

4.4. ctDNA Quantification and Gene Mutations

Assessment of the ctDNA percentage can help determine tumor dynamics, treatment response, and risk of relapse. ctDNA percentage correlated with progression-free survival in triple-negative breast cancer patients [45][46]. In addition, it can be used to assess specific gene mutations. Several genes play an important role in the management of patients with metastatic breast cancer, with TP53, PIK3CA, ESR1, GATA3, ARID1A and PTEN are the most frequently altered [47]. These mutations can be truncal, when they are found in all the patient's cancer cells, or subclonal, when they are randomly dispersed throughout the genome. The ctDNA dynamics of subclonal mutations have a limited potential to predict clinical outcome [48].

4.5. ctDNA Gene Alterations in Metastatic Breast Cancer

PIK3CA encodes for the p110a subunit of PI3K. PIK3CA mutations are associated with worse prognosis [49], although they confer sensitivity to PI3K inhibitors (PI3Ki) such as taselisib, alpelisib, buparlisib and copanlisib [41][50][51]. The majority of PIK3CA mutations are truncal mutations, including H1047R/L, N345K, G1049R, E545K and E542K, but others are subclonal [47][48]. Although there are no validated predictive biomarkers of response to CDK 4/6 inhibitors, early ctDNA dynamics of PIK3CA truncal mutations predicted sensitivity to palbociclib, a CDK 4/6 inhibitor. Palbociclib is a cytostatic drug, and its effects decrease PIK3CA-mutant ctDNA, indicating that ctDNA PIK3CA mutations may be useful as an early predictor of response, as was observed in the PALOMA-3 trial of ER-positive/HER2-negative advanced breast cancer patients who had previously progressed to endocrine therapy [52][48]. However, Razavi et al. found that in HR-positive metastatic breast cancer, PTEN loss promotes PIK3alf-independent activation of AKT, causing resistance to PI3Ki [53].

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