Liquid Biopsies for Monitoring Temporal Genomic Heterogeneity in Breast and Colon Cancers

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Keywords

Liquid biopsy · Breast cancer · Colorectal cancer · Genomic heterogeneity · Circulating tumour DNA · Cell-free DNA · Circulating tumour cells

Abstract

Cancer is a spatial and temporal dynamic disease where differently evolving genetic clones are responsible for progression. In this landscape, the genomic heterogeneity of the primary tumours can be captured by deep-sequencing representative spatial samples. However, the recognition of genetic alterations responsible for tumour evolution remains a challenging task. Recently, the “liquid biopsy” was recognized as a powerful real-time approach for the molecular monitoring of this dynamic disease. The term “liquid biopsy” generally refers to the use of circulating (cell-free) tumour DNA (ctDNA) and circulating tumour cells (CTCs) as non-invasive biomarkers for the early diagnosis, prognosis, monitoring of clinical progression, and response to treatment in different types of tumours, including the highly genomic heterogeneous breast cancer. The implementation and standardization of both approaches are still needed to achieve the required sensitivity and specificity to successfully analyze heterogeneous tumours, but pivotal studies, in

particular those concerning colorectal cancer, have shown the feasibility and usefulness of liquid biopsy for monitoring the Darwinian clonal evolution from an early to a metastatic stage.

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Liquid Biopsy for the Study of Temporal and Spatial Heterogeneity

Human tumours are characterized by genomic intra-tumoral heterogeneity, with cell populations exhibiting Darwinian clonal evolution with an accumulation of passenger and driver genetic alterations [1]. In this model, heterogeneity is achieved through the selection and expansion of tumour clones, which dynamically evolve with a branched evolution and confer growth advantage within a specific individual microenvironment [2–4]. Consequently, different tumour regions are characterized by a mixed clone population, which, under drug selective pressure, leads to heterogeneous metastases [5, 6]. These metastases are genetically different from the primary tu-

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mours and clinical monitoring can be challenging. In this landscape, genetic heterogeneity of the primary tumours can be captured by deep-sequencing representative spatial samples, but the detection of the genetic alterations responsible for tumour evolution and progression remains an unmet task. Histopathologists have been aware of morphological variations within neoplastic tissues for a long time. However, genetic heterogeneity has only recently been recognized as a common feature of different cancer types, thanks to remarkable advances in novel approaches, including “liquid biopsy.”

Breast cancer is a paradigmatic example of both a phenotypical and genomic heterogeneous tumour. In breast neoplasia, intra-tumoral or spatial heterogeneity within a single lesion and temporal heterogeneity during tumour progression or in response to treatment have been described. Intra-tumoral differences in the expression of estrogen (ER) and progesterone (PR) receptors, and in chromosomal alterations including epidermal growth factor receptor 2 (*ERBB2*) gene amplification, were first reported between 1984 and 1999 [7–9]. More recently, different patterns of chromosomal changes were observed in distinct regions of primary breast tumours, using comparative genomic hybridization [10] and genomic fingerprinting [11]. In addition, multiple genetically different metastatic cells were shown to independently colonize the axillary lymph nodes [12], and intra-tumoral heterogeneity for activated signaling pathways, e.g., the Notch, Sonic hedgehog, and Wnt pathways, were reported among populations of breast cancer stem cells [13, 14]. Genome-wide molecular approaches conclusively assessed spatial and temporal intra-tumour differences [15, 16] and variable genomic heterogeneity of the lesions in the same patient [17]. Finally, the new methods for single-cell sequencing suggested that the earliest steps of breast cancer development involve copy number changes that occur in bursts whereas point mutations evolve gradually, which leads to drug resistance [18]. Multiple biopsies, taken at different times, are needed to capture the evolving spatial and temporal genomic heterogeneity of metastatic breast cancer, as shown in the Intensive Trial of Omics in Cancer (ITOMC). This study was designed to enrol patients with triple-negative breast cancer to specific therapies on the basis of the molecular profile of their lesions, using multiple biopsies, from the primary tumour to each stage of progression [19]. The ITOMC results showed that different genetic alterations, both intra-tumour and between metastases, lead to the onset of clonal cells in response to specific administered therapy, and suggested that the best treatment should include the genomic profile of the mul-

tle biopsies for each patient. However, some drawbacks limit the large-scale adoption of parallel tumour biopsies for characterizing a neoplastic mass. First, tissue biopsies are invasive. Second, tumour tissues might be scarcely available or else not suitable for providing a high yield or good quality of DNA for the new sequencing techniques. Moreover, there is the need of repeatable methods to monitor the tumour evolution over the disease progression in real time. These issues can be overcome by the use of liquid biopsy, now recognized as a powerful real-time approach for the comprehensive imaging of genomic heterogeneous tumours at given times (Fig. 1a, b).

Liquid Biopsy for Genotyping Cancers in the Blood

The term “liquid biopsy” generally refers to the use of circulating (cell-free) tumour DNA (ctDNA) and circulating tumour cells (CTCs) as non-invasive biomarkers for the early diagnosis [20], prognosis [1], monitoring of clinical progression [21, 22] and response to treatment [23, 24]. Its successful application in oncology has been recognized only in the last 2 decades, coinciding with technological improvements affording ultrasensitive methods for ctDNA and CTC detection. At present, only ctDNA-based liquid biopsy has been implemented in routine clinical practice along with European Union approval of *EGFR* mutation testing for patients with non-small-cell lung carcinoma (NSCLC). The IFUM (Gefitinib [IRESSA] Follow-Up Measure) trial, the European study that led to this approval, reported a sensitivity of 65.7% and a specificity of 99.8% of the approach using a real-time method [25, 26]. Further clinical studies are currently ongoing to evaluate whether ctDNA-based liquid biopsy could be exploited for *RAS* mutation detection in patients with metastatic colorectal carcinoma (CRC).

Cell-free DNA (cfDNA) can be found in human blood and is released by normal cells and cancer cells by an active and a passive process (cell death) [27, 28]; the former is only partly understood, but we know that the latter occurs both via programmed cell death (apoptosis) and necrosis [29, 30]. Apoptosis generates cfDNA fragments corresponding to the size of the DNA wrapped around the nucleosomes (approx. 145/180 bp or multiples of these) whereas necrosis gives rise to irregular and longer cfDNA fragments (up to 21 kb) [31]. Notably, any necrotic or mechanically disrupted cells can release longer DNA fragments; therefore, patients with injuries [32], acute inflammation, or heart disease may also have high levels of serum/plasma DNA.

Fig. 1. The advantages of liquid biopsy in depicting the evolution of genomic heterogeneous tumours (modified from [107]). **a** Biopsies taken at different times are needed to describe the spatial and temporal genomic evolution of tumours. However, tissue biopsy can fail to capture the presence of co-existing intra-tumoral cell clones, permitting the evaluation of only a few types of DNA molecules and genetic alterations. **b** On the contrary, with liquid biopsy, it is possible to obtain snapshots of genetically different and co-occurring cell clones, thereby providing representative cfDNA and CTCs in real time.

ctDNA, which usually represents 0.01–90% of total cfDNA [33], was first described in 1994 [34, 35]. Tumours that contain approximately 50 million malignant cells release sufficient ctDNA to enable their non-invasive detection in the blood [36]. However, levels of ctDNA vary widely from person to person and can be difficult to detect in the early stages of cancer. Advances in technology, including next-generation sequencing (NGS), massive parallel sequencing, and highly sensitive quantitative polymerase chain reaction (PCR)-based tests, have increased the feasibility of ctDNA analysis that can be carried out with 2 different approaches.

The first, targeted approach, involves the identification of specific genetic alterations previously found only in the primary tumour; the second, untargeted approach, consists of searching for as yet unknown genetic changes. The targeted method includes amplification refractory mutation system (ARMS) [37], digital PCR (dPCR) [38–40], beads, emulsions, amplification, and magnetics (BEAMing) [41, 42], and pyrophosphorolysis-activated polymerization (PAP) [43]. The untargeted method is based on whole-genome sequencing and exome sequencing. Since the ctDNA half-life is <2 h [44], tumour genetic changes can be evaluated in days rather than in weeks [41], prior to changes assessed by radiological imaging studies [45, 46]. Given this evidence, ctDNA-based liquid biopsy could be integrated with tissue biopsies and imaging, or even replace them when the tumour lesions are not easily accessible. This approach is minimally invasive and can thus be repeated at multiple time points to

closely capture the dynamic tumour changes. Currently, it is still challenging to identify the tumour origin of each circulating fragment, and the standardization of collection and extraction methods is still required, but ctDNA represents the most promising biomarker for monitoring the progression and response to therapy of genomic heterogeneous tumours (Fig. 1b).

CTCs were first identified by Ashworth [47] in 1869 in a woman with metastatic breast cancer; however, for over a century, CTC research was hampered by the difficulties in detecting these very rare (usually <10 cells/mL of blood) cell population in the circulation. Similar to ctDNA, these cells can originate from either primary or metastatic tumour tissues, and specific enrichment techniques are required for their detection [48]. These techniques are based either on the biological properties of the CTCs, such as protein secretion or cell-surface antigen expression, or else rely on their physical characteristics, such as the presence of electric charges, and the size, density, or deformability of the cells. The most widely used CTC enumeration platform, Cell-Search™ (Veridex LLC, NJ, USA), was approved for clinical use by the US FDA for breast cancer patients (in 2004), CRC patients (in 2007), and prostate cancer patients (in 2008). Recently, a label-free method based on pH measurement has been proposed to detect CTCs [49]. Cristofanilli et al. [50] first defined the prognostic role of CTCs in a trial where women with metastatic breast cancer with an elevated CTC level (≥ 5 cells/7.5 mL of blood) displayed worse progression and overall survival. More recently, Aceto et al. [51]

demonstrated that, in breast and prostate cancer, CTC clusters are derived from the oligoclonal cells of the primary tumour and are associated with a 23- to 50-fold increased metastatic potential compared to a single CTC. In metastatic CRC, the Cell-Search system received US FDA clearance for routine clinical use with a threshold CTC count of $\geq 3/7.5$ mL of blood, to stratify patients into favourable and unfavourable prognostic groups [52–54]. However, due to the intrinsically low yield of CTCs, both at baseline and in the course of treatment [55, 56], ctDNA remains the preferred approach for the study of the genomic heterogeneity of CRC (Fig. 1b).

Liquid Biopsy in a Genomic Heterogeneous Landscape: From Early Diagnosis to Metastases

The Example of CRC

Several studies concerning CRC have shown the feasibility and usefulness of ctDNA analysis in monitoring the clonal evolution from an early to a metastatic stage and the response to therapy. In 2004, Wang et al. [57] reported that the detection of *APC*, *KRAS*, and *TP53* mutations in DNA from the serum of CRC patients correlated with a higher post-operative metastasis/recurrence rate. A year later, Diehl et al. [58] showed that quantification of circulating *APC* gene fragments can serve as a useful biomarker for CRC, while Umetani et al. [59] reported that the length of ctDNA could be a marker for tumour detection, since they found an association of the longer fragments (>250 bp) with cancer-derived ctDNAs.

In the last decade, studies have focused on the role of ctDNA to monitor CRC dynamics, including the post-surgery surveillance, tumour burden, and response to therapy [21, 41, 60–63]. Frattini et al. [60] described the utility of ctDNA quantification for monitoring early-stage post-operative CRC patients, reporting that ctDNA levels were significantly higher in patients with CRC, when compared to healthy controls, and then decreased during the follow-up period, but that patients with recurrence or metastasis had increasing levels. Diehl et al. [61] demonstrated that CRC patients with a detectable ctDNA level in the post-operative period had evidence of relapse within 1 year whereas those with undetectable ctDNA had no recurrence. Subsequently, Spindler et al. [62] compared 229 patients with chemotherapy-refractory metastatic CRC and 100 healthy individuals, to establish the normal ranges of cfDNA in each group, and Reinert et al. [21] correlated ctDNA levels with disease load in 151 serial plasma samples from 6 relapsing and 5 non-relaps-

ing CRC patients. Tie et al. [63] reported that ctDNA is useful for evaluating tumour burden and predicting the response to standard chemotherapy in early-stage CRC; they also highlighted a trend between ctDNA changes and progression-free survival. Some other works have focused on the assessment of hotspot alterations in frequently mutated CRC genes, mainly the *RAS* and *BRAF* genes [64–66]. The study by Bettegowda et al. [67] was the first to correlate the level of *KRAS* mutant fragments with shorter overall survival. Other landmark studies showed that, in CRC patients, the acquisition of resistance to EGFR-specific antibodies is associated with the emergence of *RAS* pathway mutations detectable in the blood before any evidence of clinical progression [36, 68]. Performing whole-genome sequencing of the plasma of CRC patients treated with anti-EGFR therapy, Mohan et al. [69] observed the presence of several copy number changes including the loss of the region harbouring *APC* on 5q22, deletions of chromosome 17p and 18q, and amplifications of genes involved in the resistance to EGFR blockade, e.g., *MET*, *ERBB2*, and *KRAS*. Accordingly, using ctDNA to track clonal evolution during treatment with EGFR inhibitors, Siravegna et al. [70] identified alterations in various genes, including *KRAS*, *NRAS*, *MET*, *ERBB2*, *FLT3*, *EGFR*, and *MAP2K*. They showed that mutated *KRAS* clones, which emerge in the blood during EGFR blockade, experience a decline upon the withdrawal of EGFR-specific antibodies, indicating that the CRC genome adapts dynamically to intermittent drug schedules. Very recently, Russo et al. [71] demonstrated that lesion-specific radiographic responses to targeted therapies can be driven by distinct resistance mutations affecting MAPK pathway genes which can arise in separate tumour lesions at different times in the same patient. This breakthrough study, which was carried out by performing an integrated analysis of multiple tissue and ctDNA biopsies with serial radiological imaging, clearly showed that a single-lesion biopsy does not represent the molecular heterogeneity of the resistant tumour clones, but rather reflects a lesion-specific response to a given therapy.

Given the low levels of CTCs in most CRC patients, especially in the non-metastatic setting, the detection of CTCs requires more cumbersome isolation and enrichment techniques; so far, the use of CTC enumeration has been the established tool for predicting poor prognosis [52, 72, 73], but CRC intra-patient mutation heterogeneity has also been reported with CTC biopsies. By profiling with array comparative genomic hybridization and NGS, Heitzer et al. [74–76] showed that most of the mutations initially found only in CTCs were present at a subclonal

level in the primary tumour and/or metastasis from CRC patients. Accordingly, Gasch et al. [77] demonstrated considerable *KRAS* mutation heterogeneity in the same patient, showing both *KRAS* wild-type and *KRAS*-mutated CTCs; Schölch et al. [78] similarly reported that patients with *KRAS* wild-type primary tumours had both *KRAS* wild-type and *KRAS*-mutant CTCs.

State of the Art in Breast Cancer

In patients with non-metastatic breast cancers, liquid biopsy can be used to detect and monitor the markers of minimal residual disease after surgery and anticipate the development of clinical or radiological recurrence. The use of different types of techniques used for analyzing ctDNA in early-stage breast cancer patients has provided variable results [20, 79–82]; however, in 2014, Bettegowda et al. [67] showed that ctDNA fragments could be identified in up to 50% of these patients. In the same year, by using droplet digital PCR, Beaver et al. [20] were able to identify *PIK3CA* mutations in ctDNA prior to and after breast cancer surgery, thereby supporting the feasibility of detecting DNA mutations in the plasma of early-stage breast cancer patients who have minimal, clinically undetectable disease. These results were further confirmed by 2 other studies that demonstrated that the identification of ctDNA alterations in the plasma of non-metastatic breast cancer patients could anticipate the diagnosis of a clinical metastatic relapse [80, 81]. Similar to in CRC, ctDNA can be a helpful tool to monitor tumour burden and study the mechanisms of response and resistance to therapy in breast cancer patients. Different types of plasma alterations have been detected in these patients, including copy number alterations and structural variations [5, 45, 46, 67, 81, 83–87]. In this regard, it has been shown that the detection of ctDNA carrying structural variations and *TP53/PIK3CA* gene mutations is a sensitive and specific circulating blood biomarker, and that the number of ctDNA copies is associated with the response to therapy [45]. More recently, the detection of *ESR1* mutations in plasma from ER-positive metastatic breast cancer patients has suggested the identification of *ESR1*-mutated ctDNA as a predictive marker of resistance to aromatase inhibitor therapy [88–90].

Much attention has been focused on the application of ctDNA for the detection of intra-patient variable genomic alterations of primary and metastatic lesions [5, 83, 84, 86]. For example, Butler et al. [91], using whole-exome sequencing on plasma DNA, have shown a significant heterogeneity between the primary breast cancer and metastases in the same patient. They reported the

clonal presence of the activating *PIK3CA* p.H1047R mutation in the primary tumour but its complete absence from both the metastasis and plasma DNA, and an *ESR1* mutation that, in contrast, was detectable in the plasma and at the metastatic site but not in the primary lesion. One year later, Murtaza et al. [5] performed parallel sequencing of sequential tumour tissue biopsies and plasma ctDNA samples in an ER+/HER2+ metastatic breast cancer patient, and demonstrated that plasma ctDNA may reflect the clonal hierarchy of breast tumours. They showed that the most represented mutations in ctDNA were identified in all tumour samples, whereas the private mutations could be found in only 1 of the metastatic samples. This study supports the role of ctDNA analysis as a powerful tool for describing genomic intra- and inter-metastatic heterogeneity and the clonal evolution of breast cancer.

On the other hand, the analysis of CTCs can be useful as a prognostic marker, particularly in breast cancers that are rich in CTCs, as assessed in the pivotal trial by Cristofanilli et al. [50]. In 2014, Bidard et al. [56] substantiated this finding by investigating 1,944 patients from 17 European centers. They reported that patients with a baseline CTC count $\geq 5/7.5$ mL of blood had worse progression-free (HR 1.92; $p < 0.0001$) and overall survival (HR 2.78; $p < 0.0001$) than patients with a CTC count of $< 5/7.5$ mL. More recently, Pestrin et al. [92] reported the feasibility of using CTCs to test the heterogeneity of metastatic breast cancer by evaluating the mutational status of the *PIK3CA* exons 9 and 20 in both the single and pooled CTCs from 18 patients. Two patients were heterogeneous for the presence of *PIK3CA* mutations when sequencing analysis was performed on >1 single CTC and 1 patient showed a negative correlation between the *PIK3CA* status in the primary tumour (wild-type) and the matched CTCs (exon 20 mutation).

Conclusions

The precise evaluation of gene mutation frequency is becoming an important parameter to evaluate genomic tumour heterogeneity. The use of plasma ctDNA analysis to distinguish the tumour DNA and the DNA derived from non-transformed cells can be affected by the availability of specific tumour mutational biomarkers and methods able to detect variant allele percentages as low as 0.01% [58, 93, 94]. In the future, the implementation of plasma NGS techniques will overcome these drawbacks. Until 5 years ago, the usefulness of NGS was only demon-

strated in patients with exceptionally high ctDNA levels (33–65% of the cfDNA of tumour origin), but some studies have suggested helpful correlations between clinical characteristics of the disease and the amount of plasma ctDNA in different cancer patients [58, 67].

Rachiglio et al. [95] recently showed that, in CRC patients, the sensitivity of the NGS plasma analysis for RAS mutations was 100% in patients without resection of the primary tumour before blood collection, and 46.2% in patients with primary tumour resected before enrollment. Their results suggest the importance of identifying specific clinical characteristics to help select patients with sufficient levels of ctDNA for plasma NGS analysis. On the other hand, not all tumours can be easily traced by their genomic alterations. In breast cancer, there are only a few frequently mutated genes that drive disease progression, and not all breast metastatic diseases appear to release ctDNA into the bloodstream in measurable quantities [46, 69, 75, 76, 96, 97]. In these cases, CTC analysis can be helpful, as the CTCs might reflect the characteris-

tics of the biologically relevant subclones, irrespective of a detailed anatomical distribution. One of the major challenges in CTC research concerns the number of CTCs that have to be analyzed to be able to outline the profile of the clones that drive disease in cancer, with conflicting results being provided by case reports involving both CTC and ctDNA analysis in metastatic breast cancer patients [96, 97]. However, in recent years, efforts have been made to develop multiple markers to increase the yield of CTCs [98–100], and to improve their amplification [101–103] and sequencing techniques [104–106].

Finally, the sequencing of isolated CTCs will also enable the detection of multiple mutations within the same cell to enable us to understand its clonal evolution. In conclusion, it is presently quite accepted that metastatic lesions and recurrent disease can differ substantially from the primary tumour. In the future, the improvement of plasma DNA and CTC sequencing will allow us to identify these differences and to better define the evolution of each neoplasia.

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