feature

Incorporating liquid biopsies into treatment decision-making: obstacles and possibilities

Nick Beije[‡], n.beije@erasmusmc.nl, John W.M. Martens[‡] and Stefan Sleijfer[‡]

Circulating tumor cells (CTCs) and cell-free DNA (cfDNA) together with newer emerging liquid biopsies have a unique potential to deal with key issues in oncology. For example, they can be used to assess prognosis, direct treatment with certain kinds of drug, or provide information about response to treatment. However, despite an overflow of literature on the subject, clinical implementation of these liquid biopsies has been scarce. This is mainly because there is a lack of preanalytical standardization, multiple different techniques or platforms are being used, and a lack of prospective studies investigating a meaningful clinical question are performed. Here, we provide an overview of the current state of liquid biopsies and make suggestions for how liquid biopsies can reach the tipping point.

Introduction

In the era of precision medicine, liquid biopsies have attracted significant interest for personalizing the treatment of patients with cancer. The premise of liquid biopsies is that, by obtaining a simple blood sample, all sorts of cancer-related characteristics can be determined in real time, and can be used to personalize cancer treatment. However, the clinical utility of liquid biopsies is yet to be confirmed. Here, we discuss the current standing of liquid biopsies in oncology, their highlights until now, as well as their pitfalls and caveats.

Liquid biopsies: lots to choose from

The current landscape involves multiple types of liquid biopsy. Most of the research has been done on CTCs and cfDNA. CTCs are intact tumor cells that have detached from a solid tumor, whereas cfDNA is fragmented DNA comprising germline DNA and potentially circulating tumor DNA (ctDNA) that originates mainly from apoptotic tumor cells (Fig. 1). Given that cfDNA only offers the possibility to analyze DNA, CTCs theoretically offer the analysis of all molecular materials, including RNA and proteins in the same cell. There are also emerging liquid biopsies, such as cell-free RNA, extracellular vesicles, such as exosomes and microvesicles, circulating proteins, and platelets exhibiting tumor-specific RNA profiles. Promising data on the use of these emerging liquid biopsies in cancer screening have been presented [1–4], but, given the relative scarcity of data on these newer liquid biopsies, we focus here on CTCs and cfDNA. For both, there are some clinical data underlining their potential relevance for personalizing cancer treatments, but robust evidence showing their clinical utility is warranted.

Circulating tumor cells

The CellSearch system is currently the only system approved by the US Food and Drug Administration (FDA) for enumerating CTCs in patients with metastatic breast, colorectal, or prostate cancer. The system enriches for all cells expressing EpCAM by using anti-EpCAM magnetic beads. Subsequently, cells are stained for anti-cytokeratin (CK) 8/18/19 (positive on CTCs), DAPI (a nucleus marker), and anti-CD45 (to exclude contaminating leukocytes) to identify CTCs. The method has shown to be highly specific, given that CTCs are rare in healthy donors [5]. The prognostic value of CTCs counted with CellSearch has been shown not only for many different metastatic epithelial malignancies, but also in patients with non-metastatic cancer. For example, in metastatic breast cancer (MBC), a CTC count of five or more
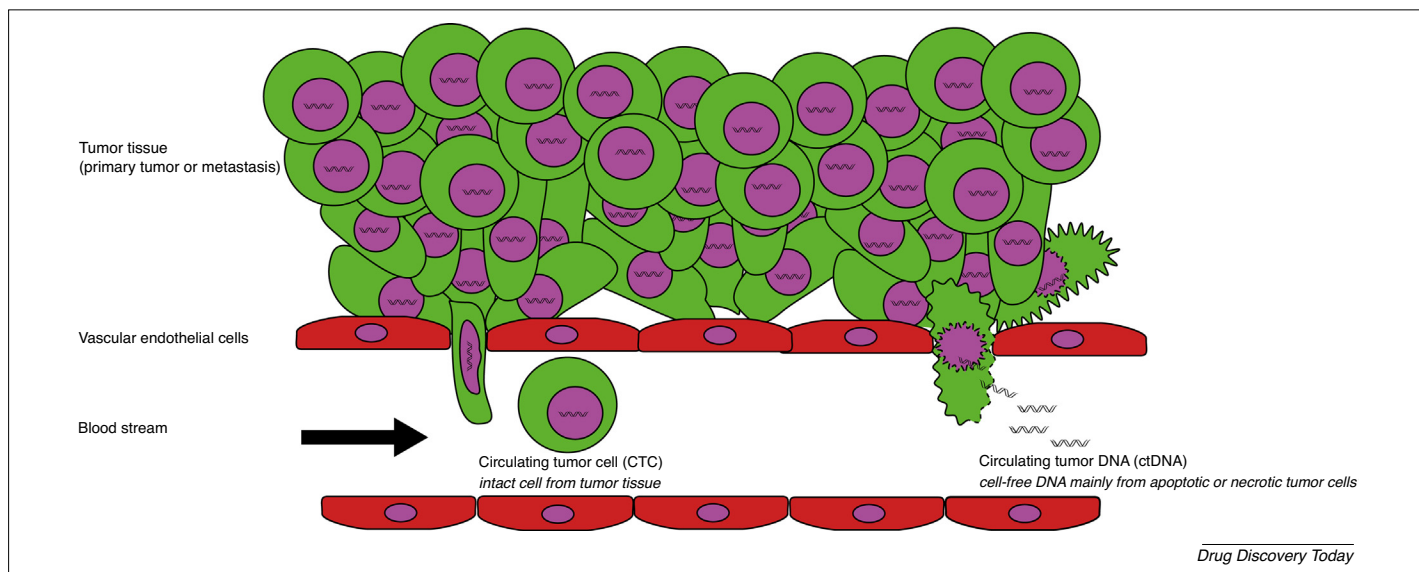
FIGURE 1

Current leading liquid biopsies: circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). From the solid tumor, which can either be the primary tumor or a metastatic site, tumor cells can extravasate and appear in the blood as CTCs or go into apoptosis and shed their DNA. CTCs are intact cells and can form distant metastases or go into apoptosis. CtDNA is cell-free DNA (cfDNA) that is mainly from apoptotic or necrotic tumor cells and has unknown biological relevance. Both CTCs and cfDNA can be isolated from whole blood using sophisticated techniques and then be quantified or subjected to multiple molecular analyses.

CTCs is strongly associated with poor prognosis; similarly, patients with primary breast cancer with one or more CTCs have decreased overall survival [6,7]. In addition, changes in CTCs have been associated with response. However, although enumerating CTC undoubtedly has clinical validity, studies investigating clinical utility have been rare and, thus, the test is seldom used by clinicians.

One study randomizing patients based on CTC changes during treatment for MBC demonstrated no survival benefit for switching to another line of systemic treatment based on CTC counts rather than on traditional means [8]. Similarly, a study in which patients with HER2-negative primary breast cancer with detectable CTCs after surgery and standard (neo)adjuvant therapy received additional trastuzumab showed no additional benefit [9]. The only study demonstrating a possible benefit of counting CTCs for treatment decision-making was recently presented and demonstrated that a baseline CTC count can be used safely to direct patients with MBC to receive either first-line chemotherapy (if five or more CTCs) or endocrine treatment (if less than five CTCs) [10]. Interestingly, approximately half of the patients who the treating physician intended to give chemotherapy based on clinical grounds could be safely de-escalated to receive endocrine therapy. However, given that patients with MBC now increasingly receive combined treatment with CDK4/6 inhibitors and endocrine therapy, it is unlikely that these findings will result in

widespread changes in the treatment of such patients. The lack of true clinical utility of counting CTCs means that baseline CTC counts and changes in CTC counts are now mainly used in clinical trials only as a prognostic marker or as early response marker.

One of the main concerns with CellSearch is its dependency on EpCAM, with the potential of missing out on EpCAM-negative CTCs [11]. Therefore, a plethora of alternative assays have become available that have tried to address this issue: for example, by using alternative markers to detect CTCs or using size-based properties of CTCs to enrich for them. However, head-to-head comparisons of the several available assays for CTC detection have been anecdotal. One of the main problems in comparing assays is the absence of a ground truth, meaning that, for some CTC detection assays, increased sensitivity compared with CellSearch has been described, although it is unclear whether this was at the expense of specificity. It is unlikely that, for counting CTCs, any other assay than CellSearch will undergo such vigorous validation of its prognostic value in so many tumor types and settings.

Beyond counting CTCs

Counting CTCs is a rather 1D use of CTCs. Most CTC detection assays have described methods to characterize CTCs at the RNA, DNA, and protein level. Initially, these types of analysis were limited to the detection of genes or mutations on a mixed pool of CTC-enriched

material and leukocytes. Although it is possible to identify somatic mutations and RNA profiles on these materials, it is labor intensive and, moreover, specificity issues arise when attempting to measure any tumor-specific signal in a background of leukocyte-derived material. One of the most clinically relevant markers that have come from CTCs is the androgen receptor splice variant V7 (AR-V7) in metastatic prostate cancer (mPC), which is a RNA variant that predicts for resistance to antiandrogen therapies in mPC [12,13]. Prospective clinical studies investigating whether the CTC AR-V7 status is useful to guide treatment decision-making in mPC are ongoing.

Given its great promise, many efforts are underway to further improve methods to molecularly characterize CTCs. For example, efforts to characterize CTCs at the single cell level by DNA or RNA sequencing have taken off with the availability of several methods to isolate single cells [14], but have so far been limited to proof-of-concept studies. Although single CTC characterization will probably offer more information on heterogeneity, it is unclear to what extent this will reflect the entire landscape of tumor heterogeneity, especially if performed in limited numbers of CTCs.

Another method to improve CTC characterization is by increasing the blood volume that is analyzed, sometimes even by using leukapheresis [15]. Although using leukapheresis does yield more CTCs to analyze [16], it somewhat compromises the minimally invasive and easy-

to-collect nature of liquid biopsies, while still requiring purification of CTCs among contaminating blood leukocytes.

Another strategy for obtaining higher numbers of CTCs for downstream analysis is culturing of CTCs, for example with the intent to subsequently run drug-sensitivity analyses on them [17]. Although multiple groups have described successful long-term cultures of CTCs, the chance of success is low and seemingly limited to patients with very high CTC counts of >300 CTCs/7.5 ml [18]. Given this low success rate and because it takes several weeks to months to culture these CTCs, broad implementation appears unlikely, other than perhaps for groups of patients with higher levels of CTCs, such as most patients with small-cell lung cancer [19].

Cell-free DNA

Even more as is the case for CTCs, many different assays to detect aberrations in cfDNA are now commercially available. Compared with CTC detection, cfDNA is more easily analyzed with tools such as next-generation sequencing (NGS) machines and PCR machines already available in most research laboratories. ctDNA is the fraction of cfDNA that is tumor derived. The percentage of ctDNA is often <1%, meaning that classical techniques, such as Sanger sequencing and quantitative PCR, often lack the sensitivity to detect tumor-specific mutations. Initially, mostly digital PCR (dPCR)-based approaches were used. However, using dPCR, only a limited amount of mutations can be detected in one run. Therefore, NGS protocols using unique molecular identifiers are now more often used, increasing sensitivity and allowing the detection of ctDNA mutations in multiple genes of interest. Newer methods even allow for the detection of copy number variations using low-coverage whole-genome sequencing [20] or methylome profiles [21].

Preanalytical and postanalytical processing

Regardless of the technique, preanalytical steps, such as sample collection and processing, have to occur and can greatly affect the final results. There are significant differences between the various protocols, as described in the literature. It is known that blood samples have to be processed within 24 h or in special tubes with a stabilizing agent (e.g., CellSearch tubes or BCT tubes) to prevent lysis of leukocytes increasing the amount of nontumor-derived cfDNA, thereby lowering the sensitivity [22]. Additionally, some cfDNA isolation kits select for smaller or longer cfDNA fragments than other kits [23], of which the significance is unknown. Besides

these factors, there is also limited insight into the effect of freezing and thawing of samples and to what extent various comedications, underlying comorbidities, or, for example, circadian rhythms influence cfDNA concentrations [24].

After sample processing, the postanalytical phase in which the cfDNA somatic mutations have to be identified is also crucial. Although commercially available cfDNA assays usually come with associated software, the subsequent analysis and calling of somatic mutations is often left to the users.

An issue related to these downstream analyses is how somatic variants should be reported. Somatic variants are most commonly expressed as variant allele frequency (VAF) or the number of mutant copies/ml. With the availability of highly sensitivity assays, we do not know whether detection of a variant is clinically relevant at a low VAF or low mutant copy number. A complicating factor is also that the VAF in particular is influenced by the background. Hence, an increase or decrease in background (e.g., increased apoptosis of leukocytes) can change the VAF, which might especially be important in monitoring of VAF during treatment. The number of mutant copy numbers appears to be more constant [22]; however, it is unclear what change in VAF or copy number is both biologically and clinically relevant.

Given all the steps that might influence cfDNA analysis, Torga and Pienta [25] recently sent blood samples from patients taken at the same moment to two CLIA-licensed commercial laboratories for cfDNA NGS sequencing for head-to-head comparison. The results were worrisome, because results from 64% of the tested samples were incongruent between the two laboratories. At this point, it is unclear how the differences between both assays can be explained.

Potential clinical use of ctDNA

Although it is clear that there is still some way to go before optimal analytical validity is reached, there are also exiting data published that open doors to all kinds of key clinical problems in oncology. This includes the use of ctDNA as a predictive marker for certain treatments, as a marker to detect disease relapse, as a marker to detect emerging resistance to a (targeted) treatment, or as a means to screen people for the presence of cancer.

Nowadays, cfDNA assays are most commonly used as predictive markers in the setting of EGFR-mutated metastatic non-small cell lung cancer (mNSCLC), because the presence of activating EGFR mutations in treatment-naive mNSCLC is a prerequisite for treatment with

EGFR tyrosine-kinase inhibitors (TKIs). The only cfDNA test that is currently approved by the FDA is the cobas test, which is approved to identify EGFR exon 19 deletions, L858R, and T790 M mutations. Overall concordance between tissue and cfDNA using this test has been high at 89–91% [26,27] and patients with mNSCLC harboring EGFR mutations in cfDNA identified using this test benefitted from treatment with EGFR TKIs compared with placebo [26]. Therefore, cfDNA is increasingly used to screen patients for first-line EGFR TKI treatment and most clinicians use it if tissue specimens are unavailable [27]. For the T790 M mutation in the EGFR, conferring resistance to TKIs in patients with mNSCLC and rendering these patients suitable for treatment with osimertinib, some groups even advocate using cfDNA initially to detect EGFR T790M followed by a tumor biopsy in case of negative results [27]. However, the FDA approved the T790M EGFR cfDNA test only when it is not possible to obtain a tissue biopsy at the time of progression on a TKI. Their cautiousness is probably because of poorer concordance rates between tissue and cfDNA for EGFR T790M mutations at ~70% [28]. However, concordance studies between tissue and cfDNA will never show perfect similarity. For driver mutations, such as EGFR exon 19 deletions and L858R mutations, which are present in most tumor cells, concordance will probably be good. By contrast, for subclonal or resistance mutations, such as EGFR T790M, concordance rates will likely be lower. These discrepancies between tissue and cfDNA might occur because of analytical issues, low numbers of ctDNA, or biological factors, such as heterogeneity.

Another important factor for cautiousness with using cfDNA without tissue-based confirmation is that there is no formal proof that cfDNA-mutant-positive, tissue-mutant-negative patients have similar responses to therapy as cfDNA-mutant-positive, tissue-mutant-positive patients. For example, patients with EGFR T790M-positive cfDNA had poorer progression-free survival (PFS) on osimertinib if they had a T790M-negative tumor than if they had a T790M-positive tumor (PFS 4.2 versus 9.3 months, respectively; $P = 0.0002$) [28].

Besides the use of cfDNA in EGFR-mutated mNSCLC, cfDNA is not yet routinely used as a predictive marker to select patients with cancer for certain targeted therapies. Although a recent report underlined its potential use to direct patients with an actionable mutation to appropriate Phase I trials within a timeframe acceptable for clinical decision-making [29], further trials are eagerly awaited.

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With respect to the use of ctDNA to detect early relapse, there have been examples in multiple non-metastasized tumors in which somatic cfDNA mutations were detected after curative operative treatments and were associated with decreased relapse-free survival [30,31]. This might mean that patients at risk for relapse can be identified early and treated accordingly, although the extent to which there might be some type of lead-time bias remains to be seen.

Mutations in cfDNA can also be used as a surrogate of drug response in longitudinal monitoring. For example, interesting data were described for *KRAS* mutations that cause resistance to monoclonal antibodies targeting the *EGFR* (EGFR-MoAbs). There is some evidence that, upon discontinuation of EGFR-MoAbs, KRAS mutations decay and drug sensitivity is regained, which might mean that these patients can be rechallenged with EGFR-MoAbs [32], which is now being tested in a proof-of-concept study (CHRONOS trial, NCT03227926).

Lastly, it has been suggested that cfDNA could be used as a cancer-screening tool. In an analysis of 1005 patients with non-metastatic cancers, a test for detecting cancer based on ctDNA mutations and protein tumor markers had a median sensitivity of 70% and specificity of >99% [33]. Although this highlighted the potential of cfDNA analyses to detect cancer at an early stage, its application as a screening tool in a healthy population still has a long way to go, especially because the positive predictive value of the test declined significantly when tested in a population in which the prevalence of cancer was low, which is typically the case when screening the general population [34].

Reaching the tipping point

Research on liquid biopsies has skyrocketed over the past decade and has led to novel insights into cancer biology. However, incorporation of liquid biopsies into clinical workflows remains a major challenge. Given the plethora of articles on liquid biopsies that have been published to date, it is disappointing that approval only exists for the CellSearch CTC assay and certain cfDNA assays able to detect mutations in the *EGFR* receptor. With respect to cfDNA, a recent ASCO and College of American Pathologist joint review even concluded that there is 'little evidence of clinical validity and clinical utility to support widespread use of ctDNA assays in most patients with advanced cancer' [35].

One of the factors contributing to the lack of clinical applicability is the current overabundance of liquid biopsy assays. This is especially the case in the cfDNA field, in which dozens of assays are now commercially available and also an important subset of assays developed in-house by academia. All of these assays have their own limit of detection, sensitivity, and specificity, meaning that findings on one particular liquid biopsy platform are not necessarily applicable to all other platforms, hampering clinical applicability and underlining the need for external quality assessment studies before implementation in routine diagnostics (general issues in cfDNA research are listed in Table 1).

Another key issue is that trials that examine a true change in clinical decision-making based on liquid biopsies are rare. From 642 trials registered at clinicaltrials.gov that are evaluating liquid biopsies in some way, only 21 trials (3.3%) are investigating a particular intervention based on liquid biopsies (supplemental information online). In almost half of these interventional studies, patients received a particular intervention (such as a targeted treatment) based on baseline CTC or cfDNA status without the inclusion of a control group that does not receive that particular intervention, meaning that, in the case of a positive study result, these studies are unlikely to result in immediate changes in clinical practice. Although observational studies are also of key importance in increasing our understanding of cancer and might be hypothesis generating, our current understanding of liquid biopsies does allow for more direct interventional research, but at this point that seems to be rare.

Concluding remarks

In conclusion, for liquid biopsies to eventually reach the tipping point, in addition to as much harmonization of preanalytical and analytical conditions as possible, the need for more clinical trials that investigate a meaningful clinical question is especially high. Whether this research is done with CTCs, cfDNA, or one of the emerging liquid biopsies is not that important: the one that proves to be of clinical utility, in combination with analytical validity, will eventually be adapted by the community.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.drudis.2019.05.028.

TABLE 1

Factors influencing the clinical validity and utility of cfDNA

Problem	Solution
Preanalytical	
Large variety in use of blood tubes and cfDNA isolation assays	Establishing evidence-based standardized protocol for blood collection and processing
Unknown influence of circumstantial biological factors (e.g., comedications, comorbidity)	Researching influence of these circumstances in large cohort studies
Analytical	
Multitude of assays used to detect and characterize ctDNA	Vigorous analytical validation of each assay and perform cross-assay comparisons
Postanalytical	
Somatic variants reported in different variables	Use of mutant copy number/ml instead of VAF
Clinical utility	
Unknown relevance of finding a somatic mutation at (extremely) low frequency	Performing clinical trials investigating at which cut-off a somatic mutation is related to response to a targeted treatment or clinical residual disease
Unknown what change in somatic mutation over time is clinically relevant	Establish biological and analytical variation for a given somatic mutation and subsequently perform clinical trials that take this variation into account

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Nick Beije[‡,*]
John W.M. Martens[‡]
Stefan Sleijfer[‡]

Erasmus MC Cancer Institute, Department of Medical Oncology and Cancer Genomics Netherlands, Erasmus University Medical Center, Rotterdam, The Netherlands

*Corresponding author.

[‡]Current address: Erasmus MC Cancer Institute, Wytemaweg 80, 3015 CN, Rotterdam, The Netherlands.

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