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Commentary

Advances in liquid biopsy: From exploration to practical application

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Liquid biopsy has received tremendous attention as a non-invasive approach for detecting and tracking cancer. Here, we discuss the latest work on circulating tumor DNA and circulating tumor cells with respect to clinical applications, including cancer screening, early detection of relapse, real-time monitoring of therapeutic efficacy, and detection of therapeutic targets and resistance mechanisms.

Introduction

Tissue biopsies are the current diagnostic standard, but they are invasive procedures with potential side effects (e.g., bleeding, infections), and some locations are difficult to access (e.g., metastases in the lung or brain). A single biopsy of an accessible metastatic lesion (e.g., a skin metastasis) may not represent the genomic composition of the brain or lung metastasis in the same patient. In contrast, blood is a pool of tumor cells, and their products are in principle released from all tumor lesions in a cancer patient. Most importantly, collecting blood is minimally invasive and can be therefore easily repeated, which allows for real-time monitoring of tumor evolution and therapy responses in individual patients. In patients treated under curative intent, blood-based surveillance enables detection and molecular characterization of minimal residual disease (MRD) as well as early detection of relapse months before current imaging modalities. Tracking cancer in liquid biopsy (LB)—a term introduced in 2010[1]—has therefore been highlighted in the latest Nature Milestones edition as one of the key discoveries of the past 20 years.[2]

Here, we will discuss the different clinical applications of LB in cancer management from diagnosis of cancer to metastatic disease (Figure 1). We will focus on circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) as the most intensively investigated LB markers to date.

Early detection of cancer

Early detection of cancer is the LB application that probably receives the highest attention from the public. A technical challenge is that very sensitive assays are necessary in order to detect small tumors that may shed only minute amounts of CTCs or DNA fragments into the blood. For CTCs, there is current development of more sensitive technologies, and the detection of intact tumor cells in the blood as transit organ for distant metastases might indicate the tumor has already disseminated. For ctDNA tests, the required assay sensitivity is at least 0.01% variant allele frequency (VAF) or less, which can be reached by several current technologies. Since the molecular composition of the occult tumor is *a priori* unknown, large panels encompassing the key drivers of a large variety of different tumor types are needed if the test is being used as a multi-cancer early detection (MCED) test in large population screening programs.[3] Alternatively, an LB test can be used to detect a specific tumor type in individuals at risk, which might require a more limited panel. Examples are patients with high-risk cysts, intraductal papillary mucinous neoplasms of the pancreas, or late onset diabetes who have an increased risk of developing pancreatic cancer and are therefore targets of the ongoing EU-funded consortium project PANCAID (https://pancaid-project.eu/) and French PANLIPSY trial (NCT06128343), as well as family members with an inherited risk to develop cancer.

Besides mutations, the detection of methylation signatures on cfDNA is a promising approach.[3] For example, the prospective multicenter PATHFINDER study (NCT: 04241796) has analyzed 6,621 patients aged 50 years or older without known tumor disease[3] and found a suspicious signature in 1.4% of them. Early cancers (stages I and II) were detected in approximately 50% of cases, which underlines room for improvement. Another approach is fragmentomics based on a comprehensive analysis of the ctDNA fragments in the plasma, which has also shown promising results as an MCED test.[4]

Despite promising results, early detection of cancer is one of the most challenging applications of LB tests. Overall, the detection of early stage (T_1) tumors still needs to be improved. The combination of different LB markers might improve assay sensitivity for detection of early or even pre-neoplastic lesions. Moreover, there is still a lack of long-term follow-up, and it remains to be shown if "early detection" is early enough to cure the respective tumor type detected by the blood test. This depends on the therapeutic options available for the specific tumor type. The clinical management varies between tumor types, and the blood test needs to be incorporated into the diagnostic workup. If the patient comes with a positive blood test to the physician, localization information will be crucial. Methylation signature can provide tumor origin information.[5] In addition, multimodal tests combined with sophisticated AI applications may help to improve

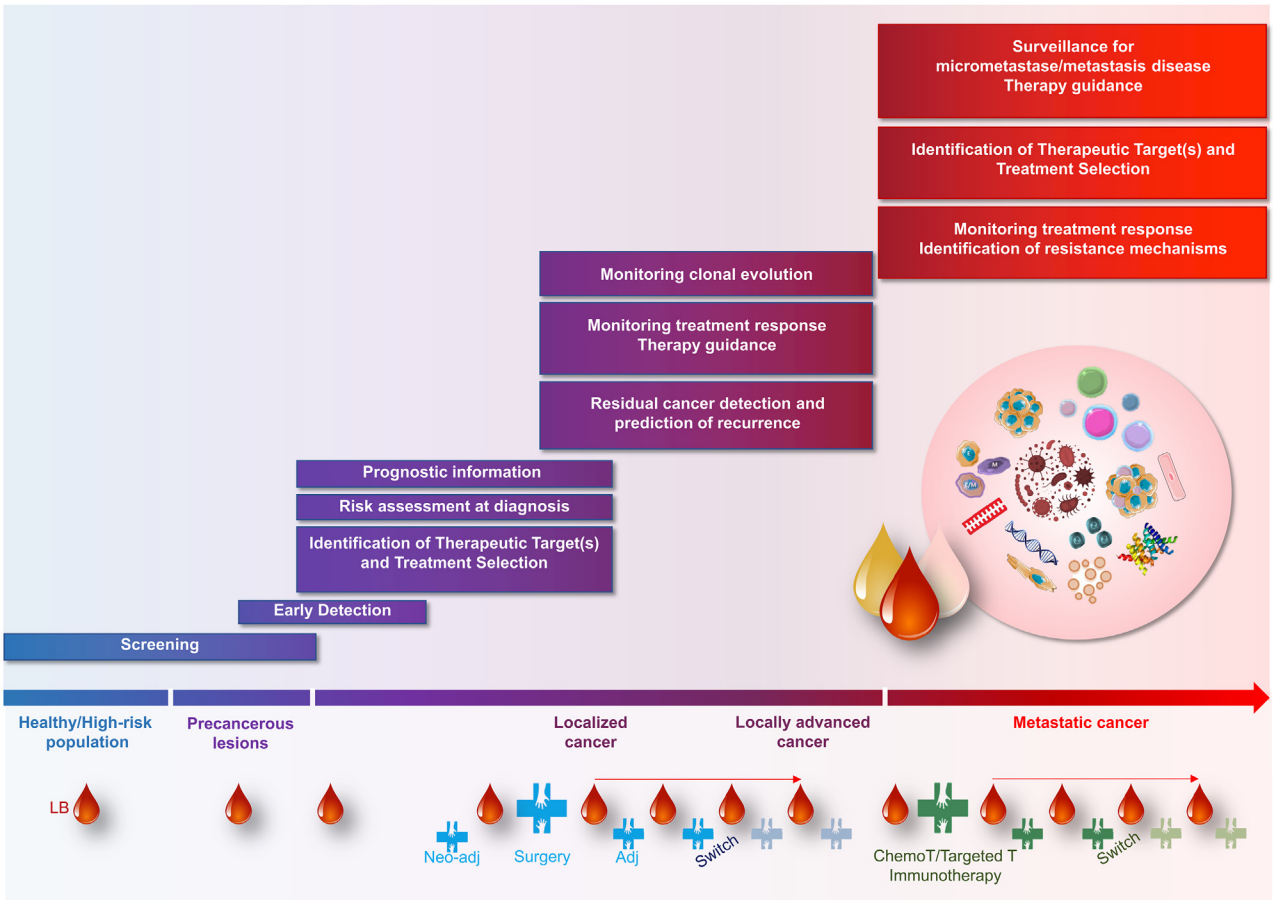

Figure 1. Current landscape of clinically used LB assays—Indications/clinical settings needing LB assays/platforms

Clinical applications of LB in cancer management, from diagnosis to death including screening/diagnosis, treatment selection and monitoring, MRD detection, and detection of resistance. The figure presents the various tumor-derived and tumor-induced circulating biomarkers (CTCs, ctDNA, EVs, tumor-educated platelets, circulating RNA, proteins, circulating endothelial cells, leukocytes, and circulating microbiome) that are detected and characterized in LB assays with potential clinical relevance alone or in combination. The LB performed at different time points can give crucial, real-time information on the cancer status as well as its evolution to propose the best individualized decision to each cancer patient. LB, liquid biopsy; Neo-adj, neo-adjuvant treatment; Adj, adjuvant treatment; Switch, change of treatment; ChemoT, chemotherapy; T, treatment.

accuracy of the LB test. Another challenge is how to deal with incidental findings (e.g., cancer signals detected during non-invasive prenatal testing in pregnant women[6]).

Surveillance of relapse after initial therapy

Another application of early detection of cancer traces in the blood is the detection of relapse after initial therapy during the follow-up of cancer patients by sequential blood analyses. The advantage over MECD tests for early detection of cancer is that the tumor type is known, which allows a more precise use of a tumor type-specific or even individualized test.

With regard to CTCs, the multicenter open phase III study SUCCESS-A included 1,087 patients with stage III breast cancer.[7] The detection of CTCs at the 24-month follow-up was an independent, statistically significant negative prognostic factor with regard to disease-free and overall survival. Similar data were provided by Sparano et al.[8] for CTC detection 5 years after initial therapy in breast cancer. Thus, CTCs signal the presence of occult micrometastases releasing cells into the circulation, and CTCs can therefore be detected before overt metastatic lesions are revealed by current imaging technologies.

For ctDNA, individual tumors can be sequenced to design patient-specific probes for ultra-sensitive surveillance of cancer signals in the blood. Multiple studies across cancer types have documented significant lead time in recurrence detection (up to >1 year) with serial ctDNA monitoring compared to standard-of-care surveillance with the most significant progress in colorectal cancer (CRC).[9] In particular, the question whether adjuvant therapy can be de-escalated in MRD-negative patients is of utmost importance, but it requires well-designed interventional studies. For example, patients with stage II CRC may not need adjuvant chemotherapy if their ctDNA blood test is negative. The precise detection of minute amounts of ctDNA fragments present in the peripheral blood during surveillance (usually <0.1% of the total amount of circulating cell-free DNA) is still technically challenging, as pointed out in the recent European Society for Medical

Oncology ctDNA guidelines,[10] which underline the need for more sensitive technologies and considerable clinical validation studies (cf. GUIding multimoDal thErapies against MRD by liquid biopsies, GUIDE.MRD [https://www.guidemrd-horizon.eu/]). Moreover, how to treat LB-positive individuals remains to be determined and is the subject of ongoing clinical studies.

Monitoring therapy response in advanced cancers (toward RECIST_2.0)

The current approach to measure response to treatment is based on radiological imaging and defined in the EORTC RECIST criteria used in most clinical studies (https://recist.eortc.org/). However, the reliability of progression-free survival as an outcome parameter has been debated, and recent perspective publications have suggested developing extended RECIST criteria based on LB measurements (in particular ctDNA). Besides ctDNA, assessment of CTC counts in metastatic breast cancer patients has shown clinical utility for treatment decision.[11]

Despite a wealth of studies demonstrating significant correlations between ctDNA measurements and clinical outcome in several tumor entities, there is a high level of heterogeneity between the studies regarding the number of included subjects; the types of clinical cohorts; and the methodology adopted to measure ctDNA, different sensitivity thresholds, and quantification strategies. Notably, most of the studies so far have only demonstrated the clinical validity of ctDNA as a biomarker. The use of ctDNA value as response evaluation biomarker will therefore necessitate establishing precise pre-treatment and on-treatment ctDNA cut-off points for each particular assay and for each particular tumor type. Moreover, interventional studies are needed to demonstrate the clinical utility of these ctDNA cut-off points. In addition, a better knowledge of ctDNA intra-day variation and the reproducibility of the methods is necessary.

Besides differences in sensitivity, one potential source of discrepancy between ctDNA assays and imaging can be pseudo-progression caused by various immune cells infiltrating the tumor mass, thus contributing to increased tumor volume. Pseudo-progression is a specific challenge associated with ICI treatment, and ctDNA measurements could be helpful to discriminate it from real progression.

Profiling of therapeutic targets and resistant mechanisms

The aim of precision oncology is to adapt the therapeutic approach according to the LB result, in particular in patients with advanced disease where metastatic tissue is frequently not available. In addition, cost, time, morbidity, and mortality can be reduced compared to when using tissue biopsies. Moreover, conventional tissue biopsy may not reveal the heterogeneity of a tumor disease (e.g., intrapatient heterogeneity of metastases at different locations in the same patient). However, blood is a pool of tumor-derived cells, and DNA released from different locations and should provide more comprehensive information than a single tissue biopsy of a metastatic lesion.

Additionally, targeted immunotherapies (in particular immune checkpoint inhibition, ICI) have changed the landscape of clinical trials in oncology and have been already implemented into clinical practice for a variety of tumor entities such as non-small cell lung cancer, melanoma, and urothelial bladder cancer. Nevertheless, only a fraction of patients responds to these new forms of immunotherapy, and research on resistance mechanisms and LB tests may contribute to the early identification of responders and non-responders with the opportunity to switch treatment before it is too late to change the course of the disease. Besides tumor characteristics, the host (patient) also contributes to response or failure of therapy of immunotherapy (e.g., the coagulation system affects resistance to ICI therapy in advanced melanoma patients[12]).

So far, ctDNA assays have limited ability to identify the transcriptional programs that govern cancer phenotypes and their dynamic changes during the course of disease. Several teams described assays that provided a robust proxy for transcriptional activity, allowing us to infer the expression levels of diagnostic markers and drug targets.

Besides analyzing cfDNA, the phenotyping of CTCs for therapeutic targets has also opened a new field for interventional studies. Such phenotyping might be in particular useful in tumor types where metastatic relapse occurs years after primary tumor resection, leaving enough time for a switch in phenotype, which could be only detected by obtaining metastatic tissue. Here, CTCs released by metastatic lesions could be an easy to obtain, minimally invasive source of metastatic tumor cells, which also has the advantage that the CTC counts in metastatic patients are higher than in early-stage patients. This new concept has been tested in the randomized interventional DETECT III trial: patients with initially $HER2^{-}$ primary breast carcinomas, who had $HER2^{+}$ CTCs in the blood at the time of metastatic recurrence, were additionally treated with the dual EGFR/HER2 inhibitor lapatinib in addition to the standard therapy, which resulted in a survival benefit compared to standard therapy alone.[13]

Conclusions and perspectives

LB has opened a new avenue for understanding the evolution of tumor progression in individual patients with important implications for the management of cancer patients.

For early detection of primary cancer or MRD/relapse, ultra-sensitive assays need to be used to detect minute amounts of ctDNA. However, increased sensitivity can be associated with decreased specificity. E.g., the mutational "background" in aging individuals affects blood cells (so called clonal hematopoiesis of indeterminate potential mutations), but cancer mutations are also present in epithelial cells classified as "normal" by the pathologist.[14] Moreover, DNA methylation can change with age, inflammation, and other chronic disease conditions. Thus, it cannot be excluded that a "background" of tumor-associated genomic and epigenetic aberrations may exist that may affect the specificity of ctDNA assays. Comprehensive analyses of large age-matched non-cancer control groups are therefore required to avoid false positive findings.

Insights into the biology behind LB markers are important to improve the clinical use of these biomarkers. In particular, biomarkers with a function in tumor development and progression might be of interest. To date, it cannot be excluded that cfDNA fragments might also interfere with processes relevant for cancer progression (e.g., affecting the generation of neutrophil extracellular traps or transfection of host cells through extracellular vesicles [EVs]).

The biology of ctDNA shedding and clearance is still under investigation with recent findings suggesting that cancers might have a systemic impact on cell turnover or DNA clearance.

CTCs are by their nature functional biomarkers. Research has indicated that the journey of CTCs through blood circulation is not just a passive shedding but rather an active process that supports the selection of the fittest clones. Moreover, mathematical modeling suggested that the survival of CTCs in the bloodstream seems to be a key parameter determining metastatic progression.[15] In addition, EVs, as another functional biomarker, play an important role in long-range communication through the bloodstream and participate in, for example, setting up the pre-metastatic niches in distant organs or interfering with the anti-tumor immune response.

There is and most likely will be not one assay that fits all clinical demands, but rather the selected LB test might be dependent on the specific tumor type, stage, or treatment. Today, there is much focus on single LB biomarkers; however, a few studies already suggest that a combination of different LB biomarkers may lead to higher accuracy. Moreover, most LB investigators focus on the tumor burden or characteristics, but natural tumor progression and resistance to therapy may depend on both tumor and host responses (both local and systemic). To what extent the LB measurements in the peripheral blood mirror the tumor microenvironment is the subject of ongoing investigations.

Given the molecular complexity of cancer and its clonal evolution that leads to the emergence of increasingly aggressive clones, we believe that the complementarity of a whole panel of circulating biomarkers is required to obtain comprehensive and real-time information on the natural and treatment-induced evolution of cancer. Despite the overwhelming interest in ctDNA, we strongly believe that a combination of additional LB markers will provide more information than a single marker class, and we are also confident that cellular biomarkers with a functional role in tumor biology (such as CTCs or EVs) should not be left behind.

Most importantly, interventional clinical studies are important to demonstrate the clinical utility of LB assessment to demonstrate that the blood test has a significant benefit to the cancer patients. For example, it is widely accepted that LB tests can detect the progression of a tumor disease many months before imaging, but can this lead to a therapeutic consequence that prolongs patients' survival and/or increase quality of life? The first reports are published on interventional clinical trials using LB to make treatment decisions that show a survival benefit. These types of studies are also relevant to regulatory agencies that decide on reimbursement for new diagnostic tests. Reimbursement is, of course, critical to making LB available to all cancer patients. To bring LB assays from the discovery and validation stage into clinical routine, the assays need to be validated and harmonized, including reliable quality assurance measures such as standardized DNA fragments with defined genetic alterations. Several consortia worldwide are active in this arena such as the ELBS (https://www.elbs.eu).

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AUTHOR CONTRIBUTIONS

C.A-P. and K.P. conceptualized the commentary, conducted the literature search and drafted the initial manuscript, provided critical revisions and contributed to the structure and content of the manuscript, wrote specific sections, reviewed and edited the manuscript for important intellectual content, supervised the project, and provided final approval of the version to be published.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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