Opinion Paper

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Liquid biopsy: novel technologies and clinical applications

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Abstract: “Liquid biopsy” was introduced as a new diagnostic concept in 2010 for the analysis of circulating tumor cells (CTCs) and has been now extended to material (in particular DNA) released by tumor cells in the peripheral blood of cancer patients. Over the past decade, various methods have been developed to detect CTCs and ctDNA in the peripheral blood of cancer patients.

Keywords: circulating tumor cells; liquid biopsy; minimal residual disease.

Metastasis in cancer patients can occur after long latency periods of up to more than 10 years, a process called “cancer dormancy”. The sources of these late relapses are disseminated tumor cells (DTCs) or small micrometastases undetectable by current imaging procedures. Recent advances in the development of sensitive and specific immunoassays and PCR-based technologies have made this stage of “minimal residual cancer” (MRC) become visible. Indeed, the rate of subsequent distant recurrence is significantly higher in patients with detectable DTCs than in those without [1]. In this context, the high plasticity of cancer cells plays an important role. Carcinoma cells can undergo an epithelial-to-mesenchymal transition, which is associated with cancer cell stemness leading to an increased invasiveness and resistance to chemotherapy. Another key area of investigation was cancer dormancy, which is controlled by the features of the DTCs and the surrounding microenvironment (e.g. changes in the BM environment may induce the escape from dormancy). Most DTCs in cancer patients are initially in a dormant (i.e. non-proliferative) stage, and they can display a cancer stem cell phenotype, which might help DTCs to survive systemic chemotherapy and evade immune recognition and eradication [2]. After the induction of proliferation, DTCs can be eradicated by the surrounding immune cells until some mutations occur (e.g. LOH in MHC genes) that enable the DTCs to escape immuno-surveillance.

Given the ease and comfort of a simple blood test, attention has turned to the analysis of a so-called “liquid biopsy”, a new diagnostic concept introduced in 2010 [3] for the analysis of circulating tumor cells (CTCs) and now extended to material (in particular DNA) released by tumor cells in the peripheral blood of cancer patients [4, 5] (Figure 1). Metastatic progression starts with local invasion and intravasation. Once tumor cells invade blood vessels, they can become CTCs, but most will not survive these steps because of anoikis, shearing forces of blood flow and immune cell attack. The key challenges in CTC isolation are their rarity in blood (1–10 CTCs per 10 mL) and lack of cancer-specific surface markers. Although reliable information can be easily obtained in patients with advanced disease, early stage cancer patients usually present with very low concentrations of CTCs and ctDNA. Over the past decade, various methods have been developed to detect CTCs and ctDNA in the peripheral blood of cancer patients [5, 6], and some have already reached the clinic (for example, the FDA-approved cobas® EGFR mutation test v2 and CellSearch®-based CTC enumeration). However, the majority of the new tests suffer from insufficient clinical and technical validation and lack clinical utility [7, 8]. At present, most CTC assays rely on epithelial markers, and the majority of CTCs detected are single isolated cells. For the detection of epithelial CTCs, antibodies against EpCAM and cytokeratins (CK8, CK18 and CK19) are frequently used. The current standard for CTC detection is the FDA-cleared CellSearch. CTC enrichment is based on positive selection via EpCAM antibody-coated ferromagnetic beads with subsequent staining with DAPI, anti-CD45 and anticytokeratin. CTCs are defined as nucleic acid+, CD45− and cytokeratin+. The enumeration of CTCs in the peripheral blood of cancer patients, surrogate of the dissemination process, was standardized for years in international studies and has demonstrated its
clinical validity at metastatic stage in many cancer types [9–11], particularly in breast and prostate cancers [5, 12]. Although most published studies have been performed on patients with carcinomas and melanomas, CTCs have been also detected in the peripheral blood of patients with primary brain tumors (glioblastomas) despite the blood–brain barrier [13].

An international meta-analysis shows that CTC dissemination is affecting the outcome of treated patients and that this process cannot be predicted precisely enough by the current assessment of the primary tumor characteristics and response. Bidard et al. [14] found that the best postneoadjuvant survival models included prespecified clinicopathological prognostic markers at baseline, CTC detection at baseline and pathological complete response, demonstrating the relevance of such metastasis-associated biomarkers for outcome prediction [15].

The clinical relevance of “mesenchymal” CTCs lacking any epithelial markers as well as CTC clusters are still under investigation. CTC clusters either contain just a group of neoplastic cells or are associated with fibroblasts, leukocytes, endothelia cells and plates. In breast and prostate cancers, CTC clusters were shown to consist of oligoclonal cells from the primary and seems to be associated with higher metastatic potential than single CTCs [16]. However, clusters are rare and occur almost exclusively in patients with advanced disease. Thus, the strong prognostic data in early stage breast cancer patients are based on the detection of single CTCs [14, 15, 17].

There are strong interests for detecting viable CTCs and for expanding them ex vivo in different cancer types, and it remains a crucial challenge in this field of expertise. For functional analysis of CTCs, the development of in vitro and in vivo test systems has started, which might also serve as models for drug testing. In particular, the development of CTC lines and xenografts derived from CTCs can provide novel insights into the biology of tumor cell dissemination and may be used to discover new pathways to target specifically metastatic cells [18–21].

Liquid biopsy assays are currently being validated for the early detection of cancer, which is supposed to reduce cancer-related mortality. Despite remarkable progresses, the liquid biopsy-based detection of early stages of cancer remains a challenge, in particular in breast cancer [4].

These limitations are illustrated by the recent work of Cohen et al. [22] who introduced the Cancer-Seek panel for the detection of the eight most common cancers. This complex approach combined the evaluation of eight soluble tumor biomarkers (including standard tumor markers such as CEA) with ctDNA analysis of cancer-related mutations in 16 genes. The panel reached an overall median sensitivity of 70% with specificity at ≥99%, but significant differences in sensitivities were observed among the tumor types analyzed (for example, 98% in ovarian cancer, 60% in lung cancer and 33% in breast cancer) [22]. Moreover, the authors analyzed only healthy controls; thus, the high specificity of the Cancer-Seek approach requires further validation with a non-cancer control with comorbidities such as inflammatory diseases that are common in older individuals.

In patients with diagnosed cancer, CTCs and ctDNA analyses can obtain independent information on prognosis in early and advanced stages of disease. In particular, CTC counts at initial diagnosis are able to refine the current risk stratification by TNM staging in early stage breast cancer [14, 15, 17]. Moreover, the early detection of relapse by sequential ctDNA (or CTCs) analysis of blood samples obtained postsurgery during the follow-up is possible and may be used in future trials to stratify patients to “postadjuvant” therapies [22].

Besides CTCs and ctDNA, the analysis of circulating micro-RNAs, exosomes or tumor-educated platelets may provide complementary information as “liquid biopsy”. Currently, the validation in clinical trials of this new blood-based biomarkers for early detection was carried out. RNA molecules present in extracellular vesicles such as exosomes or in platelets reveal relevant molecular information in cancer patients [23, 24]. Exosomes are
nanscale vesicles shed by most types of cells. Exosomes were first isolated 30 years ago. Finding proteins RNA and DNA unique to tumor-derived exosomes is the subject of intense research [25–27]. Exosomal material, floating in virtually all bodily fluids, has potential for liquid biopsy and may represent a novel therapeutic delivery system [4]. Moreover, exosomes can also affect tumor biology, and the integrin composition of exosomes seems to determine the organ site of metastatic niches [26]. These findings suggest that exosomes prime future metastatic sites, thus facilitating metastasis, and that exosomal proteins can be reliable biomarkers for cancer development and metastasis. Encouraging proof-of-principle studies demonstrating clinical relevance have been performed in patients with solid tumors such as pancreatic or ovarian cancer [28]. The primary literature on RNA molecules in exosomes rapidly growing and in particular the area of non-coding RNAs has drawn substantial attention [29, 30].

Recently, tumor-educated blood platelets (TEPs) have emerged as an alternative source of tumor-related biological information [23]. Tumor-associated biomolecules are transferred to platelets. Moreover, external stimuli, such as activation of platelet surface receptors and lipo polysaccharide-mediated platelet activation, induce specific splicing of pre-mRNAs in circulating TEPs. TEPs may also undergo specific splice events in response to signals released by cancer cells and the tumor microenvironment – such as stromal and immune cells. mRNA sequencing of TEPs from patients with different types of localized and metastasized tumors and healthy individuals detected tumors with 96% accuracy [31]. The combination of specific splice events in response to external signals and capacity of platelets to directly ingest circulating mRNA can provide TEPs with a highly dynamic mRNA repertoire, with potential applicability to cancer diagnostics [23, 32].

Another key application of liquid biopsy is to identify therapeutic targets or mechanisms of resistance of metastatic cells in individual patients. Although the analysis of ctDNA focuses on mutations relevant for cancer therapy (e.g. EGFR, KRAS or ESR1 mutations), CTCs offer a wide spectrum of analyses at the DNA, RNA and protein levels [4, 5]. Metastatic cells might have unique characteristics that can differ from the bulk of cancer cells in the primary tumor currently used for stratification of patients to systemic therapy. Moreover, monitoring of CTCs and ctDNA before, during and after systemic therapy (e.g. chemotherapy, hormonal therapy, antibody therapy) might provide unique information for the future clinical management of the individual cancer patient and might serve as a surrogate marker for response to therapy. In the context of recent success in antibody-mediated blockade of immune checkpoint control molecules, the expression of the PD-L1 on CTCs might be of interest as a potential predictive marker [33, 34]. Moreover, the expression of androgen receptor variant 7 in CTCs may predict resistance to antianrogen therapy in prostate cancer, whereas mutations in the estrogen receptor gene (ESR1) provides information on resistance to hormone therapy in breast cancer [35, 36]. Additional therapeutic targets detected on CTCs in cancer patients include estrogen receptor and HER2 oncogene [5]. Single-cell RNAseq analysis of CTCs may provide more comprehensive information on relevant pathways.

Tissue biopsies presently remain the gold standard or reference for liquid biopsy analyses, and this concept is even adopted by regulatory agencies to approve tests. However, the use of tissue biopsies as a reference standard is questionable. Besides the intratumor heterogeneity of the lesion biopsied and used as a reference, ctDNA or CTCs can be derived from lesions that were not biopsied and may contain a divergent genomic composition. Even in patients with no metastatic lesions detected by current imaging modalities (stage MO), occult micrometastases may contribute to the pool of ctDNA or CTCs and, therefore, lead to different genomic landscapes than the primary tumor used as a reference.

In conclusion, liquid biopsy analysis can be used to obtain new insights into metastasis biology, and as companion diagnostics to improve the stratification of therapies and to obtain insights into therapy-induced selection of cancer cells. Different approaches such as CTC or ctDNA analysis will provide complementary information. Technical and clinical assay validation is very important and can be achieved in international consortia such as the European IMI Cancer-ID network (www.cancer-id.eu).

“To bring liquid biopsy into routine monitoring of cancer patients more interventional studies aimed to demonstrate clinical utility in order to address the key questions ‘how does the biomarker analysis change treatment and does this biomarker-induced change results in a better clinical outcome?’ Besides the FDA-approved use of EGFR mutation testing in lung cancer, several interventional clinical trials are ongoing (e.g. STIC CTC METABREAST study on the use of CTCs to decide about chemotherapy vs. hormone therapy in metastatic estrogen-positive breast cancer [37]), and it can be envisaged that a positive result of these trials will open new avenues for introducing liquid biopsy into standard care.”

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