Review

Cancer Research

Real-time Liquid Biopsy in Cancer Patients: Fact or Fiction?

Klaus Pantel[1] and Catherine Alix-Panabières[2,3]

Abstract

Distant metastases harbor unique genomic characteristics not detectable in the corresponding primary tumor of the same patient and metastases located at different sites show a considerable intrapatient heterogeneity. Thus, the mere analysis of the resected primary tumor alone (current standard practice in oncology) or, if possible, even reevaluation of tumor characteristics based on the biopsy of the most accessible metastasis may not reveal sufficient information for treatment decisions. Here, we propose that this dilemma can be solved by a new diagnostic concept: liquid biopsy, that is, analysis of therapeutic targets and drug resistance–conferring gene mutations on circulating tumor cells (CTC) and cell-free circulating tumor DNA (ctDNA) released into the peripheral blood from metastatic deposits. We discuss the current challenges and future perspectives of CTCs and ctDNA as biomarkers in clinical oncology. Both CTCs and ctDNA are interesting complementary technologies that can be used in parallel in future trials assessing new drugs or drug combinations. We postulate that the liquid biopsy concept will contribute to a better understanding and clinical management of drug resistance in patients with cancer. *Cancer Res; 73(21); 6384–8. ©2013 AACR.*

Introduction

The prognosis of patients with carcinoma, even with small primary tumors, is mainly determined by the blood-borne dissemination of tumor cells from the primary site to distant organs such as bone marrow, liver, lungs, or brain and the subsequent outgrowth of a largely unknown subset of these cells ("metastasis-initiator cells") into overt metastases in their new microenvironment (1). The colonization of distant organs by disseminated tumor cells (DTC), resulting in the appearance of clinically detectable metastases, can take many years in breast cancer (and other solid tumors; ref. 2) and the mechanisms behind this "cancer dormancy" are largely unknown (3–5). This stringent selection process together with a potential independent genomic progression of DTCs may explain why overt metastases can harbor unique genomic alterations different from the bulk of the original primary tumor cells (6–8). Thus, the direct analysis of metastatic cells will reveal important information for systemic cancer therapy targeting metastatic disease (9, 10). However, biopsy of overt metastases is an invasive procedure limited to certain locations and not easily acceptable in the clinic. Moreover, recent work has shown that different metastatic sites harbor different genomic aberrations (11) and biopsy of one or two accessible metastases may not be representative.

An alternative approach is the analysis of blood samples for circulating tumor cells (CTC) or circulating tumor DNA (ctDNA), which can be conducted repeatedly and might allow real-time monitoring of cancer therapies in individual patients (10). Recent reports indicated that CTCs and ctDNA give important complementary information on therapeutic targets and drug resistance mechanisms in patients with carcinoma (7, 12). The peripheral blood is a pool of cells and/or DNA derived from the primary tumor and different metastatic sites and may, therefore, provide a comprehensive real-time picture of the whole tumor burden in an individual patient (Fig. 1). At present, there is an ongoing discussion whether CTCs or ctDNA are better suitable as "liquid biopsy" (Annual ACCR Meeting 2013, Washington, DC) and we will briefly discuss the main advantages and disadvantages of both the approaches.

CTCs

Clinical relevance and biologic implications

The detection of CTCs in the peripheral blood of patients with solid epithelial tumors (e.g., breast, prostate, lung, and colon cancer) holds great promise, and many exciting technologies have been developed over the past years (13). In addition, there is a wealth of information on the clinical validation of CTC detection and enumeration (e.g., large-scale pooled analysis of thousands of patients with breast cancer; ref. 14), which resulted in the inclusion of CTCs in the new edition of the tumor–node–metastasis (TNM) cancer staging manual in 2010 as classification $cM_0(i+)$. Currently, more than 400 clinical trials use CTCs as biomarkers and PubMed lists more than 14,000 publications on CTCs. Many publications deal with the patients in advanced stages, but there is also an increasing number of publications on patients at earlier

Authors' Affiliations: [1]Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [2]University Medical Centre, Saint-Eloi Hospital, Institute of Research in Biotherapy, Laboratory of Rare Human Circulating Cells, Cell and Tissue Biopathology of Tumors Department; and [3]University Institute of Clinical Research UM1-EA2415-Epidemiology, Biostatistics & Public Health, Montpellier, France

Corresponding Author: Klaus Pantel, Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, Hamburg 20246, Germany. Phone: 49-40-7410-53503; Fax: 49-40-7410-55379; E-mail: pantel@uke.de

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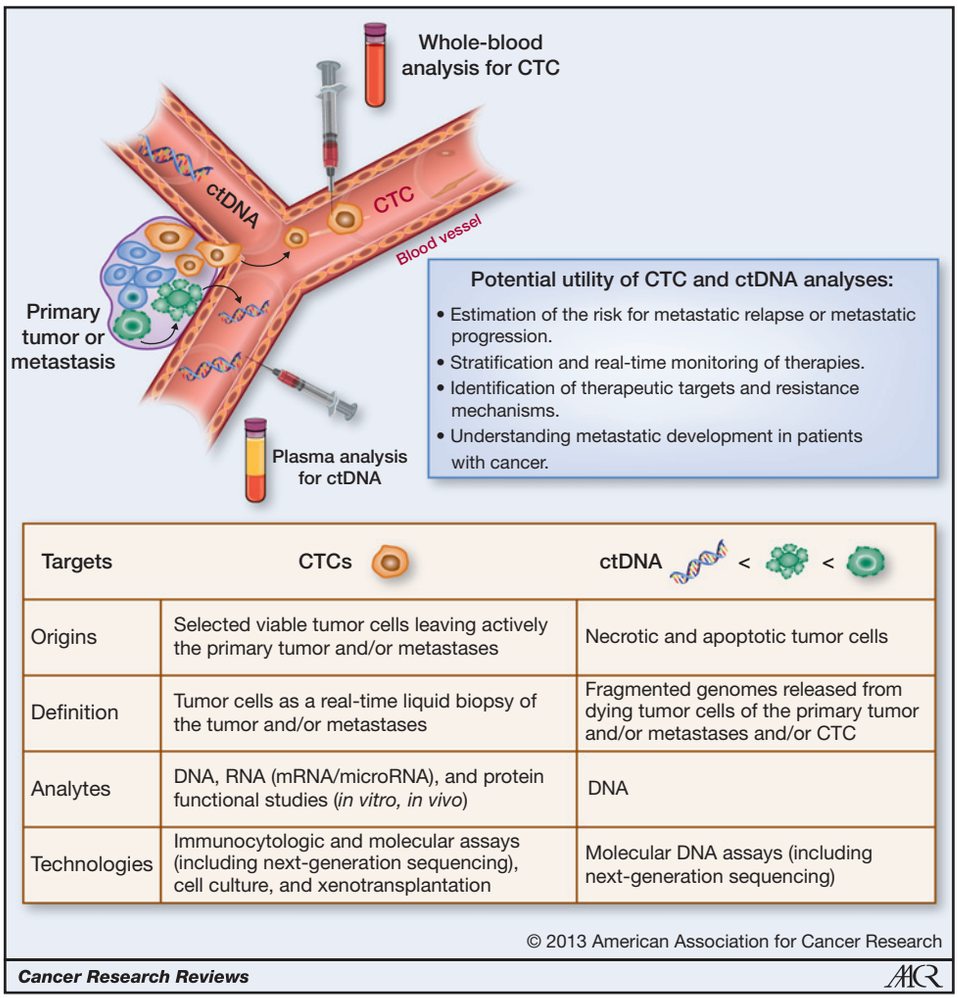

Figure 1. Scheme of blood analysis for CTCs and ctDNA in patients with cancer. The figure describes the origin of CTCs and ctDNA, provides a comparative listing of the technical possibilities of analyses of CTCs and ctDNA, and summarizes their potential use in regard to clinical applications and basic research.

disease stages without clinical and radiologic signs of overt metastases, in particular in breast cancer (14, 15), but also in other tumor entities such as bladder cancer (16). These publications show a significant correlation between the CTC counts and prognosis of patients with cancer, suggesting that CTCs are either surrogates of metastatic activity or causally involved in the metastatic process. Interestingly, CTCs are observed months and years after surgical resection of the primary tumor. Regarding the short life span of CTCs in the circulation (17), this observation suggests that DTCs or micrometastases replenish the pool of CTCs over a long period of time (17, 18). Thus, the analysis of these CTCs may provide unique information about the molecular characteristics of occult minimal residual disease (MRD) in patients with cancer without clinically detectable overt metastases. Detection and molecular analysis of MRD is of utmost importance for tailoring systemic adjuvant therapy aimed to prevent the progression of DTCs/micrometastases to overt metastases, but this decision is usually based on statistical risk assessment and the analysis of a very small sample of the primary tumor. This is a severe limitation in view of the pronounced genomic and phenotypic heterogeneity of the primary tumor and the fact that metastatic cells may harbor unique molecular characteristics not detectable in the bulk of the corresponding primary cancer cells.

Potential and challenges

However, detecting CTCs remain technically challenging. CTCs occur at very low concentrations of one tumor cell in the background of millions of blood cells. Their identification and characterization require extremely sensitive and specific analytic methods, which are usually a combination of complex enrichment and detection procedures (9, 13, 19). In particular, early-stage patients present with very low concentration of CTCs and might, therefore, require more sensitive CTC assays and/or analysis of large blood volumes (13).

Nevertheless, recent studies have documented the potential of CTC analyses for an in-depth assessment of viable metastatic tumor cells at various levels (DNA, RNA, and proteins) and functionally (*in vitro*/*in vivo*), including next-generation sequencing analyses (7) and xenotransplantation of CTCs into immunodeficient mice (20). This may contribute to the identification of metastases-initiator cells; these cells are the prime targets of antimetastatic therapies and they may have

developed special resistance mechanisms. At present, more sensitive technologies are being developed, also capturing CTCs that underwent an epithelial–mesenchymal transition (EMT), a process linked to cancer stemness and chemotherapy resistance (21, 22). Several studies have shown that CTCs express various EMT-related transcription factors (e.g., Twist and Snail) and vimentin as the typical mesenchymal intermediate filament protein (23, 24). Recently, it has been suggested by experimental studies that CTCs "frozen" in a mesenchymal state might not be able to form solid metastases, but these cells need to revert to an epithelial phenotype (25). If this is also true in patients with cancer, CTCs with the highest EMT/mesenchymal–epithelial transition (MET) plasticity might be the most aggressive ones, supporting both tumor cell dissemination and initiation of metastatic outgrowth.

ctDNA

Clinical relevance and biologic implications

Elevated concentrations of cell-free ctDNA fragments have been found in blood plasma and serum of patients with cancer with various tumor types and associated with unfavorable outcome in some clinical studies (26). ctDNA fragments mainly originate from apoptotic or necrotic tumor cells that discharge their DNA into the blood circulation (26). The use of ctDNA as an abbreviation may not be optimal and should be considered carefully as this is likely to be adopted widely in the scientific literature on this topic. At present, there is no way to isolate tumor DNA specifically from other circulating DNAs and only the detection of tumor-specific mutations on circulating cell-free DNA indicates the presence of ctDNA.

With the development of next-generation sequencing technologies, the field of ctDNA analysis, which originally started almost 20 years ago (27–29), has revived and focused on genomic aberrations relevant to therapy resistance in patients with metastatic cancer [e.g., *KRAS* mutations for EGF receptor (EGFR) inhibition in colorectal cancer; ref. 30]. Besides targeting specific gene mutations, several groups have developed assays to screen the genome of ctDNA. Leary and colleagues analyzed four colorectal and two breast cancers with massively parallel sequencing and revealed an average of nine rearranged sequences (range, 4–) per tumor (31). PCR with primers spanning the breakpoints was able to detect mutant DNA molecules present at levels lower than 0.001% and readily identified mutated circulating DNA in patient plasma samples. More recently, Murtaza and colleagues have established proof-of-principle that the exome-wide analysis of ctDNA can identify mutations associated with acquired drug resistance in advanced cancers. The authors serially analyzed ctDNA for 1 to 2 years in 6 patients with cancer with very high ctDNA concentrations in their blood plasma. Even though exome sequencing revealed numerous mutations in ctDNA, the authors were able to identify specific mutations in the post-therapy samples that are known to confer drug resistance (32). In another study from the same research group, Dawson and colleagues used targeted or whole-genome sequencing to identify somatic genomic alterations and designed personalized assays to quantify ctDNA in serially collected plasma samples (12). ctDNA was detected in 29 of the 30 (97%) patients with metastatic breast cancer in whom somatic genomic alterations were identified.

Taken together, detailed genomic information relevant to cancer therapy can be obtained from ctDNA present in the peripheral blood of patients with cancer with advanced disease. However, it should be noted that patients with high ctDNA concentrations were selected for these interesting proof-of-principle studies.

Potential and challenges

Although the analysis of plasma samples (ctDNA) seems to be more convenient than the analysis of whole blood (CTC), preanalytic conditions for ctDNA analysis must be also standardized (26). For example, normal DNA from dying blood cells after blood collection will contaminate the specimens and dilute ctDNA. However, immediate plasma separation, storage, and shipment on dry ice make multicenter trials more complicated.

Besides these technical considerations, the key question about the biology and clinical relevance of ctDNA analyses is, why cell-free DNA mainly released from dying tumor cells should give important information on resistant clones? Possible hypotheses that need to be tested in future studies are that resistant viable tumor cells may release ctDNA and/or that a fraction of these cells might undergo apoptosis and release fragmented ctDNA into the blood.

Studies on combined analyses of CTCs and ctDNA

Investigations on combined analyses of CTCs and cell-free DNA have just begun and show an interesting relationship between ctDNA and CTCs in blood and DTCs in bone marrow of patients with cancer. Previous studies in patients with prostate cancer showed that the presence of CTC was significantly correlated with the frequencies of LOH at certain chromosomal loci on ctDNA (33). Another previous investigation in patients with early breast cancer indicated that both CTC counts and ctDNA (*HER2* amplification) were predictors of metastatic disease (34). In non–small cell lung cancer, Maheswaran and colleagues analyzed mutations in EGFR in CTCs and ctDNA in the same patients and concluded that genotyping of CTCs seemed to be more sensitive than analysis of free plasma DNA ($P = 0.009$) and the concomitant quantification of CTCs provided an important context in which to interpret genotyping results (35).

In more recent studies, modern next-generation sequencing technologies have been applied for a more in-depth genomic analysis. Dawson and colleagues claimed that ctDNA levels showed a more dynamic range and more correlation with changes in tumor burden than did CTCs (12). However, they have used the EpCAM-based CellSearch system with its known sensitivity problems for CTC analysis. Interestingly, they could show a good correlation between ctDNA and CTC levels in patients with higher CTC counts, which is consistent with other reports in colon cancer (33, 36). In patients with metastatic colon cancer (36), Heitzer and colleagues found a significant correlation between the detection of CTCs and the presence of circulating DNA fragments with a particular length

that contained tumor-specific mutations (e.g., in the *KRAS* gene). Comparing the sequences of DNA extracted from CTCs with ctDNA of the same patients with colon cancer revealed striking similarities in the few index patients analyzed (7). However, these investigations were so far restricted to a small group of metastatic patients with high CTC counts and ctDNA concentrations.

Another set of comparative investigations targeted epigenetic alterations on ctDNA. In patients with advanced stage melanoma, the number of CTCs significantly correlated with the methylation of cell-free *RASSF1A* and *RAR-β2* DNA molecules and both CTC and ctDNA concentrations were prognosticators for response to therapy and clinical outcome (37). Two other studies in patients with breast cancer also showed a significant correlation between cell-free methylated DNA and the detection of CTCs and both parameters were associated with more aggressive tumors (38, 39).

Nevertheless, a caveat needs to be added because the quoted studies were conducted with various types of CTC and ctDNA assays and relatively small cohorts of patients with cancer were analyzed. Thus, larger studies with standardized assays are required to validate these interesting preliminary findings.

Conclusions and Perspectives

The term liquid biopsy originally introduced for the analysis of CTCs (13) has been also used now for ctDNA analysis (12, 30, 32). The current definition of a biopsy is, however, "the removal of cells or tissues for examination by a pathologist" (National Cancer Institute, NIH, Bethesda, MD). Although this term is suitable for CTCs, it seems somewhat misleading when applied to ctDNA, a fragmented cell component released mainly by dying tumor cells.

Thus far, proof-of-principle data in small cohorts of metastatic patients with high CTC counts and ctDNA amounts have been published (7, 12, 30, 40), but larger prospective trials are needed to show clinical use. Future analysis of blood samples from M_0-patients with much lower amount of CTCs and ctDNA will be a real challenge. Dilution with normal DNA released, for example, from cells during infections or injuries, and mutations of tumor-associated genes in normal tissue of aging patients and in frequent benign diseases (e.g., nevi or prostate hyperplasia) may cause sensitivity and specificity problems.

Moreover, future studies need to show whether CTCs or ctDNA detected in blood are representative of all relevant metastatic cell clones located at different sites, which need to be further explored in prospective intervention trials testing whether changes in therapy based on ctDNA and/or CTC analyses using standardized tests will improve patient's outcome by enabling an individualized therapy. Prospective multicenter trials have been initiated for HER2-directed therapies (e.g., DETECT-III and EORTC-sponsored TREAT-CTC trials) randomizing lapatinib or trastzumab based on the HER2 status of CTCs in patients with metastatic breast cancer (41).

The outcome of the current race between CTCs and ctDNA is open. Both CTCs and ctDNA are interesting complementary technologies that can be used in parallel in future trials assessing new drugs or drug combinations. Considering the rapid developments in CTC research and sequencing technologies, it might become possible in the near future to identify the metastasis-initiator subpopulation of CTCs by immunostaining and specifically analyze the genome of these important cells. Sequential applications of different targeted drugs based on real-time liquid biopsy analyses in individual patients might become a novel strategy for personalized medicine in oncology. We postulate that this strategy will contribute to a better understanding and clinical management of drug resistance in patients with cancer.

Disclosure of Potential Conflicts of Interest

K. Pantel is a consultant/advisory board member of Veridex, Alere, and GILUPI. No potential conflicts of interest were disclosed by the other author.

Authors' Contributions

Conception and design: K. Pantel, C. Alix-Panabières
Development of methodology: K. Pantel
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Pantel
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Pantel
Writing, review, and/or revision of the manuscript: K. Pantel, C. Alix-Panabières
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Pantel
Study supervision: K. Pantel

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