Molecular Detection of Circulating Tumor Cells and Cell-Free Nucleic Acids

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Abstract

One of the key roles performed by pathologists is determination of the presence or absence of tumor in clinical samples. This is the basis for most approaches to staging, monitoring response to treatment, and detecting relapse of neoplasia and, as such, is a critical step in determining the course of patient management. Pathologists have utilized a variety of methods, continually seeking to improve assay performance and thus patient outcome. The literature reflects this quest, including reports assessing the increased sensitivity afforded by immunohistochemistry (IHC), flow cytometry, and, more recently, molecular approaches for the detection of tumor cells and nucleic acids in blood and bone marrow samples. The goal is, of course, the more accurate detection of disease spread and, ultimately, better patient care.

This chapter addresses some of the recent work in tumor detection, focusing on molecular and, to some degree, immunofluorescent approaches for the detection of circulating tumor cells and free nucleic acids in clinical samples. A synopsis of the hundreds of articles published to date is beyond the scope of this chapter; instead, more general issues and findings are addressed, along with presentation of selected work. Several reviews are available for more detailed reading (Alix-Panabières and Pantel, Clin Chem 59:110–118, 2013; Pinzani et al., Methods 50:302–307, 2010; Pratt et al. Chem Eng Sci 66:1508–1522, 2011; Schwarzenbach et al., Nat Rev Clin Oncol 11:145–156, 2014).

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Introduction

One of the key roles performed by pathologists is determination of the presence or absence of tumor in clinical samples. This is the basis for most approaches to staging, monitoring response to treatment, and detecting relapse of neoplasia and, as such, is a critical step in determining the course of patient management. Pathologists have utilized a variety of methods, continually seeking to improve assay performance and thus patient outcome. The literature reflects this quest, including reports assessing the increased sensitivity afforded by immunohistochemistry (IHC), flow cytometry, and, more recently, molecular approaches for the detection of tumor

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Circulating Tumor Cells

Cancer metastasis occurs when tumor cells acquire the ability to escape their local environment, enter the circulation to reach distant sites, attach at the distant site, and proliferate to form a metastatic cancer lesion. Depending on the type of tumor, cells enter either the venous or lymphatic circulation (or both) and thus are spread to distant tissues (such as the lung, liver, or bone marrow) or local lymph nodes, respectively, prior to the development of clinically detectable metastatic lesions. The fact that significant proportions of patients with organ-confined tumors who undergo theoretically curative surgery later have recurrence of their disease argues that current approaches to cancer staging are, to some degree, inadequate. Sensitive detection of circulating tumor cells could lead to improved staging and monitoring of cancer patients. Such techniques can also be applied to the study of stem cell harvests and assessment of body fluids.

Available Assays

Methods for detection of circulating tumor cells (CTCs) incorporate multiple technologies and platforms. At their most basic, information can be obtained about the quantity of tumor cells in blood and the antigens they express. Some systems also allow for captured CTCs to be examined morphologically, be cultured in vitro, or be used for cytogenetic or molecular analysis. While only one assay is currently FDA-approved for CTC enumeration, work is being done to create simpler and more sensitive instruments for CTC detection that allow for additional analysis to be performed on isolated CTCs and their extracted nucleic acids.

Immunohistochemistry
IHC methods for tumor cell detection have been applied to preparations of cells from the bone marrow, lymph node aspirates, and peripheral blood mononuclear cells. Cells can be smeared or centrifuged by cytospin onto slides, or sections taken from frozen or formalin-fixed, paraffin-embedded (FFPE) tissue. IHC is performed using antibodies to specific proteins associated with tumor type and standardized methods. Slides can be scanned under the light microscope by the eye, though to achieve a high level of sensitivity, thousands to millions of cells must be screened, which is tedious and time consuming. Thus, image analysis has become a popular approach for screening [5]. Alternatively, flow cytometry is utilized to achieve assessment of high numbers of cells rapidly [6].

In general, the interpretation of occult disease detection assays consists of either a positive or negative result, given that the assay controls are appropriate. For IHC markers, nonspecific or aberrant expression of protein markers requires that careful evaluation of the cytologic characteristics of the positive cells be performed. The ability to visualize cell morphology with IHC can reduce false-positive results because the interpretation of a positive result can be limited to IHC positive cells with appropriate tumor cell morphology.

Reverse Transcription-Polymerase Chain Reaction
Reverse transcription-polymerase chain reaction (RT-PCR) was the earliest molecular method by which detection of circulating tumor cells was explored [7]. RT-PCR assays rely on the detection of mRNA transcripts specific to a tumor type, such as tyrosinase in melanoma [8] or prostate-specific antigen for prostate cancer [9]. Unfortunately, such methods detect all cells expressing the marker of interest, which may include benign circulating epithelial cells [10]. More specific RT-PCR detection of CTCs may be possible through detection of markers of malignancy such as chromosomal translocations or point mutations. However, translocations are more common in sarcomas and hematologic malignancies such as lymphoma and leukemia, limiting their utility when applied to the detection of carcinoma CTCs.

Antibody Capture
Normal hematopoietic cells do not express the surface epithelial cell proteins, such as epithelial cell adhesion molecule (EpCAM), seen on carcinoma-derived CTCs. Therefore, many methods rely on positive selection strategies based on CTC expression of antigen markers such as BER EP4 to facilitate separation and identification, as well as increase the sensitivity and specificity of detection. The most common techniques use magnetically labeled antibodies. While automated immunomagnetic cell separation is currently used clinically for applications such as chimerism testing after stem cell transplant [11], CTC detection requires a higher level of sensitivity due to the small numbers of CTCs present. The only assay currently FDA approved, the CellSearch® System (Janssen Diagnostics, LLC, Raritan, NJ) (Fig. 39.1) [12], utilizes antibody-coated ferrofluids to separate CTCs
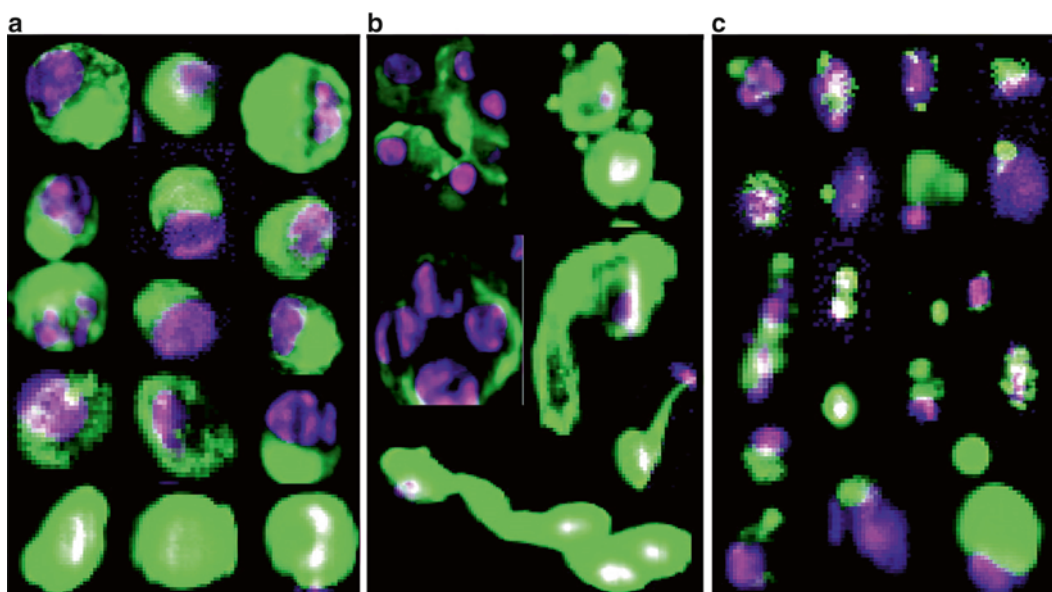
Figure 39.1 Gallery of CTC images from the CellSpotter Analyzer (Janssen Diagnostics LLC, Raritan, NJ) Diagnostics obtained from 7.5 mL of blood from cancer patients. (**a**) Examples of typical intact CTCs. (**b**) Examples of intact CTCs present as clusters or with odd shapes that are present less frequently. (**c**) Examples of CTC fragments and apoptotic CTCs. Images presented in **c** were not included in the CTC counts but are frequently observed in CTC analysis of carcinoma patients. From Allard WJ, Matera J, Miller MC et al. (2004) Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with non-malignant diseases. Clin Cancer Res. 47:6897–6904. Reprinted with permission from American Association for Cancer Research

from normal blood components. To increase specificity, the isolated cells are then stained with 4′,6-diamidino-2-phenylindole (DAPI) and a fluorescently-labeled antitumor antibody prior to imaging. Automatically captured images are reviewed by a pathologist to determine the number of tumor cells present, based on a combination of fluorescent immunostaining and morphology [13].

To increase sensitivity, alternative methods of detection are being explored. One recently described ultrasensitive CTC detection device relies on detection of changes in electrical conductance caused by the presence of magnetic immunoparticles on cells instead of image analysis of separated cells to detect CTCs. Similar to flow cytometry, this system could allow multiple antigens to be simultaneously bound by multiple fluorescently labeled antibodies and detected on the same cell for increased specificity [14]. Such methods would require rigorous quality control and validation prior to clinical usage.

Negative cell selection strategies have successfully utilized CD45 immunomagnetic labeling to remove hematopoietic cells and enrich for CTCs in head and neck cancer patients [15]. These methods have possible advantages in that CTCs may express cytokeratins or other epithelial-specific markers weakly or not at all, limiting the ability to adequately tag CTCs for positive selection. Negative cell selection methods would allow for a wider spectrum of CTCs to be captured, including those that have undergone epithelial-to-mesenchymal transformation and thus might lack typical epithelial markers.

Size-Based Selection

CTCs of interest from solid tumors are often larger in size than normal blood components. This has led to the development of filter-based methods which collect larger cells for use in targeted analysis. One such device utilizes a parylene membrane to isolate possible CTCs from diluted blood. Once the blood has been passed through the filter, the trapped cells are analyzed directly on the membrane via light or electron microscopy, as well as by IHC [16]. These captured cells also are viable for cell culture, which is not possible for cells that have been fixed and stained [17]. Additional devices utilizing the principles of microfluidics have been successfully used to isolate CTCs [18, 19].

CTC chips combine the principles of both antibody capture and filter devices. Patient blood samples are filtered through silicon chips made up of microposts coated with antibodies to EpCAM; the chips are engineered to reduce cellular shear forces and allow a reasonable volume of blood to be analyzed. The captured epithelial cells are analyzed similarly to those captured on a filter and also remain viable for cell culture [20].

Clinical Utility

Blood

Many studies evaluating CTCs have been published. In a variety of tumor types, a correlation between the detection of blood-borne cells and tumor stage has been demonstrated [21–25]. Unfortunately, the data do not support the conclusion that the absence of CTCs indicates absence of metastatic disease with enough precision for clinical application [26]. Ultimately, the presence of CTCs may provide valuable information on the systemic spread of tumor in a manner different from conventional staging approaches. Small but aggressive tumors may shed cells into the circulation, while a more indolent but larger tumor may not; such a situation may appear to correlate poorly with tumor stage but may more accurately reflect tumor aggressiveness. The detection of circulating breast cancer cells is correlated with vascular invasion in the primary tumor. Larger studies using appropriate markers are needed to determine how to integrate these molecular test results for CTCs into cancer staging strategies.

While a relationship between CTCs and tumor stage is evident, studies demonstrating a correlation between circulating cells and cancer recurrence or progression have shown mixed results. A correlation between blood-borne cells and disease-free survival following radical prostatectomy has been reported [27]. Early studies correlated the presence of CTCs in patients with metastatic breast cancer to progression-free survival and overall survival [13]. However, in newly diagnosed breast cancer patients, the presence of CTCs prior to surgery was associated with an increased risk of cancer-associated death, but not a decreased risk of recurrence free survival [28]. Similar relationships have been established in colorectal [29] and prostate [30] cancers. These findings support the use of CTCs as prognostic markers that can help stratify patients into risk categories based on multiple clinical parameters.

CTC characterization may also be used to monitor response to chemotherapy. Prostate cancer patients treated with androgen deprivation therapy demonstrating the continuing presence or re-emergence of CTCs with androgen receptor signaling have poorer outcomes than those patients whose CTCs do not demonstrate androgen receptor signaling [31].

Bone Marrow

In general, better correlation has been observed between patient outcome and molecular or IHC detection of tumor cells in the bone marrow compared to blood [24, 25, 32, 33]. It is possible that circulating cells have the ability to get into the bloodstream but lack the ability to survive at a metastatic site, while cells in the marrow are at an "advanced" stage in the metastatic continuum, having acquired the capacity to survive in the circulation, attach, and grow in a remote environment. This, however, is a speculation until the molecular events underlying the metastatic process are better understood.

While few studies using single markers have shown results that correlate with clinical or pathologic parameters, correlation with survival/outcome was observed using IHC to detect cytokeratin-positive cells in bone marrow samples from breast cancer patients [32]. Similar findings have been reported for prostate [33] and colorectal cancer [34]. In the USA, such studies can be hampered by clinical practices that do not include bone marrow sampling as part of routine staging for many tumors.

Circulating Epithelial Cells in Benign Disease

While detection of CTCs has prognostic value when identified in cancer patients, CTC detection methods may show positivity in patients with benign diseases, particularly in disease processes in which the vascular integrity may be compromised, such as by inflammation, or by surgery. Patients with benign colonic diseases, including diverticulosis, Crohn disease, ulcerative colitis, endometriosis, and benign polyps, showed the presence of circulating epithelial cells identified as "circulating tumor cells" by the CellSearch (Janssen Diagnostics, LLC) and CK19-EPISPOT (Mabtech, Cincinnati, OH) assays in up to 18.9 % of patients. Up to 41 CTCs were detected in these patients, compared to no CTC detection in any of the healthy controls. Both platforms use antibodies to cytokeratin expression to identify circulating epithelial cells; again, these markers are not specific to malignant cells [35]. Further work is needed since patients with cancer may also be affected by benign disease of any organ and be falsely classified into poor prognosis groups based on circulating benign epithelial cells that are falsely identified as CTCs, regardless of the method used.

Circulating Nucleic Acids

Circulating tumor nucleic acids have multiple origins. They can exist as cell-free nucleic acids (cfNA) or be extracted from intact circulating tumor cells (see previous section). The presence of circulating nucleic acids has long been noted from peripheral blood samples [36], and the presence of circulating fetal DNA is routinely used for prenatal diagnosis [37, 38]. Increased levels of cfNA are often seen in patients with cancer [39]. In addition, since the advent of massively parallel sequencing, the molecular characterization of tumors has greatly expanded. Studies suggest that the majority of tumors carry somatic alterations [40, 41] that could be used to identify cfNA originating from tumors. This raises the possibility of "liquid biopsies," where peripheral blood is

analyzed for the presence of tumor-specific mutations. This is particularly significant as cell-free tumor DNA can originate from the patient's primary tumor, metastases, and CTCs that have undergone apoptosis within vessels. The contribution of cfNA from each would then provide information about tumor heterogeneity that would be unable to be obtained from a traditional biopsy and at lower risk to the patient [42].

Detection Methods

Compared to CTCs, cfNA are relatively easy to extract. The cellular component of the blood is spun down and the acellular plasma or serum used for extraction of nucleic acids, using either manual or automated methods. However, this apparent ease of isolation may adversely impact the implementation of cfNA analysis. Because of the ubiquity of blood-based specimens, a wide variety of extraction methods are available, each with its own performance characteristics. While general increases in cfNA are observed in patients with cancer [43], no standards are available to determine the efficiency of a specific cfNA extraction process. In addition to varying quantities of cfNA, the length of cfNA varies, from 20 bp-long microRNAs to DNA fragments over 80,000 bp long [44].

Exosomes

A more recent finding of relevance to the detection and isolation of cfNA is the existence of exosomes, which are small membrane-encoated vesicles ranging from 40 to 100 nm in diameter. Exosomes contain mRNA, miRNA, and proteins and are able to influence cell function when taken up by the cells [45, 46]. Exosomes have been shown to be stable in various body fluids and can be separated intact from serum and their contents used for subsequent analysis [47]. Exosomes appear to have important functions in normal immune regulation and also appear to have a role in regulation of immunoreactivity to cancer cells, as well as intercellular communication between cancer cells and stroma [46]. A better understanding of exosomes will be critical to understanding the metastatic process as well as the potential use of exosomes as cancer biomarkers.

Clinical Utility

Three main categories of cfNA are being explored for utility in patients with cancer: DNA, mRNA, and microRNA.

Cell-Free DNA (cfDNA)

If tumor-specific mutations are known, the presence of detectable point mutations in patient plasma over time could be used to monitor relapse and/or progression. The percentage of total cfDNA that is derived from tumor has been seen to track with the amount of disease present [42, 48]. A recent study showed that the percentage of cfDNA with TP53 mutation detectable in ovarian carcinoma patients tracked over time with CA125 levels. The same study also demonstrated that levels of cfDNA with tumor-specific mutations tracked with clinical presentation in a breast cancer patient with relapsed disease [42]. Tumor-derived cfDNA may be present in large amounts, as up to 52 % of cfDNA from a patient with a 13 cm hepatocellular carcinoma originated from the tumor. In this study, patients with smaller tumors had lower fractional concentrations of tumor-derived DNA. When the tumors were resected, the amount of tumor DNA in the circulation decreased [48].

High-sensitivity assays could also be used to look for mutations to help guide therapy, as demonstrated by the ability to detect 56 % of patients positive for *BRAF* V600 mutations using amplification refractory mutation system (ARMS) PCR in cfDNA [49]. Copy number changes and chromosomal rearrangements can be detected in cfDNA by genome sequencing (Fig. 39.2) [50], as noted in case reports of maternal malignancies detected by the presence of multiple aneuploidies on noninvasive prenatal testing using cfDNA [51].

Cell-Free mRNA (cfmRNA)

Because cfmRNA must be transcribed from DNA, it may provide more specific information about the pathways activated in a patient's tumor. Multiple studies have associated cfmRNA detection with clinical outcomes. The presence of cyclin D1 mRNA in the plasma of tamoxifen-treated breast cancer patients has been associated with poorer overall survival and lack of response to tamoxifen [52]. Expression levels of circulating hTERT cfmRNA have been studied in multiple tumor types. Increased levels above those seen in healthy individuals are associated with reduced disease-free and overall survival in patients with gastric cancer [22]. Elevated values have also been associated with shorter recurrence-free survival in patients with prostatic carcinoma [53].

Cell-Free microRNA (cfmiRNA)

MicroRNAs are involved in regulation of gene expression and are frequently dysregulated in cancer [54], and miRNA expression profiles have been established for many tumor types, increasing their potential clinical utility [55]. While most biomarker studies utilize multiple cell-free microRNA (cfmiRNA) targets, such as the combination of miR-21, -210, -155, and 196a in pancreatic adenocarcinoma [56], a recent study found that patients with stage IV breast cancer had higher concentrations of miR-21 [57]. Of note, miR-16, which is often used as a normalizer when evaluating expression levels of cfmiRNA, is present in high quantities in red blood cells. Therefore, the presence of hemolysis may interfere with the interpretation of cfmiRNAs in the plasma [58]. While measurement of cell-free miRNA has
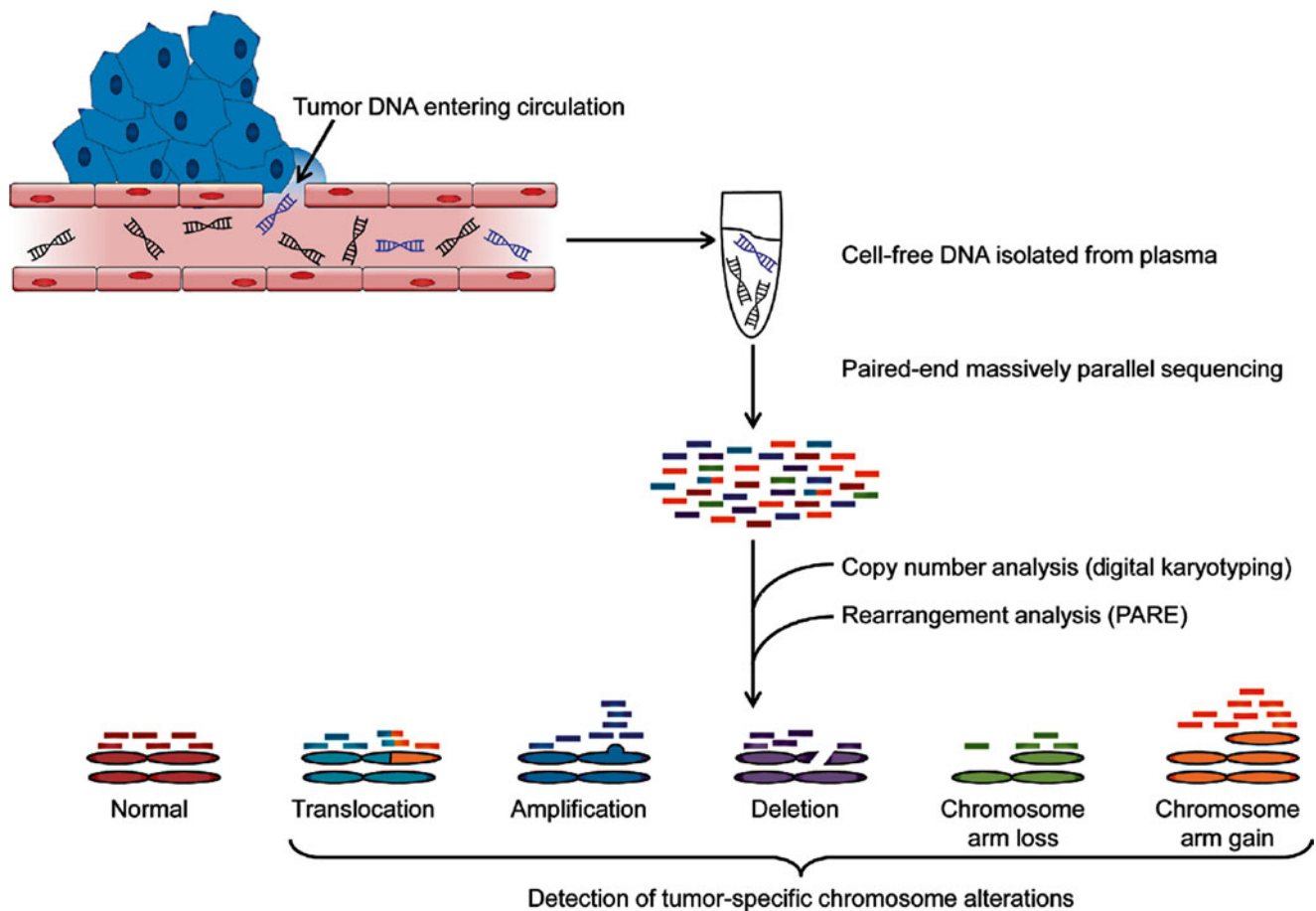
Figure 39.2 Schematic of analyses for direct detection of chromosomal alterations in plasma. The method uses next-generation paired-end sequencing of cell-free DNA isolated from plasma to identify chromosomal alterations characteristic of tumor DNA. Such alterations include copy number changes (gains and losses of chromosome arms) as well as rearrangements resulting from translocations, amplifications, or deletions. From Leary RJ, Sausen M, Kinde I, et al. (2012). Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. *Sci Transl Med.* 4:162ra154. Reprinted with permission from AAAS

great potential, a more thorough understanding of normal and disease-related miRNA variation, as well as more straightforward analytic methods, will be needed.

Conclusions and Future Directions

Since 1991, when the first report of detection of circulating cells in melanoma was published [7], investigation of the clinical relevance of CTCs has been pursued by teams of investigators. To date, the only well-developed clinical parameter is the number of CTCs present in breast, colon, and prostate cancer patients, to be used for risk stratification. However, as the technology used for molecular analysis becomes capable of utilizing ever smaller quantities of input nucleic acids, the information that can be gained

from cfNAs and isolated CTCs will continue to increase. In addition, these alternative tumor detection methods are beginning to be directly compared. For example, a recent study demonstrated a relationship between the amount of cfDNA in the serum of patients and overall survival, with those patients with higher levels of cfDNA demonstrating poorer survival. In addition, both levels of cfDNA and detection of CTCs are tracked with disease progression (Fig. 39.3) [30]. In addition, these more sensitive analysis methods will allow for elucidation of tumor heterogeneity, and it may therefore become possible to combine targeted therapies which block all of the oncogenic pathways utilized by a specific patient's cancer, much like antiretroviral therapies are currently tailored to the group phenotype of all HIV virions present in a patient, rather than one specific subclone.
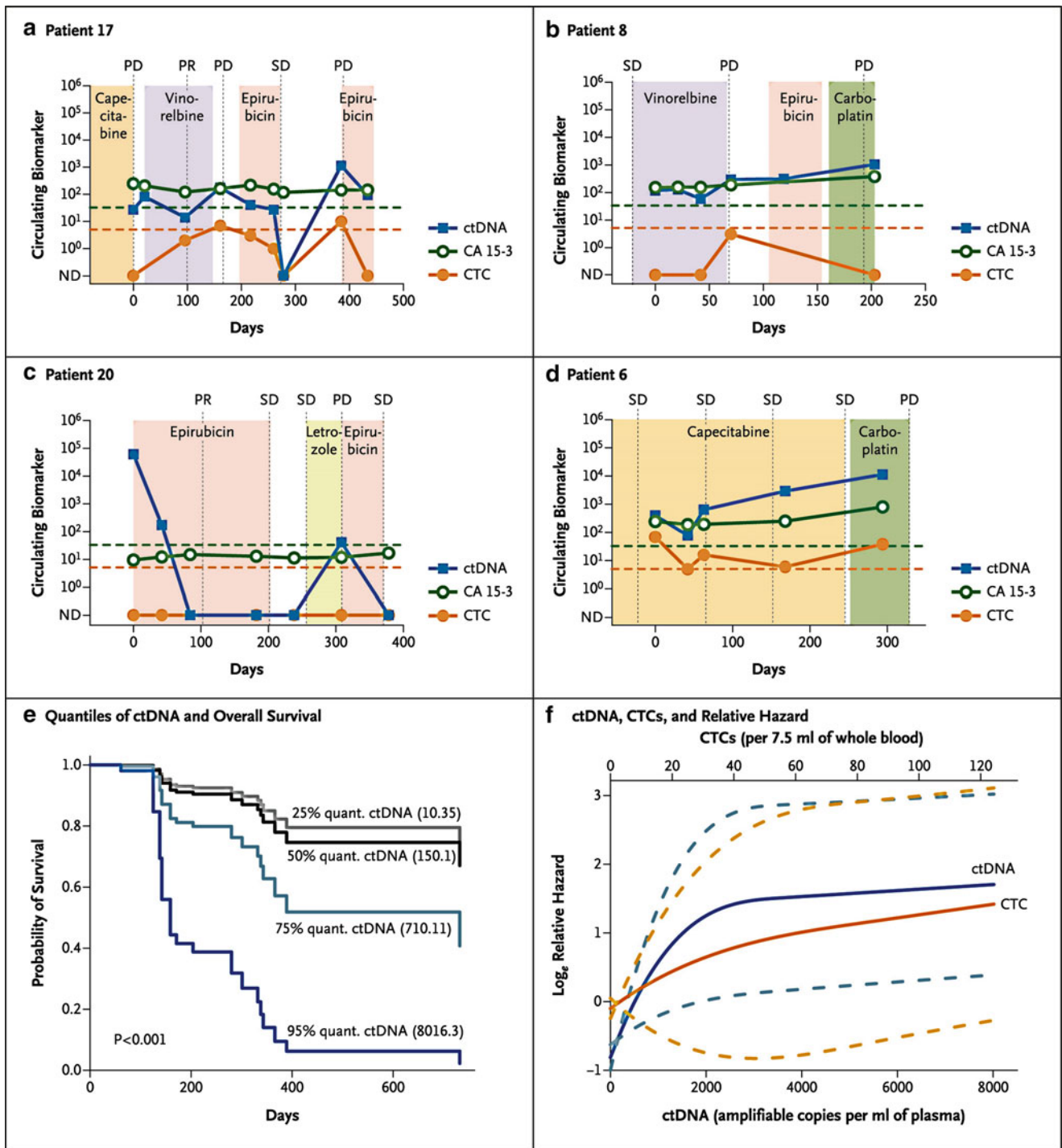
Figure 39.3 Comparison of circulating biomarkers to monitor tumor dynamics and predict survival. Panels **a–d** show serial circulating tumor DNA (ctDNA) levels (number of copies per milliliter of plasma), circulating tumor cell (CTC) numbers (per 7.5 ml of whole blood), CA15-3 levels (U per milliliter), and disease status as ascertained on computed tomography (*vertical dashed lines*) for four patients (one in each panel). Details of endocrine or cytotoxic therapy are indicated by *colored shading*. The *orange dashed line* indicates the threshold of five CTCs per 7.5 ml of whole blood. The *green dashed line* indicates the CA15-3 threshold of 32.4 U per milliliter. *ND* not detected, *PD* progressive disease, *PR* partial response, and *SD* stable disease. Panel **e** shows the results of a Cox regression model, which identified an inverse relationship between quantiles (quant.) of ctDNA (indicated in copies per milliliter of plasma) and overall survival, with increasing levels significantly associated with poor overall survival (*P*<0.001). At 200, 400, and 600 days, a total of 23, 8, and 3 patients were at risk, respectively. Panel **f** shows that increasing ctDNA levels (copies per milliliter), as indicated on the *bottom x* axis, and increasing numbers of CTCs (per 7.5 ml of whole blood), as indicated on the *top x* axis, were associated with an increased log$_e$ relative hazard. The prognostic discrimination power of circulating tumor DNA level was greatest with levels up to 2,000 copies per milliliter. Patients with levels of more than 2,000 copies per milliliter were uniformly found to have the worst prognosis. The prognostic power of CTCs increased according to the number of cells. *Dashed lines* represent 95 % confidence intervals. From New England Journal of Medicine, Dawson SJ et al., Analysis of circulating tumor DNA to monitor metastatic breast cancer, 368:1199–1209. Copyright © (2013) Massachusetts Medical Society. Reprinted with permission from Massachusetts Medical Society

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