Circulating DNA and Liquid Biopsies in the Management of Patients with Cancer

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Forty-five years ago, *Cancer Research* published the study by Leon and colleagues in which the authors described the observation of increased levels of cell-free DNA (cfDNA) in the serum of patients with cancer as compared with healthy individuals, with the highest serum levels in patients with metastatic cancer. Most interestingly was the correlation between serum DNA concentrations and therapy outcome: Increased serum DNA levels were associated with poor response to treatment, whereas decreases in DNA levels during treatment appeared to be a sign of better prognosis. Since the discovery of the prognostic value of blood DNA, much research has been focused on the characterization of cfDNA to understand its origins and increase the sensitivity and specificity of using cfDNA as a prognostic and predictive marker in the battle against cancer. Tumor-specific cfDNA markers that were discovered include genetic alterations, chromosomal aberrations, epigenetic modifications, and DNA fragmentation size. In recent years, due to the development of highly sensitive molecular technologies, cfDNA-based assays are now being introduced into the clinic as the so called "liquid biopsy." The advantages of a liquid biopsy over traditional biopsy and imaging have led to the implementation in the clinic for early cancer detection, improved cancer staging, early detection of relapse, real-time monitoring of therapeutic efficacy, and detection of therapeutic targets and resistance mechanisms. Despite Leon and colleagues' initial skepticism about the potential diagnostic value of serum DNA, cfDNA-based liquid biopsy has become one of the most important tools for personalized cancer treatment.

See related article by Leon and colleagues, Cancer Res 1977;37:646–50

The landmark paper by Leon and colleagues from 1977 (1) described the measurement of serum DNA in 173 patients with 11 different types of cancer before and during radiotherapy, and 55 healthy individuals as the control group. DNA concentrations were measured using a ^{125}I-based radioimmunoassay to reach the sensitivity required for detecting nanogram quantities of DNA. The majority (93%) of the investigated healthy individuals were found to have serum DNA concentrations between 0 to 50 ng/mL, whereas half of the patients with cancer had concentrations above this level before therapy. Patients with cancer with confirmed metastasis had the highest levels of up to 5,000 ng/mL and a mean DNA concentration that was two times higher than patients without evidence of metastatic disease. On average, the patients with cancer showed a 14-times higher DNA concentration as compared with the healthy individuals, which was statistically significant in all tumor types except for "unknown primary" and "miscellaneous." Finally, the authors compared the serum DNA concentrations of the patients with cancer before and during radiotherapy and showed that a temporal decrease correlated to a reduction in tumor size and pain, whereas constant high levels of serum DNA correlated with poor survival. No correlation could be found between the serum DNA concentrations and individuals' characteristics age, sex, and smoking status or tumor characteristics such as size, location, or histologic composition. Because half of the patients with cancer had serum DNA concentrations similar to healthy individuals, in their discussion, the authors questioned whether their findings were of diagnostic value, either for screening high-risk individuals or to predict relapse.

Since the publication of the study highlighted here, DNA measurement techniques have become radioisotope-free and are now able to accurately detect down to single DNA molecules. Furthermore, blood-based DNA assays now rely on using blood plasma rather than serum to keep contamination of leukocyte DNA from lysis during clotting at a minimum and to increase the sensitivity of downstream molecular assays. Despite the advances in DNA detection methods, the average concentrations of cell-free DNA (cfDNA) in the blood of healthy individuals and patients with cancer currently being detected in plasma are similar to what is reported by Leon and colleagues, although different cfDNA isolation methods have been shown to exhibit variable efficiencies in their recovery (2).

In the last 45 years, much has been discovered about cfDNA and its origins that made the development of circulating tumor DNA (ctDNA)–directed liquid biopsy assays possible (2). The most important development in cfDNA-based liquid biopsy was the detection of tumor-specific features such as point mutations, chromosomal aberrations, epigenetic modifications, and DNA fragmentation size (**Fig. 1**). These cancer-specific data have led to the implementation of cfDNA in the clinic to guide personal treatment (3).

The origin of cfDNA can be traced back to different mechanisms in the body (4). In healthy individuals, more than half of the cfDNA originates from normal white blood cells that underwent apoptosis, about a third comes from erythroblasts that lose their nucleus (enucleation) during maturation to red blood cells, and the remaining cfDNA can come from neutrophils trapping microorganisms through extracellular traps of disintegrated chromatin (NETosis), autophagy, mitochondrial cfDNA, and extracellular vesicles (EV) including apoptotic bodies. Due to the rapid degradation of DNA in the blood circulation as well as the degradation processes of apoptotic cells, cfDNA is highly fragmented and has lengths of around 166 or 320 bp. These specific lengths are the result of the digestion enzymes being able to cleave DNA between nucleosomes only, leaving the DNA wrapped around one or two nucleosomes undigested. ctDNA present

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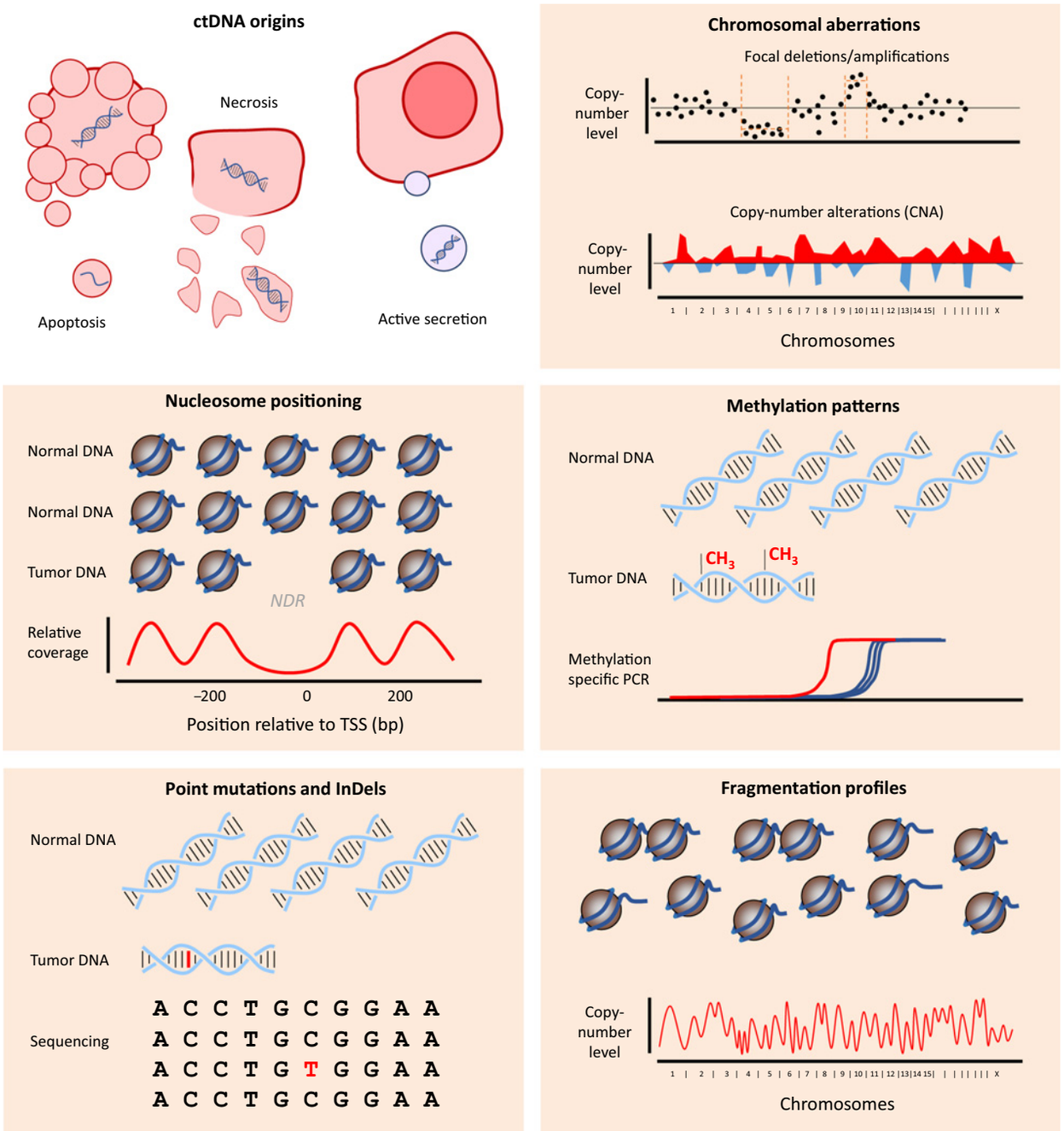

Figure 1.

ctDNA origins and tumor-specific markers. ctDNA originates mainly from tumor cells that underwent apoptosis and necrosis but is also secreted into the blood circulation in EVs (top, left). Tumor-specific markers of cfDNA currently under investigation are: (i) chromosomal aberrations such as focal deletions and amplifications, as well as genome-wide copy-number alterations; (ii) gene expression by nucleosome positioning can be determined by looking for nucleosome-depleted regions (NDR) in front of the transcription start site (TSS); (iii) methylation patterns in gene promoters regions; (iv) point mutations and indels; (v) genome-wide fragmentation profiles.

in nonapoptotic EVs is protected from degradation and has been shown to have average length of 150–250 bp, ranging up to 10 kbp. These microvesicles and exosomes may transfer DNA to other cells, which can be integrated, into the genome of the receiving cells and affect gene expression. Still, most cfDNA found in the blood of patients with cancer usually originate from normal cells. In patients with early-stage disease (e.g., without distant metastases), the ctDNA concentration is usually below 1% of total cfDNA (5). Only in patients with a very high tumor load can the cfDNA originating from the tumor cells exceed the concentration of the normal DNA.

Although the higher concentrations of total cfDNA in patients with cancer compared with healthy individuals have been confirmed in many other studies, only detection of specific tumor–derived DNA could improve the diagnostic, prognostic, and predictive value of ctDNA (**Fig. 1**). On a genetic level, tumor-specific mutations identified in the primary site can be achieved by PCR-based technologies with allele-specific primers. If a larger panel of mutations is required to be screened, targeted next-generation sequencing can be applied. Whole genome approaches target the detection of chromosomal aberrations, mainly copy-number alterations. In addition, determining methylation patterns of gene promoters has been shown to be cancer-associated as well. Due to these recent advances of cfDNA-based assays, detection and thereby diagnosis of early-stage cancer will be possible in the near future. For instance, by determining cfDNA fragmentation profiles, lung cancer can be detected in high-risk individuals with 98% accuracy (6). Furthermore, epigenetic signatures on ctDNA are also a promising analyte under investigation for early detection of all cancer, so far achieving a specificity of 99.3% and sensitivity of 67.3% while accurately predicting the tissue of origin in 96% of cases (7). Not only is cfDNA-based liquid biopsy useful for diagnosis, but patient stratification and resistance mechanism identification is also possible through this assay. Due to the dynamic nature of epigenetics, monitoring of epigenetic modifications during a patient's treatment can uncover tumor heterogeneity and the development of treatment resistance (8). Focal amplifications, deletions, and other copy-number alterations in the genome of tumors can be detected if the ctDNA percentage is >5% of the total cfDNA. Stratification of patients can be made possible based on amplifications of, e.g., *ERBB2* or *AR*. Through coverage analysis of ctDNA, the positioning of nucleosomes can be determined in genomic regions in which the nucleosome positioning is highly defined and thereby identify highly expressed genes.

Since the discovery of cfDNA, five tests have so far been approved by the FDA. These tests include the identification of point mutations in the cancer-related genes such as *KRAS*, *EGFR*, and *PIK3CA*, tumor mutation burden, microsatellite instability, *ALK* rearrangement, insertions and deletions, and methylation patterns (9). The result of these tests can have direct impact on the treatment of the patient. Current clinical applications include early cancer detection, improved cancer staging, early detection of relapse, real-time monitoring of therapeutic efficacy, and detection of therapeutic targets and resistance mechanisms (3), especially in the context of immunotherapy (10). Although we are closer than ever to implementing personalized treatment into clinical practice, it's essential to harmonize and standardize our assays as conducted by international consortia like the EU/IMI Cancer-ID, its successor network European Liquid Biopsy Society (ELBS; www.elbs.eu), and the International Liquid BIOPSY Standardization Alliance. Moreover, interventional clinical studies are required to demonstrate that the ctDNA test result leads to changes in patient management (e.g., individualized imaging controls for relapse or change in therapy) that result in a benefit for the patients (e.g., longer survival). In future studies, combinations of ctDNA with other liquid biopsy analytes detectable in the blood of patients with cancer such as circulating tumor cells, EVs, miRNAs, or proteins may lead to an artificial intelligence–guided composite biomarker test with high accuracy.

Authors' Disclosures

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