A hitchhiker’s guide to cell-free DNA biology

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**Abstract**

Liquid biopsy provides a noninvasive window to the cancer genome and physiology. In particular, cell-free DNA (cfDNA) is a versatile analyte for guiding treatment, monitoring treatment response and resistance, tracking minimal residual disease, and detecting cancer earlier. Despite certain successes, brain cancer diagnosis is amongst those applications that has so far resisted clinical implementation. Recent approaches have highlighted the clinical gain achievable by exploiting cfDNA biological signatures to boost liquid biopsy or unlock new applications. However, the biology of cfDNA is complex, still partially understood, and affected by a range of intrinsic and extrinsic factors. This guide will provide the keys to read, decode, and harness cfDNA biology: the diverse sources of cfDNA in the bloodstream, the mechanism of cfDNA release from cells, the cfDNA structure, topology, and why accounting for cfDNA biology matters for clinical applications of liquid biopsy.

**Key Points**

- Cell-free DNA (cfDNA) can be used as a liquid biopsy in neuro-oncology.
- New applications harnessing the biological patterns of cfDNA improved detection.
- This review will provide the basis to understand and use cfDNA biological signatures.

Liquid biopsy, and cell-free DNA (cfDNA) in particular, have been intensively investigated for diagnostic applications in oncology. Tumor-derived mutations can be detected on cfDNA molecules using molecular biology or sequencing methods. Detecting genetic alterations noninvasively with cfDNA can help clinicians to guide treatment choice, identify resistance to treatments, or monitor treatment efficiency.

But the current extent of clinical applications and performance of liquid biopsy assays are hampered by technical and biological limitations. Mutation assays are predominantly employed due to their ability to inform and guide treatment decision in oncology. However, the number of mutant cfDNA molecules is relatively limited and ultimately finite in a tube of blood. In addition, such a signal can be contaminated by mutations linked to clonal hematopoiesis released from normal cells in the bloodstream. This limited sensitivity and specificity has a direct impact for the most challenging applications in oncology: detecting early-stage cancer, tracking minimal residual disease (MRD), and analyzing brain cancers with liquid biopsy.

Innovative techniques have partly resolved these limitations. Selective sequencing of mutant DNA strands, sequencing of phased variants, and tumor-guided approaches improved the detection of minute numbers of tumor-derived mutations in the bloodstream, allowing improved MRD tracking. Nevertheless, these approaches are conceptually constrained by the number of mutations initially present in cancer cells, rendering them more efficient in cancers with high rates of mutations. The amount of information that they provide on cancer biology, hallmarks, or on the remote immune processes occurring in a patient is limited.

Our knowledge of cfDNA biology is expanding and could be used to unlock new applications for liquid biopsy, or improve the performance of current liquid molecular assays. Here, we will present the current state of knowledge allowing to decode and mine cfDNA biology. Specific attention will be devoted to the neuro-oncology and pathology context.
cfDNA Concentration and Composition in Healthy Individuals and Cancer Patients

cfDNA concentration in plasma can vary depending on the gender, age, physiology, and potential pathologies of an individual. Meta-analysis of previous reports indicates an increase in cfDNA concentration in cancer patients in comparison to healthy individuals. The cfDNA concentration in plasma has been frequently reported using a range of methods (from spectrofluorometer assays to digital PCR systems) further increasing potential bias when comparing results from different studies. This questions the implementation of cfDNA concentration alone as a specific liquid biopsy marker, notably for applications in neuro-oncology.

A fraction of these cfDNA molecules is released by cancer cells as circulating tumor DNA (ctDNA) in plasma (Figure 1). ctDNA exhibit the genetic alterations from their cell of origin and can be used for the molecular characterization of cancers. The ctDNA concentration increases in later stage cancers in comparison to earlier stages, but also depending on the cancer type, with gliomas being the most challenging malignancy to detect using ctDNA. This difference can be caused by a range of factors, from cellular biology, to the impact of cancer hallmarks and compartmentalization (eg, blood–brain barrier separation for gliomas).

Using methylome or fragmentome data, the cell of origin can be deduced from cfDNA analysis, revealing the contribution of the different types of cells to the pool of cfDNA molecules in plasma (Figure 1). These methods highlight the predominantly hematopoietic origin of cfDNA in the blood of healthy individuals. Using genome-wide bisulfite sequencing, Sun et al. observed a median 64.3% of cfDNA coming from neutrophils, 12.6% from T cells, 9.4% from B cells, and 10.7% from the liver in the plasma from healthy individuals. Studies focusing on the recovery of differentially methylated regions from selected CpG sites draw similar conclusions regarding the hematopoietic origin of cfDNA in healthy individuals, even if the relative contribution of each cell type is markedly different. Moss et al. observed a median 55% of cfDNA originating from white blood cells, 30% from erythrocyte progenitors, 10% from vascular endothelial cells, and 1% from the liver. Using a nonmethylation approach, Snyder et al. demonstrated that the nucleosome densities that can be extracted from high-depth genome-wide sequencing data could give clues about the tissue releasing cfDNA in the bloodstream. The position of the cfDNA fragment-ends, as well as their orientation, can also be leveraged to inform about the nucleosome coverage and subsequently the tissue type releasing cfDNA in biofluids.

Taken together, these studies highlighted that the majority of cfDNA that can be isolated from human plasma of healthy individuals is originating from the blood, despite clear differences in the relative contribution of each cell to this pool.

In brain-related pathological conditions, the relative contribution of different tissues to the pool of cfDNA in the bloodstream can be altered. Methylation assay has demonstrated increased concentration of cfDNA released by oligodendrocytes in relapsing multiple sclerosis, and has identified cfDNA derived from brain cells in patients after traumatic or ischemic brain damage. Using a similar approach, Lubotzky et al. recently identified elevated neuron-, oligodendrocyte-, and astrocyte-derived cfDNA in a subpopulation of patients with brain metastases compared with cancer patients without brain metastasis.

Beyond the DNA originating from cell nuclei, additional DNA can be detected in liquid biopsy (Figure 1). DNA released by mitochondria has been described in the plasma and biofluids from healthy individuals, animal models, and cancer patients. Circulating mitochondrial DNA is comprised of short linear DNA (<100 bp) and long circular DNA, and can be released in blood, both by circulating extracellular mitochondria, and by intracellular mitochondria, including from cancer cells. In addition, cfDNA from bacterial and viral sources can also be detected in the bloodstream. These bacterial cfDNA could inform on the
infection status after transplantation, but can also be used for cancer diagnostic purposes, by detecting tumorigenic pathogens.39–42 Nonnuclear DNA only represent a minor fraction (<1%) of the overall pool of cfDNA in the plasma from healthy individuals. Their proportion can be increased in the case of physiological modification or pathology (including cancer), even if the extent of such an increase and their potential as cancer biomarkers remain to be explored.32,41,43,44

**How cfDNA Is Released in Circulation**

cfDNA molecules are marked by their cell and tissue of origin.28 Their biology could also reflect the mechanisms involved in their release into the bloodstream (Figure 2).45 Apoptosis, necrosis, and active secretion have long been suspected as potential contributors to the pool of cfDNA in blood.46 The typical distribution of cfDNA fragment sizes observed in the plasma of healthy individuals is centered at 167 bp and multiples thereof, pointing toward an apoptotic origin of these fragments.47,48 Additional multivariable correlations indicate cfDNA concentration is also associated with typical protein markers of cell death (including cleaved-caspase 3).32,49

Additional mechanisms of cell death, for example necrosis, have been suggested to be involved in cfDNA release (Figure 2). Necrosis is characterized by a random disorganized cleavage of the nuclear DNA. Data from paired-end short-read sequencing technologies did not confirm the presence of long fragments of randomly cleaved cfDNA in healthy individuals or other conditions. More recently, cfDNA size analysis from long-read sequencing technologies seems to confirm that the majority of cfDNA fragments are short, at least in healthy individuals and cancer patients at baseline. Irradiation-induced necrosis is an important contributor of cfDNA release in preclinical models of cancer.32,50 Cellular senescence has recently been demonstrated to delay release of cfDNA, and therefore could bias the interpretation of some liquid biopsy results (Figure 2).50 The impact of other cell-death mechanisms on cfDNA release, as well as alteration from cancer hallmarks remain unknown so far.51,52 Other mechanisms of cell death (eg, pyroptosis, autophagy, ferroptosis, NETosis) can be involved in the release of cfDNA in bloodstream, even if their contribution remain to be determined.51 The relative contribution of each mechanism of cfDNA release in blood (or other biofluids), as well as their impact on cfDNA biology is still unknown.

**How cfDNA Is Structured in Blood**

The high level of fragmentation of cfDNA, in comparison to nuclear DNA, has long been identified in healthy individuals and cancer patients.46,53,54 The irruption of high-throughput paired-end sequencing methods opened a
more refined analysis of the size, structure, and topology of cfDNA in biofluids. They revealed that the majority of cfDNA fragments are distributed around 167 bp (and multiples of this) in healthy individuals. Oscillations every 10 bp can be observed below 167 bp, reflecting a potential cleavage by enzymes, likely exonucleases, in the open strand of DNA. This suggests that cfDNA can be wrapped around the nucleosome in plasma and thus opened and closed chromatin regions could be differently represented by cfDNA (Figure 3).

In cancer, and other conditions, there is an overall shortening of cfDNA fragments in the bloodstream. This shortening can be exploited via size-selective approaches: selecting specific size ranges can lead to a relative enrichment in tumor signal via in vitro or in silico approaches (Table 1). Beyond this canonical description, recent reports using alternative sequencing technologies have highlighted the presence of previously hidden cfDNA fragments. The use of single-stranded library preparations has uncovered more single stranded or heavily damaged DNA in plasma. By combining high-affinity magnetic beads with single-stranded sequencing, Hudecova et al. highlighted the presence of ultrashort organized fragments around 50 bp. These ultrashort cfDNA fragments map to accessible chromatin regions of blood cells, particularly in promoter regions, with the potential to adopt G-quadruplex DNA secondary structures (Table 1). Conversely, using long-read sequencing, long fetal cfDNA fragments can be detected in the plasma of pregnant woman. Capturing long cfDNA could improve the analysis of extrachromosomal circular DNA in plasma.

cfDNA is enzymatically cleaved during release into the bloodstream and the end of cfDNA molecules retain signatures from these enzymes (Figure 3). A range of nucleases can be involved in both cfDNA generation (DFFB and DNASE1L3) and clearance (DNASE1L3 and DNASE1). These nucleases, and end position of the cfDNA fragment also mark the fragment-ends with variable jaggedness. Using a metric to quantify the

Figure 3. cfDNA fragmentomic signatures in biofluids. The signatures observed can be altered by exogenic factors (pre-analytical and analytical conditions, sequencing method used, computational pipelines). cfDNA, cell-free DNA.
diversity of 4-mer end motifs, Jiang et al. observed an increase in the diversity of cfDNA fragment-ends in patients with hepatocellular carcinoma. The biological properties of cfDNA in the neuro-oncological context have been barely explored but seem to mimic those observed in other malignancies. Finally, beyond cfDNA, DNA can be observed linked or within extracellular vesicles released by cells, with conflicting reports regarding their tumor fraction. The boundary between cfDNA directly released by cells and cfDNA subsequently released by extracellular vesicles in the circulation is unclear (as multiple cell-death mechanisms involve formation of extracellular vesicles like apoptotic bodies). The interplay of apoptotic nucleases...
with other potential sources of circulating cfDNA, such as necrosis, NETosis, and secretion of extracellular vesicles, represents an area for future exploration.72

**cfDNA Biology and Liquid Biopsy Applications**

The biological properties of cfDNA (fragmentation, half-life) have often been mentioned as an intrinsic limitation toward greater clinical implementation of cfDNA. However, approaches focusing on the sole technological augmentation have not unlocked the most challenging liquid biopsy applications yet, notably for gliomas.73 cfDNA biology could be exploited to boost the potential of existing liquid biopsy or to unlock novel methods (Table 1).

First, by decoding the hidden patterns of cancer in plasma, we could increase potential targets to improve the classification of cancer using machine learning (on a much larger scale than by using genomic features alone). Here, different tactics are possible: combining multiple analytes from the same sample or multiple signals from the same data. The combination of multiple analytes (eg, cfDNA mutations and proteins) have reported high sensitivity for detecting cancer in early-stage cancer.74 With adapted computational approaches, such multianalyte integration is performing well for challenging scenarios in oncology.75 They are however marked by an increase in logistical burden and cost, linked to the multiplication of tests. The combination of multiple signals from a single analysis could by-pass these limitations. In a pan-cancer cohort, Mouliere et al. demonstrated using low-coverage whole genome sequencing (WGS) that, by combining specific cfDNA fragment sizes with the detected genetic alterations and using machine learning, we can classify cancer cases from healthy controls with a high performance.76 This approach applied to the plasma samples of patients with gliomas accurately detected 65% of cases (area under the curve = 0.914) (Table 1). Other machine learning models combining features from regional fragmentation, local fragmentation, fragment-end positions, and diversity, also exhibit high classification performance using plasma cfDNA on other malignancies, including early-stage cancer.63,70,77 Recently, another fragmentomic-based method using urine cfDNA, exhibited high performance in classifying gliomas from healthy individuals or patients with other brain pathologies.17 Despite their potential, fragmentomic approaches (alongside with other methylation tests) do not directly and specifically capture tumor-derived ctDNA molecules, and thus will require an in-depth characterization of potential preanalytical and physiological bias.78

Beyond the detection of cancer, fragmentomic methods have the potential to awaken new clinical applications for liquid biopsy. By harnessing a large number of cfDNA features, supervised classification of cancer types is possible allowing multicancer detection. Using sequencing coverage near transcription factor-binding sites and local cfDNA fragmentation, Cristiano et al. showed a potential to distinguish different cancer types in a supervised manner with machine learning at 61% accuracy.74 cfDNA fragmentomic features are correlated with epigenetic background patterns which are known to be cell-type specific, inferring cfDNA tissue (and cell) of origin. By analyzing nucleosome protection patterns of cfDNA with high-depth WGS, Snyder et al. deconvoluted the tissue of origin of cfDNA in plasma samples.26 These works confirmed a predominant contribution of hematopoietic cells to the pool of cfDNA molecules in the plasma of healthy individuals, as observed with methylation analysis.24,38 Using information from nucleosome depleted regions, the tumor burden in plasma could be estimated.79 Mining gene regulation via fragmentomic approaches has also recently showed promise for detection and localization of various malignancies, either via genome-wide or targeted sequencing.35,80

Therefore, using the biological properties of cfDNA the sensitivity and specificity of existing sequencing methods can be improved.57,81 Mutation analysis using cfDNA is hampered by variants released by normal cells in the circulation (clonal hematopoiesis of indeterminate potential, CHIP). Normal cells are not affected by the same set of nucleases and biological hallmarks as cancer cells. Thus, their size distribution of mutant cfDNA from CHIP is also different from mutant cfDNA of cancerous origin.82 Sequencing methods leveraging hundreds of mutations (eg, tumor-guided or personalized sequencing) are recovering enough mutant-derived cfDNA to filter out or weight variants depending on their size, leading to an improvement in sensitivity for applications related to MRD tracking.16,83,84 An application to a small cohort of glioblastoma detected consistently tumor-derived cfDNA in plasma and urine samples,17 when standardized panels or PCR methods exhibited limited sensitivity.85

**Concluding Remarks**

cfDNA is commonly used as a liquid biopsy for a broad and increasing range of clinical applications in oncology, and we can forecast a large-scale clinical implementation in the coming decade. The integration of features from cfDNA biology into liquid biopsy assay has driven a renewed traction toward a deeper understanding of cfDNA biology. New methods accounting for elements of cfDNA biology, for example its fragmentation, have exhibited increased performance for detecting minute amounts of tumor signal in plasma. cfDNA biology could be a key source of innovation for liquid biopsy by unlocking new applications for the clinician’s armamentarium.

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