Chapter 3
Cell-Free DNA Testing

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Key Points

- Cell-free DNA (cfDNA) is currently in clinical use for noninvasive prenatal testing, for monitoring of solid organ transplantation, and as a predictive biomarker for treatment selection in non-small cell lung cancer.
- Pre-analytical assay optimization strategies are driven by the unique biology of cfDNA and include specimen type, collection method, storage, and extraction techniques.
- Assays in use for analysis of cfDNA are broadly based either on polymerase chain reaction or next-generation sequencing technologies that may be employed based on clinical requirements for sensitivity versus breadth of target detection.
- Rapid evolution in chemistry, informatics, and sequencing technology will drive further expansion of clinical applications of cfDNA testing to include disease monitoring and screening.

Key Online Resources

- AMP Liquid Biopsy Webinar Series: https://educate.amp.org/store/seminar/seminar.php?seminar=128701
- Liquid biopsies come of age: towards implementation of circulating tumour DNA. Wan, et al.: https://www.ncbi.nlm.nih.gov/pubmed/28233803

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Introduction

Cell-free DNA (cfDNA) is genetic material that is within the body but found outside of viable cells. Cell-free DNA is released from tumor cells by a variety of mechanisms [1]. The majority of cell-free genetic material is thought to derive from nuclear breakdown in the setting of apoptosis or necrosis. DNA can also be secreted from cells in the form of exosomes. When cfDNA diffuses from the site of origin, it can become a solute in proximate body fluids, such as sputum, cerebrospinal fluid, urine, or stool. Cell-free nucleic acids can also enter the bloodstream, where they become circulating cfDNA. Once in a compartment amenable to collection, purification, and evaluation, cfDNA can be quantified and characterized as a measure of health and disease.

Circulating nucleic acids were first discovered in the bloodstream in 1948 [1]. It was subsequently noted that the quantity of cfDNA varies from one healthy individual to another. Furthermore, it was found that cfDNA changes are also observable in the setting of numerous physiologic or pathologic conditions, including trauma, myocardial infarction, stroke, transplantation, pregnancy, and cancer (Table 3.1).

In traumatic or ischemic events, the raw quantity of cfDNA may correlate with the extent of injury [2]. Posttransplant quantification of donor-derived cfDNA correlates with acute and chronic rejection of transplanted organs [3–6]. In pregnancy, the FDA has approved cfDNA as a biomarker in noninvasive prenatal testing, wherein relative quantification of cfDNA mapped to their origin in the reference genome identifies chromosomal aneuploidy, as in Down syndrome [7].

Cancer is one of the most complex, yet potentially most clinically impactful, applications of cfDNA testing. All malignancies are caused by genomic alterations that dysregulate cell biology. These genetic changes include single-nucleotide

Table 3.1 Selected clinical applications of cfDNA testing

Application	Aim	Technology	References
Trauma	Quantification of total cfDNA	Quantitative PCR	[2]
Noninvasive prenatal testing	Detection of fetal aneuploidy in the maternal circulation	Random whole-genome sequencing Targeted SNP profiling	[7]
Solid organ transplantation	Evaluation of graft rejection	Y-chromosome gene PCR (sex-mismatched transplants) HLA-mismatched PCR SNP genotyping	[3] [4] [5] [6]
Solid tumor biomarker testing	Predictive biomarker testing Diagnostics Monitoring Screening	Allele-specific quantitative PCR Digital PCR BEAMing Targeted hybrid capture panel NGS Targeted amplicon NGS Whole-exome sequencing Ultralow-pass whole-genome sequencing	[17]

mutations, small insertion/deletion mutations, larger copy number changes, structural rearrangements, and epigenetic alterations, all of which are specific to neoplastic cells and drive the aberrant growth of the tumor. The ubiquity of these genetic changes across all cancer types facilitates their use as biomarkers specific to the oncogenic forces at work within an individual patient's tumor.

Current Applications: Noninvasive Prenatal Testing

Noninvasive prenatal testing (NIPT) represents the first widespread clinical application of cfDNA. NIPT exploits the fact that cfDNA, which is generally representative of the fetal genome, is released from placental trophoblasts into the maternal circulation. NIPT has largely replaced maternal serum biochemical screening and fetal ultrasound for detection of fetal aneuploidies. The high specificity and positive predictive value of cfDNA testing for detection of fetal aneuploidy have enabled a substantial drop in the rates of false positive results as compared to multiple-marker screening. As a consequence, the routine use of NIPT in high-risk maternal populations in the last decade is estimated to have halved the frequency of invasive confirmatory testing including amniocentesis and chorionic villous sampling [7].

The most common NIPT assays examine fetal sex and trisomies 13, 18, and 21. Dominant methods for quantifying fetal chromosomes include random whole-genome and targeted sequencing. In random whole-genome sequencing, representative random cfDNA fragments derived from fetal and maternal genomes are sequenced, mapped, and counted; proportionally higher numbers of sequencing reads from one chromosome will point to a fetal trisomy [8]. In targeted sequencing, characterized SNPs are amplified and sequenced; skewing of SNP allelic ratios may indicate the presence of aneuploidy [9].

NIPT is feasible starting at week 10 of gestation; before this, the levels of fetal DNA are typically too low to generate informative results. After 10 weeks, low fetal fraction may occur in a variety of scenarios, including high maternal body mass index, pregnancies resulting from assisted reproductive technologies, or as a result of some aneuploidies. Low fetal fraction may lead to false negative results; therefore, a testing step that evaluates the fraction of placental versus total cfDNA is recommended [10]. False positive results may occur in the context of confined placental mosaicism, particularly for chromosomes 18 and 21. Other reasons for false positivity include autosomal trisomies, vanishing twin syndrome, or maternal factors, such as copy number variants, prior transplant, or subclinical neoplasm.

Although assessment of fetal aneuploidies is the dominant indication for NIPT, this technology has been extended to assess micro-deletions and micro-duplications as well as noninvasive detection of fetal single-gene disorders. The use of NIPT has been reported for the prenatal detection and management of a variety of inherited disorders including blood group incompatibility, skeletal dysplasias, congenital adrenal hyperplasia, and hemoglobinopathies [7].

Current Applications: Solid Organ Transplantation

Periodic rejection surveillance of transplanted solid organs is used to titrate immunosuppressive regimens in order to prevent or slow graft failure. Traditional surveillance approaches employ invasive biopsy sampling of the engrafted organ and histopathologic evaluation; this process is prone to sampling bias and pathologist variability, as well as morbidity related to invasive biopsies. Donor-derived cfDNA is detectable in transplant recipients and has been exploited as a biomarker of graft rejection in a variety of organ contexts. Donor-derived cfDNA increases as a fraction of total cfDNA in the setting of acute rejection following heart, liver, kidney, and lung transplantation, and levels correlate with biopsy-proven rejection. The fraction of donor-derived cfDNA can be determined using Y-chromosome measurements in sex-mismatched transplants, SNP genotyping, human leukocyte antigen mismatch, or comparison of copy number polymorphisms. Many approaches rely on a priori knowledge of donor and recipient genotypes; however, SNP-panel NGS tests that exploit bioinformatics tools to assign recipient and donor status have also been validated for detection of rejection in cardiac and kidney allograft patients [6, 11].

The positive predictive value of elevated donor-derived cfNDA for rejection is relatively low, as cfDNA can be released due to a variety of pathologies affecting the graft, such as infection or inflammatory conditions [5]. Therefore, current assays are unlikely to replace tissue biopsy as a gold standard for rejection evaluation, but may have value as a noninvasive monitoring tool to inform the timing and/or interpretation of posttransplant biopsies.

Current Applications: Cancer

In the setting of malignancy, the subset of cfDNA in the systemic circulation that is derived from tumor cells is called circulating tumor DNA (ctDNA). Qualitative or quantitative assays designed to query ctDNA is one type of “liquid biopsy,” a concept that has long been considered a “holy grail” of cancer testing, because a fully optimized, targeted test could minimize the risk and invasiveness of diagnostic procedures for some cancer types.

Assays targeting ctDNA have numerous potential advantages. Many studies have demonstrated significant genetic heterogeneity both within a primary tumor, as well as between the primary tumor and metastatic site(s). For example, in lung cancers, mutations associated with treatment resistance have been identified in some tumor cell populations, but not in others. Stochastic and sampling factors inherent to tissue biopsy can inhibit full characterization of system-wide disease. This missing information can adversely affect clinical management. In contrast, a liquid biopsy represents multiple tumor sites in proportion to the amount of ctDNA produced by each focus, potentially yielding a more complete account of the patient’s cancer genome.

The absolute and relative quantity of ctDNA is highly variable and depends on the tissue of origin, disease burden, exposure to therapy, extent of treatment

response, and other aspects of tumor biology [12]. Plasma cfDNA originates from increased cell death, via both apoptosis and necrosis, of tumor tissue, and circulating tumor DNA (ctDNA) typically represents a minor fraction of overall cfDNA. Nevertheless, there is a reproducible correlation between cancer burden and levels of cfDNA [13, 14]. In non-small cell lung cancer (NSCLC), patients with disease confined to the lungs tend to have very low levels of ctDNA – often undetectable using standard clinical techniques, such as droplet digital PCR or targeted NGS – whereas those with metastatic disease have detectable ctDNA in 60–100% of cases [14]. The prevalence of ctDNA in patients with metastatic cancer enables the use of cfDNA for detection of cancer-specific molecular biomarkers, such as oncogene single-nucleotide or indel variants, including a subset that can be used for treatment selection and monitoring, via targeted PCR-based assays. Detection of a broader array of variants, as well as amplifications and rearrangements, can be accomplished by use of NGS-based technologies [13, 15, 16].

Ultrasensitive assays, such as allele-specific quantitative PCR or bespoke amplicon sequencing, can be used to detect ctDNA even in those patients with early stage/organ-confined disease [17]. Pathologic features that predict the presence of detectable ctDNA in surgically resectable NSCLC include squamous morphology, high tumor cell proliferation rate, and lymphovascular invasion [18]. These exceptionally sensitive assays could be used for detection of tumor-specific cfDNA alterations following surgery or other definitive therapy, enabling longitudinal monitoring for disease recurrence prior to development of radiographic or clinical evidence of relapse [18, 19].

As of 2018, only one cfDNA assay for predictive molecular biomarkers was approved in the United States by the Food and Drug Administration; the approved assay assesses *EGFR* hot spot activating mutations (exon 19 deletion mutation and L858R) as well as the resistance mutation T790M in patients with advanced NSCLC. As such, it may be used in patients with an established NSCLC diagnosis to guide choice of therapy when tissue is insufficient or unavailable for molecular profiling or in the setting of relapse to detect the T790M variant [20]. In either setting, a negative result should prompt follow-up tissue testing prior to therapy decisions, because of the lower sensitivity of cfDNA-based profiling relative to tumor tissue [21]. While ctDNA profiling has gained the most traction in the context of biomarker assessment in NSCLC, applications have been described in numerous other tumor types (Table 3.2).

Pre-analytical Considerations

Specimen collection and handling are critical for cfDNA assays. Factors that accelerate cfDNA degradation or dilute cfDNA concentration can decrease assay sensitivity. Pre-analytical variables including cfDNA biology, specimen type, collection and processing protocols, and DNA purification strategies can all affect the quality and quantity of starting material [22, 23].

Table 3.2 Applications for cfDNA testing in solid tumors

Diagnosis	Molecular target(s)	Clinical implications	Reference
Bladder cancer	*ERBB2*, TSC genes, *PIK3CA*, and others	Targeted therapy selection/ clinical trials	[39]
Breast and ovarian cancer	BRCA reversion events	Selection of PARP inhibitors	[40]
Gastric cancer	*ERBB2* amplification, others	HER2-targeted therapy, surveillance, identification of mechanisms of relapse	[41]
Hepatocellular carcinoma	Methylation marker panel	Diagnosis, prognosis	[42]
Melanoma	*BRAF* codon V600	BRAF targeted therapy	[43]
Oropharyngeal cancer	HPV	Disease monitoring	[44]
Pancreatic adenocarcinoma	*KRAS* mutation	Prognosis in surgically resected patients	[45]
Prostate cancer (relapse)	*BRCA2* reversion, *AR* mutations	PARP inhibitor resistance, androgen blockade resistance	[46–48]
Sarcoma	*EWSR1* fusions, *TP53* mutations, others	Disease monitoring, prognosis	[49, 50]
Small cell lung carcinoma	*TP53*, *RB1*, *PTEN*, *NOTCH* genes, MYC genes, others	Response assessment, disease monitoring	[51]

cfDNA Biology

cfDNA is rapidly removed from the bloodstream, via both nuclease degradation and renal clearance, with a half-life of approximately 16 minutes to 2 hours [24, 25]. In one study, quantification of ctDNA before and at time points following surgical resection show that the concentration decreases by 96.7% at 24 hours following surgery, with the drop in ctDNA concentration preceding decreases in protein biomarker levels [24]. This turnover and the possibility of early, high-sensitivity interpretation suggest that ctDNA may represent a multi-cancer posttreatment biomarker for minimal residual disease and disease recurrence.

Because cfDNA is derived from apoptotic and necrotic cells and is subsequently exposed to unfavorable extracellular conditions, the fragment length of cfDNA is generally shorter than DNA isolated from tissue or whole blood. DNA harvested from lymphocytes or fresh tissue can extend to the tens or hundreds of kilobases in length. DNA extracted from formalin-fixed, paraffin-embedded tissue degrades with storage time, but tissue archived within months to several years has been shown to yield DNA fragment sizes in the kilobases [26]. While these other sources of genetic material generally conform to a normal distribution of fragment length, cfDNA exhibits a multimodal length distribution with peaks at approximately 150, 300, and 450 bp. This ~150 bp fragment length periodicity reflects the number of bases wrapped around, and thereby protected from degradation, by a single nucleosome or chromatosome [27]. The ~300 bp and ~450 bp peaks represent cfDNA fragments wound around two and three nucleosomes, respectively. In samples from

most cancer types, ~90% of ctCNA falls in the ~150 bp band, with decreasing representation of sequentially larger fragment size peaks [28].

Assays must be consciously designed to account for the short fragment length of cfDNA. In particular, PCR-based assays must utilize small amplicons in order to produce sufficient amplification for detection.

Specimen Type

Whole blood samples contain nucleated white blood cells (WBCs). If these WBCs are lysed prior to cfDNA extraction, released enzymes may accelerate cfDNA degradation. Furthermore, WBC lysis results in the liberation of cellular non-tumor DNA, diluting the ctDNA and reducing assay sensitivity. Quantification of unique Y-chromosome sequences in cfDNA from sex-mismatched bone marrow transplant recipients demonstrates that this process is a major source of cfDNA [29, 30].

Serum and plasma are acellular subsets of whole blood, with serum representing plasma that has been depleted of clotting factors. In practice, plasma is derived via centrifugation, whereas serum isolation is accomplished via in vitro induction of clotting prior to centrifugation. The clotting step intrinsic to serum collection induces greater release of WBC-derived cfDNA compared to centrifugation alone, resulting in a higher total cfDNA concentration in serum than plasma [30]. However, due to the dilution effect, the ctDNA:cfDNA ratio is greater in plasma. Therefore, consensus recommendations favor the use of plasma as the source material for ctDNA testing [21, 31].

Collection and Processing

Collection tube type, time to centrifugation, storage, and DNA isolation approaches can also affect the degradation and relative concentration of ctDNA.

Current ctDNA assay technologies use PCR to amplify, and in some cases analyze, genetic material. Heparin is widely used to prevent coagulation in blood collection tubes, but this drug has been shown to interfere with the biochemistry of the PCR reaction [32], and, accordingly, heparin-containing tubes should not be used to collect material for ctDNA testing. Instead, tubes with alternative anticoagulants, such as EDTA, standard lavender-top tubes, or tubes with leukocyte stabilization compounds are preferred for downstream ctDNA analysis. One study directly compared ctDNA yield for EDTA, Streck (Streck, Inc., La Vista, NE), and CellSave (Menarini Silicon Biosystems, Inc., Huntington Valley, PA) tubes, including with different post-collection handling protocols [33]. The authors found that these tube types had similar ctDNA yields when plasma was isolated within 6 hours of collection. By 48 hours, however, the yield from EDTA tubes was less reliable, whereas the CellSave and Streck tubes did not show a significant decrease in harvested

ctDNA. Thus, plasma should be produced within hours of collection for EDTA tube samples. The use of leukocyte stabilization tubes can extend the plasma isolation window to days; there are now numerous commercially available options, and optimal protocols are highly dependent on the properties of each tube type [34].

Short-term storage at 4 °C versus room temperature has been shown to have negligible effect on ctDNA harvest [33]. However, depending on the tube type, higher temperatures and longer intervals can increase contamination with leukocyte-derived DNA. In general, it is ideal to refrigerate samples as soon as possible until plasma is isolated. Freezing of plasma prior to cfDNA isolation does not affect the quantity or analytical quality of the genetic material. However, as with all types of DNA samples, multiple freeze-thaw cycles cause degradation, so plasma or isolated cfDNA should be stored as aliquots in order to avoid this problem.

The need for additional and/or specialized blood collection tubes, specific sample handling, and rapid DNA isolation requires equipment, education, and, crucially, buy-in from institutions, clinicians, administration, and staff. In the inpatient or cancer center setting, it may be most economical to implement the use of already widely utilized EDTA tubes, followed by DNA isolation within 6 hours. However, stabilizing tubes may be more practical in outpatient settings where staffing and infrastructure for rapid DNA isolation are more challenging.

cfDNA Detection and Quantification Technologies

cfDNA detection and quantification assays fall into two general groups: sequencing based and PCR based. Sequencing-based assays enable discovery of the spectrum of circulating somatic alterations in the genome, exome, or targeted regions. In contrast, PCR-based tests specifically target hot spot or previously identified mutations, but generally have faster turnaround time and higher analytical sensitivity. For either approach, assay validation should be carried out via comparison against the appropriate gold standard based on the specific application (e.g., tumor tissue in cancer, donor tissue for transplant, karyotype for noninvasive prenatal diagnosis, etc.). A summary of technologies and their optimal cancer-related applications are found in Table 3.3.

Limitations

Clinical ctDNA testing has several potential limitations compared to direct tissue testing. The principal limitation is the low concentration of overall cfDNA, and ultimately the low level of ctDNA, present in most patients with solid tumors. On average, cfDNA is reportedly present at a concentration of 20–25 ng/mL (or 3000 genomic equivalents) in the plasma of patients with metastatic carcinoma [35]. For variants present at 0.1% allele fraction (1 in 1000 genomic equivalents) and input amount of 3000 genomic equivalents, there is an approximately 5% chance that this

Table 3.3 ctDNA detection and quantification technologies

Platform	Scope	Underlying technology	Example implementation	Detectable variant type(s)	Mutant allele sensitivity	Number of testable loci	Optimal applications
Sequencing based	Genome-wide	Whole-genome sequencing	Plasma-Seq [52]	Aneuploidy, structural variants	5–10%	Whole genome	ctDNA quantification, disease burden estimation, detection of aneuploidy or structural variants
		Tiled amplicon sequencing	Fast-SeqS [53]	Aneuploidy		Genome-wide targeted LINE-1 elements	
	Targeted panel	Hybrid capture	Exome sequencing [54, 55]	Aneuploidy, targeted structural variants, SNVs/indels	5%	Whole exome	ctDNA quantification, disease burden estimation, cancer gene profiling, identification of actionable mutations, monitoring treatment resistance mutations
			CAPP-Seq [16]	Targeted structural variants, SNVs/indels	0.1–0.5%	Targeted panel	
		Amplicon-based	TAm-Seq [56]	Aneuploidy, targeted structural variants, SNVs/indels	<0.5%	Targeted panel	
			Safe-SeqS [57]			Targeted panel	

(continued)

Table 3.3 (continued)

Platform	Scope	Underlying technology	Example implementation	Detectable variant type(s)	Mutant allele sensitivity	Number of testable loci	Optimal applications
PCR based	Single locus or multiplexed panel	Emulsion PCR	Digital PCR [58]	Targeted structural variants, SNVs/indels	<0.01%	Single locus or multiplexed panel	Disease burden estimation, identification of actionable mutations, quantification of hot spot mutations, monitoring treatment resistance mutations
			BEAMing [59]				
	FDA approved	Companion diagnostics	Therascreen EGFR (approved for cfDNA testing in EU only) [60]	Targeted structural variants, SNVs/indels	25–100 copies/mL	Multiplex panel in EGFR gene	Identification of actionable mutations, quantification of hot spot mutations, monitoring treatment resistance mutations, FDA-approved testing
			Cobas EGFR [20]		Variable, median 1.4%		

Table 3.4 Factors contributing to false positive or nonrepresentative results in ctDNA testing

Factor	Potential limitation (compared to tissue testing)	Mitigation strategies
Germ line variants	Mosaicism or some inherited variants could be interpreted as somatic mutations	Parallel testing of germ line DNA (at least once for each patient)
Multiple primary cancers	Difficult to determine tissue of origin of a specific somatic mutation	Parallel testing of tissue biopsy (at least once for each primary tumor)
Multifocal disease	Differential contribution to ctDNA of different tumor sites, obscuring diversity	Serial testing, selection of appropriately sensitive method
Contamination	Low-level contamination can mimic somatic mutations	Bioinformatics evaluation for evidence of contamination
PCR artifacts	Requires increase in lower limit of detection and decreased sensitivity	Use of unique molecular identifiers to identify strand-specific artifacts
Other clonal proliferations	Presence of mutant cfDNA arising from a source other than tumor cells of interest (e.g., *TP53* mutation in clonal hematopoiesis of indeterminate potential)	Parallel testing of buffy coat Future consideration: healthy baseline testing

variant will go undetected in 1 mL of tested plasma. Obviously, lower cfDNA concentrations elevate the possibility of false negative results, purely by chance. This limitation can be at least partially mitigated by testing for multiple variants, where feasible [1].

Additional challenges in the interpretation of detected variants derive from the fact that it is not possible to ascribe an origin to a specific fragment of cfDNA, and it is difficult to ascertain, a priori, the relative contribution of ctDNA to the overall cfDNA population. There is evidence, however, that the distribution of cfDNA fragment lengths shifts to the left in samples with high levels of ctDNA, with a peak length of less than 145 base pairs in samples with detectable tumor mutations [36]. These differential fragment length distributions may enable better assessment of the levels of ctDNA in a sample.

Factors that may contribute to false positive results or lead to incorrect attribution of a variant to patient's known cancer are summarized in Table 3.4.

Future Directions

Though few cfDNA tests currently have regulatory approval for general use, numerous novel analysis approaches are currently under development for clinical applications, such as generalized and tissue of origin-specific gene panel sequencing and bespoke, personalized testing. Advances in technology employing cost-effective, ultrasensitive detection of DNA variants are likely to shift clinical practice models

to enable monitoring of patients with early stage disease; permit use of alternative body fluids, such as saliva, as a source of cfDNA; and ultimately allow for early cancer detection in high-risk populations [37, 38]. Application-oriented validation standards and reference materials, clinician buy-in, and comparative clinical studies will be required for these technologies to achieve wide use in a variety of practice settings.

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