CELL-FREE DNA (cfDNA) BASED MARKERS FOR CANCER THERAPY

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Abstract:

This chapter discusses the challenges of treating cancer, particularly the variability in responses to radiotherapy and the limitations of traditional diagnostic methods. It introduces the concept of liquid biopsies using cell-free DNA (cfDNA) as a non-invasive alternative for cancer diagnosis and monitoring. cfDNA, found in the bloodstream, can carry tumor-derived genetic material, allowing the detection of cancer-associated mutations. This method offers insights into the genetic landscape of tumors and their heterogeneity, facilitating personalized medicine. Various cfDNA-based markers and techniques are explored, such as targeted sequencing, DNA copy number analysis, DNA methylation patterns, and the detection of Microsatellite Instability (MSI). The chapter highlights the potential of cfDNA in predicting treatment responses, monitoring progress, and tailoring cancer therapy regimens.

1. Introduction:

Cancer remains one of the most challenging diseases to treat, with radiotherapy being an important component of the therapeutic arsenal. However, the effectiveness of radiotherapy can vary widely among cancer patients, and the ability to predict individual responses is limited. Cancer is a complex and heterogeneous disease characterized by uncontrolled cell growth. It can originate in any tissue of the body and has a potential to spread to another part of the body (metastasis), posing a significant challenge to early diagnosis and treatment. Traditional methods for cancer diagnosis and monitoring, such as tissue biopsies and imaging techniques, have limitations in terms of invasiveness, cost, and the ability to detect cancer at an early stage. In recent years, there has been growing interest in the use of liquid biopsies

wherein cell-free DNA (cfDNA) acts as a promising biomarker to enhance the precision of diagnosis and efficacy of cancer therapy[9,29]. cfDNA-based analysis offers a non-invasive alternative to traditional tissue biopsies.

Circulating cell-free DNA is found in the bloodstream and other body fluids (**Figure 1A**) that is released from both healthy and disease cells (**Figure 1B**). The information it carries can provide insights into the genetic landscape of the individual. In cancer patients, a small fraction of cfDNA can contain tumor-derived genetic material, making it a valuable resource for cancer diagnostics. Mutations in cancer-associated genes can be detected in cfDNA, allowing for monitoring of treatment response or disease progression. cfDNA analysis can also capture the genetic heterogeneity of tumors, which is critical for personalized medicine[12]. By analyzing multiple tumor-derived mutations, clinicians can tailor treatment strategies to individual patients. Liquid biopsies using cfDNA are less invasive and can be performed repeatedly, providing real-time information about the tumor genetic profile.

Several cfDNA-based markers (**Figure 1C**) and techniques have been developed for cancer detection, diagnosis, and monitoring. Targeted sequencing of specific cancer-associated genes in cfDNA allows for the identification of driver mutations of either germline or somatic in nature. The various types of driver and passenger mutations related to cancer are reviewed by Pon and Marra (2015)[48]. These include mutations in BRCA1, BRCA2, APC, EGFR, BRAF, KRAS, etc., which can provide crucial clues for targeted therapies.

Changes in DNA copy number, indicative of genomic instability in cancer, can be assessed using cfDNA. High-resolution techniques like digital PCR or next-generation sequencing enable the detection of Copy Number Variations (CNVs). Aberrant DNA methylation patterns in cfDNA have been linked to cancer. Methylation markers can differentiate between normal and cancerous tissues, aiding in early detection. Microsatellite Instability (MSI), a hallmark of certain cancers, can be detected in cfDNA. MSI status helps guide treatment decisions, particularly in colorectal cancer. Serial monitoring of cfDNA can track changes in tumor burden and genetic alterations, allowing for the early assessment of treatment response or disease recurrence. This chapter aims to provide an in-depth exploration of the emerging field of cfDNA-based markers for cancer therapy. We will discuss the potential applications of cfDNA in predicting treatment responses, monitoring treatment progress, and personalizing cancer therapy regimens for patients.

2. Cell-Free DNA: An Overview:

2.1 Sources of cfDNA: cfDNA is a valuable and versatile biomarker, found in the different body fluids, offering insights into various aspects of health and disease. These cfDNA molecules originate from diverse sources within the body (**Figure 1B**), as mentioned below, each contributing to the pool of circulating genetic material. Understanding the different sources of cfDNA is crucial for harnessing its diagnostic potential across various medical disciplines.

1. Apoptosis and Necrosis: A significant proportion of cfDNA results from programmed cell death (apoptosis) and cellular injury (necrosis). This natural turnover of cells in the body releases DNA fragments into the bloodstream, contributing to the overall cfDNA pool[26].
2. Tumor Cells: Cancer patients release circulating tumor DNA (ctDNA) into their bloodstream as tumor cells proliferate and die. ctDNA carries genetic alterations specific to the cancer, serving as a promising source for non-invasive cancer detection and monitoring[7].
3. Foetal DNA:During pregnancy, a fraction of cfDNA in maternal blood originates from the developing foetus cfDNA that is an indicative of placental health and is instrumental in detecting conditions like preeclampsia[58]. This foetal cfDNA provides an invaluable source for non-invasive prenatal testing (NIPT), aiding in the screening of genetic abnormalities[3,8].
4. Immune Cells: Immune cells, particularly white blood cells (leukocytes), release cfDNA as part of the body's immune response to infections, inflammation, or tissue injury. Immune cell-derived cfDNA can reflect the body's ongoing health challenges[33].
5. Organ-Specific cfDNA: Diseases or injuries affecting specific organs can release cfDNA fragments indicative of the organ's condition. For example, liver damage can lead to the release of liver-specific cfDNA, which may be associated with liver disease or injury[10].
6. Microbiome-Derived cfDNA: The human microbiome, comprised of various microorganisms inhabiting the body, contributes to the cfDNA pool. Analyzing microbiome-derived cfDNA may provide insights into the microbiome's composition and its potential role in health and disease[46].
7. Exercise and Physical Activity: Intense physical activities, such as endurance exercise, can lead to increased cfDNA levels in the bloodstream, possibly due to muscle cell damage and the subsequent release of cellular contents[19].

Origin and release of cfDNA in cancer patients:

cfDNA in cancer patients primarily originates from tumor cells and is released into the bloodstream through various mechanisms involving either passive release or active secretion. Active secretion involves direct release of DNA from cells via cellular mechanisms like exocytosis[64], whereas apoptosis and necrosis[26] are the mechanisms known for passive release of DNA in the extracellular environment. For example,[64] demonstrated that the cfDNA release in case of breast cancer cell lines occurs primarily via active secretion. Understanding the origin of cfDNA and its release mechanisms in cancer is essential for its clinical applications in cancer diagnosis, monitoring, and treatment response assessment. Following are few cfDNA release related observations.

1. Tumor Cells: One of the primary sources of cfDNA in cancer patients is tumor cells. Tumor cells release cfDNA into the bloodstream as they undergo cell death, including apoptosis and necrosis. This cfDNA carries genetic alterations specific to the tumor, such as point mutations, copy number variations, and epigenetic changes[7].
2. Apoptosis: Apoptosis is a programmed cell death mechanism. Tumor cells can undergo apoptosis, releasing fragmented DNA into the circulation. These apoptotic bodies can contribute to the pool of cfDNA and contain information about the genetic profile of the tumor[56].
3. Necrosis: In cases of rapid tumor growth, nutrient deprivation, or physical disruption, tumor cells may undergo necrosis, a form of uncontrolled cell death. Necrotic cells release intact DNA fragments, including longer cfDNA fragments, into the bloodstream[56].
4. Active Secretion: Recent research has suggested that some tumor cells actively secrete cfDNA into the bloodstream as a means of communication with the surrounding microenvironment. This released cfDNA may play a role in tumor progression and immune evasion[56].
5. Tumor Vasculature and Angiogenesis: Tumor angiogenesis, the formation of new blood vessels to supply the tumor with nutrients and oxygen, can result in the shedding of cfDNA into the bloodstream through the leaky tumor vasculature[4].
6. Infiltrating Immune Cells: Immune cells that infiltrate the tumor microenvironment can release cfDNA as they interact with tumor cells. This immune cells-related cfDNA can carry information about the immune response to the tumor[18].

2.2 Characteristics of cfDNA:

Size distribution, fragmentation patterns, half-life and stability of cfDNA in bodily fluids

The assessment of cfDNA's size profile is a fundamental biological characteristic, leveraged for the development of non-invasive screening and diagnostic assays[38].The mechanism of cfDNA release in the bodily fluids governs the cfDNA size profile. The distinctive size pattern of DNA fragments originating from cancer cells diverges from the cfDNA fragments released by non-cancerous cells, shedding light on their tissue of origin and conveying significant insights into genetic profiles of tumors. For instance, Cristiano et al. (2019) developed an approach (DELFI) that can evaluate fragmentation patterns of cell-free DNA across the genome and identify the tissue of origin of cancers using fragmentation profiles of cfDNA[13].

Another critical clinical aspect of cfDNA analysis is its stability and clearance in bodily fluids. Several studies have shed light on this topic, emphasizing the robust and dynamic nature of cfDNA. For instance, a study by Norton et al. (2013) demonstrated that cfDNA in plasma is remarkably stable, with minimal degradation over time with defined storage conditions[43]. The dynamicity of cfDNA might be accounted to very short life (mean 16.3 mins) of cfDNA in the circulation resulting in real-time monitoring of pathological condition[37]. These properties makes cfDNA an ideal candidate for liquid biopsy applications, enabling the detection of genetic and epigenetic alterations in cancer and other diseases.

Nonetheless, factors such as sample handling and processing methods still need careful consideration to ensure the integrity of cfDNA in bodily fluids.

3. Methodologies for cfDNA Analysis:

Circulating free DNA (cfDNA) analysis has become a valuable tool in various fields, including cancer diagnostics, prenatal testing, and monitoring treatment responses. This section provides an overview of the key methodologies used for cfDNA analysis, highlighting their strengths and applications (**Figure 2**).

Isolation of cfDNA :cfDNA is typically isolated from plasma or serum using commercial kits based on methods like silica-based column purification or magnetic beads, phenol-chloroform-based method and filtration-based method. The most commonly used method is the column kits containing silica membranes. New isolation methods like, centrifugation-free integrated microfluidic chip for cfDNA isolation from plasma samples are under active development[34].

Quantification and Size Profiling: Quantitative Polymerase Chain Reaction (qPCR) and DNA binding sensitive Fluorescent-dye based assays are commonly used to quantify cfDNA concentration in samples[27]. qPCR can also assess the integrity and fragmentation of cfDNA.Techniques like agarose gel electrophoresis or capillary electrophoresis can assess the size distribution of cfDNA fragments, providing insights into their origins and potential clinical applications[63].

Mutation Detection and Genetic Profiling: Next-Generation Sequencing (NGS) allows for comprehensive profiling of cfDNA, including the detection of single nucleotide variants (SNVs), copy number alterations (CNAs), and structural variations (SVs). It is particularly useful in oncology for identifying tumor-specific mutations[6]. Digital PCR techniques, such as digital droplet PCR (ddPCR) or BEAMing, offer high sensitivity and precision for detecting rare mutations in cfDNA[23].

Epigenetic Analysis: Bisulfite conversion followed by PCR or NGS allows for the assessment of DNA methylation patterns in cfDNA. This is crucial for studying epigenetic alterations associated with cancer and other diseases[54]. Techniques like chromatin immunoprecipitation followed by sequencing (ChIP-seq) can be adapted for cfDNA to study gene expression of the cells of origin[49].

Liquid Biopsy Applications: Non-invasive prenatal testing (NIPT) relies on cfDNA analysis to detect foetal chromosomal abnormalities[3]. Post-transplantation, cfDNA can be used to monitor graft health and detect rejection episodes[55]. The use of cfDNA analysis in clinical management of cancer has been summarized in **Table-1**.

4. Clinical Applications:

This section explores the clinical applications of cfDNA in various aspects of cancer, including early detection, monitoring treatment response, identifying resistance mechanisms, and assessing tumor heterogeneity, backed by key research findings.

Early Cancer Detection: cfDNA analysis has significantly enhanced early cancer detection as it enables the identification of cancer-specific mutations or epigenetic alterations in blood samples even before the development of detectable tumor. For example, studies have demonstrated the utility of cfDNA in the early detection of lung cancer[24] and colorectal cancer[25] by studying the altered methylation marks on certain cancer specific genes (SOX17, TAC1, HOXA7, CDO1 and ZFP42 for lung cancer; NDRG4 and BMP3 for colorectal cancer).

Monitoring Treatment Response: cfDNA analysis allows for real-time monitoring of treatment response in cancer patients. Changes in cfDNA levels and the presence of specific genetic alterations can provide insights into the effectiveness of therapy. Research has shown the potential of cfDNA in assessing treatment response in various cancer types, including breast cancer[35] and glioblastoma[40]. They evaluated mutations in the tumor derived ctDNA (e.g., TP53 and PIK3CA, and amplification of EGFR and ERBB2) that provides a non-invasive way for disease monitoring in response to therapy.

Detection of Resistance Mechanisms: Resistance to cancer therapies is a significant challenge. cfDNA analysis can help to identify resistance-associated mutations, enabling timely adjustments in treatment plans. Studies have demonstrated the detection of resistance mutations in cfDNA in patients with metastatic cancers[53]. A study by Murtaza M et al. (2013), identified series of treatment specific mutations conferring resistance towards therapy in the plasma of cancer patients. For e.g. they found mutations in PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha), a truncating mutation in RB1 (retinoblastoma 1) and resistance-conferring mutation in EGFR (epidermal growth factor receptor; T790M); followed by treatment with paclitaxel, cisplatin and gefitinib, respectively[41].

Assessing Tumor Heterogeneity: Tumor heterogeneity poses challenges in cancer treatment. cfDNA analysis can provide a comprehensive view of intra-tumor heterogeneity by profiling various genetic alterations within a tumor[1]. This information aids clinicians in tailoring personalized treatment strategies[2].

Liquid Biopsy-Guided Targeted Therapy: Liquid biopsies based on cfDNA analysis are increasingly used to guide targeted therapy. They identify specific mutations or biomarkers that inform treatment decisions, particularly in lung cancer[6] and melanoma (BRAF mutations)[20].

Minimal Residual Disease Detection: cfDNA analysis can detect minimal residual disease, even when conventional imaging methods show no signs of cancer. This capability is vital for assessing the risk of recurrence and guiding post-treatment surveillance[52]. Cho WK et al. (2023) monitors treatment response through the measurement of chromosomal instabilities to detect minimal residual disease after radiation therapy (RT) using cfDNA[9].

5. The Role of cfDNA in Radiotherapy:

Radiotherapy is a cornerstone in cancer treatment, and the ability to monitor treatment response and detect early recurrence is crucial. Several studies have investigated the potential

of cfDNA as a non-invasive tool for assessing treatment efficacy and predicting outcomes in patients undergoing radiotherapy. From these studies, cfDNA has emerged as a promising biomarker with a growing role in the field of radiotherapy. For instance, in a study by Park et al. (2018), cfDNA analysis allowed for the early detection of tumor response to radiotherapy, providing valuable insights into treatment effectiveness[45]. Work by Lockney et al. (2021) demonstrated the utility of cfDNA in predicting radiotherapy-related toxicity, helping clinicians tailor treatment plans to minimize side effects[39]. Additionally, the analysis of specific mutations or genetic alterations in cfDNA can also aid in identifying radio-resistance mechanisms, enabling the development of personalized treatment strategies. While the field is still evolving, cfDNA holds great promise in optimizing radiotherapy outcomes and improving the overall management of cancer patients undergoing radiation treatment.

5.1 Predicting Radiotherapy Response:

Nygard et al in 2020 studied the presence of ctDNA as chromosomal aberrations using shallow-Whole Genome Sequencing (sWGS) subsequent to chemo-radio therapy[44]. They further noted that size selection improves the detection sensitivity of genomic alterations in the cfDNA. Similarly, another study measures the extent of genome-wide copy number instability in cfDNA, calculated I-score and shown the feasibility to predict the radiation response in cancer patients[9]. Studies such as these have highlighted the potential of cfDNA to serve as an early predictor of radiotherapy response, allowing clinicians to assess treatment effectiveness sooner and potentially modify treatment plans for non-responding patients. As our understanding of the role of cfDNA in radiotherapy response prediction continues to grow, it holds great promise for improving the precision and outcomes of radiation therapy in cancer patients.

5.2 Monitoring Treatment Progress:

By analyzing cfDNA extracted from bodily fluids like blood, researchers and clinicians can gain real-time insights into the dynamics of a patient's disease and response to treatment. Monitoring treatment progress using circulating free DNA (cfDNA) has been adopted as a valuable strategy in various clinical contexts, especially in cancer management. For instance, in a study by Chaudhuri et al. (2017), cfDNA analysis was employed to track the genetic mutations of localised lung cancer patients during treatment, revealing the mutation spectra of 128 cancer-related genes, pre- and post-treatment[5]. Based on the mutation spectra they distinguished ctDNA from cfDNA. They found patients detected with ctDNA after treatment have significantly lower survival. An interesting study by Bettegowda et al. (2014) demonstrated the utility of ctDNA to determine the underlying mechanism of resistance to epidermal growth factor receptor blockade in patients who responded to therapy but relapsed subsequently[2]. In these patients they found one more mutation in genes involved in Mitogen-Activated Protein Kinase (MAPK) pathway. This non-invasive approach allows for the early identification of treatment success or failure and provide insights in molecular mechanisms of treatment resistance, enabling clinicians to adjust therapeutic strategies promptly.

6. Personalizing Radiotherapy Based on cfDNA:

Radiotherapy plays a pivotal role in the treatment of cancer, with its effectiveness hinging on accurate targeting and dosage. Personalizing radiotherapy based on cfDNA has emerged as an exciting frontier in oncology. cfDNA, fragments of DNA released into the bloodstream by cancer cells, can offer unique insights into tumor biology, treatment response, and the development of resistance mechanisms. Telekes and Horváth (2022) recently reviewed the role of cfDNA in cancer treatment decision making[59]. The following paragraphs explore the potential of cfDNA as a tool for personalizing radiotherapy and improving cancer treatment outcomes.

Early Detection of Radiotherapy Response: cfDNA analysis has been found to provide early indications of a patient's response in hepatocellular carcinoma subsequent to treatment with radiotherapy (RT)[45]. Research by Nygard et al., in 2020 demonstrated the stability of cfDNA levels post chemo- and radiotherapy in the circulation from baseline to 2 hours, resulting in reliable sampling of patients for predicting radiotherapy response[44]. These findings allow clinicians to adapt treatment strategies promptly, ensuring that patients receive the most effective therapy from the outset.

Identifying Radioresistance Mechanisms: Understanding the mechanisms behind radioresistance is equally critical for optimizing radiotherapy. By analyzing cfDNA, researchers can detect mutations or genetic alterations associated with resistance. The acquired resistance is manifested as multiple resistant subclones- occurring either in the primary tumor or in a distant metastatic site[36]. In this situation, the cfDNA derived from a single sample collection has the capability of profiling alterations observed in multiple metastatic sites which can obviate the need for multiple tumor biopsies[31] (2021), discusses the involvement of KRAS mutations towards cellular and clinical radio-resistance but due to heterogeneity the mechanism responsible for cellular radio-resistance appear variable[14]. cfDNA have potential to pick up these mutations in heterogenous population and enables the development of personalized treatment plans to overcome resistance and enhance treatment outcomes changes.

Real-Time Monitoring and Adaptive Radiotherapy: Real-time monitoring of cfDNA during radiotherapy can guide treatment adaptation. Chaudhuri et al. (2017) used ctDNA analysis to identify post treatment Minimal Residual Disease (MRD) in localized lung cancer patients, allowing for timely adjustments in treatment regimens[5]. This personalized approach of identifying residual/recurrent disease earlier than radiologic imaging holds the potential to maximize treatment efficacy at early time points when disease burden is lowest while minimizing the harm to healthy tissues.

Minimizing Radiation-Induced Toxicity: Personalizing radiotherapy based on cfDNA can also minimize treatment-related toxicity. Lockney et al. (2021) showcased the potential of cfDNA in predicting radiotherapy-related toxicity[39]. Their preliminary study suggests that the cfDNA levels can predict the subset of prostate cancer patients destined to develop GI toxicity during the radiotherapy. Further studies are required to develop models that will allow dose adjustments to reduce side effects while maintaining treatment effectiveness.

7. Challenges and Future Directions:

Technical Challenges: Circulating free DNA (cfDNA) analysis is gaining importance in the field of radiotherapy as a potential tool for predicting treatment response, monitoring tumor dynamics, and personalizing treatment plans. However, several technical challenges must be overcome to harness the full potential of cfDNA in cancer diagnosis and treatment as a standard clinical procedure. This section explains these challenges and discusses recent advances and research findings in addressing them.

Sample Quality and Handling: The quality and handling of cfDNA samples are critical for accurate analysis. Pre-analytical factors, including sample collection, storage, and processing, can significantly impact cfDNA yield and integrity[16]. Standardized protocols are needed to ensure consistency.

Sensitivity and Detection Limits: One of the primary challenges in cfDNA analysis for radiotherapy is achieving the required sensitivity to detect low levels of cfDNA, particularly in early-stage cancers or minimal residual disease. The lack of sensitivity towards low allele-fraction genotypes in turn is dependent on availability of sufficient quantity and quality samples. Methods such as ddPCR and highly sensitive NGS have shown promise in improving sensitivity[23].

Specificity and Biomarker Identification: Identifying relevant biomarkers in cfDNA for predicting radiotherapy response can be challenging due to the complex and dynamic nature of tumor-derived DNA. Improved techniques for identifying specific mutations, copy number alterations, or epigenetic changes associated with cancers occurring in the wide spectrum of tissues radio-resistance are necessary[5].

Quantification and Normalization: Accurate quantification and normalization of cfDNA are essential for comparing samples across different time points or patients. Standardized quantification methods, such as digital PCR or allele-specific qPCR, can help ensure reliable data[63].

Bioinformatics Analysis: The analysis of cfDNA data can be complex, requiring advanced bioinformatics tools and pipelines to distinguish tumor-derived cfDNA from background noise and other non-tumor cfDNA. Robust algorithms for variant calling and data interpretation are crucial[21].

Tumor Heterogeneity: Tumor heterogeneity poses a significant challenge when analyzing cfDNA for radiotherapy. The complexity of heterogeneity increases particularly in case of metastatic tumors. The tumors may consist of multiple subclones with distinct genetic profiles, making it essential to capture the full genetic diversity of the tumor[1].

Temporal Changes in cfDNA: As stated earlier, the release of cfDNA shows a marked change within one week of receiving radiation fraction[28]. Such temporal changes in cfDNA levels and genetic alterations during and subsequent to radiotherapy regime may provide valuable insights into treatment response. Developing methods to track and interpret these dynamic changes is an ongoing challenge.

Validation and Clinical Integration: Clinical validation of cfDNA-based biomarkers for radiotherapy response prediction is critical before widespread clinical adoption. Collaborative

efforts and large-scale clinical trials are essential to demonstrate the clinical utility of cfDNA in radiotherapy.

Ethical and Regulatory Considerations:

Ethical and regulatory considerations play a pivotal role in the integration of circulating free DNA (cfDNA) into cancer radiotherapy. While cfDNA analysis offers promising clinical benefits, several ethical issues must be addressed. These include patient consent for cfDNA testing, ensuring data privacy and security, and transparency in communicating the uncertainties associated with cfDNA results to patients and clinicians. Additionally, regulatory oversight is crucial to ensure the quality and reliability of cfDNA assays used in radiotherapy, as well as to establish guidelines for reporting and interpreting cfDNA findings. Ethical frameworks, such as those outlined in the Declaration of Helsinki and the Belmont Report, emphasize the principles of beneficence, autonomy, and justice, which should guide the responsible use of cfDNA in radiotherapy (World Medical Association, 2013; National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research, 1979). Moreover, regulatory agencies like the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) provide guidance on the development and approval of cfDNA-based tests, ensuring their safety and efficacy (FDA, 2016; EMA, 2017). Striking a balance between harnessing the potential of cfDNA in radiotherapy and safeguarding patient rights and data privacy is crucial for its ethical and regulatory integration into clinical practice.

8. Future Prospects:

The future prospects of circulating cell-free DNA (cfDNA) in cancer therapy are highly promising and transformative. As technological advancements continue to improve the sensitivity and specificity of cfDNA analysis methods, its application in therapy is poised to become more precise and clinically impactful. The integration of cfDNA into therapy protocols holds the potential to enable real-time monitoring of tumor dynamics, the early detection of treatment response or resistance, and the development of personalized treatment regimens. Furthermore, the growing understanding of cfDNA's role in tumor heterogeneity and clonal evolution will likely aid in tailoring radiotherapy to target specific genetic vulnerabilities within tumors. Collaborative research efforts, ongoing clinical trials, and regulatory developments will be pivotal in harnessing the full potential of cfDNA for optimizing cancer radiotherapy outcomes.

9. Summary:

Cell-free DNA-based markers for cancer radiotherapy represent a cutting-edge approach with significant potential to transform cancer treatment strategies. These markers, derived from cfDNA shed by tumor cells, offer non-invasive insights into various aspects of cancer management, including early detection, monitoring treatment response, and identifying resistance mechanisms. Analysing specific genetic alterations in cfDNA, such as mutations, copy number variations, and epigenetic changes, clinicians can tailor therapy regimens to

target tumor vulnerabilities more effectively. However, the successful integration of cfDNA-based markers into therapy protocols requires overcoming technical challenges, ensuring data privacy and ethical considerations, and addressing regulatory aspects. Nonetheless, as technology advances and our understanding of cfDNA deepens, these markers hold promise for enhancing the precision and outcome of cancer therapy.

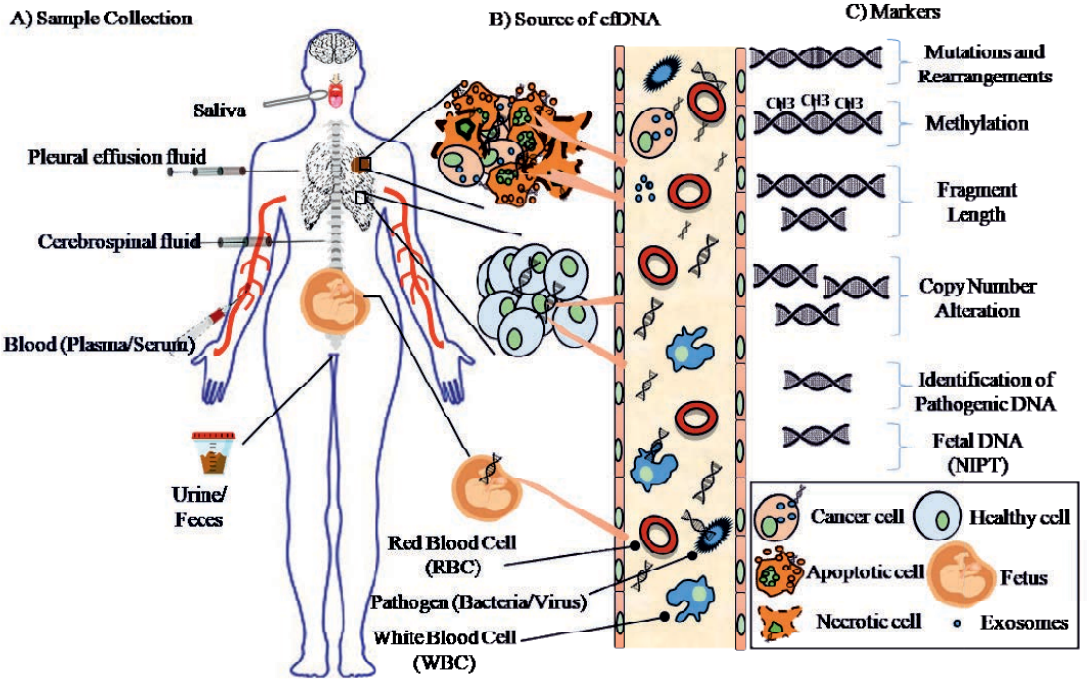

Figure1A) Various body fluids that can be collected for cfDNA analysis. Cell-free DNA (cfDNA) is released from cells into various body fluids. Blood is the most common source of cfDNA as it circulates throughout the body and contains cfDNA molecules from various tissues. Other body fluids like cerebral spinal fluid (CSF) and pleural fluid can be used as cfDNA source for studying specific conditions like neurological conditions in case of CSF and pleural effusion fluids for lung and pleural-related diseases. Saliva, urine and feces are easily accessible body fluids that can be used for non-invasive genetic testing for monitoring disease.

Figure1B) Origin of cfDNA in the circulation. cfDNA is released (passively by apoptosis and necrosis; and actively by secretion) by both healthy cells and cells that are involved in pathological activities such as inflammation (neutrophils and other immune cells) or neoplastic processes. Circulating tumor DNA (ctDNA) is a subset of cfDNA released by tumor cells, and may encompasses a range of genetic variations like point mutations, chromosomal rearrangements, copy number variations. A very small fraction of ctDNA can be contributed by the circulating tumor cells (CTCs). In females, at the time of pregnancy DNA derived from fetus can also be found in the circulation along with maternal cfDNA that can be utilized for Non-Invasive Prenatal Testing (NIPT). In addition to human-origin cfDNA, there exists another subset of cfDNA in the circulation that originates from pathogens. This pathogen-derived cfDNA can be detected in various bodily fluids and serves as a valuable source for conducting tests to diagnose infections and their clearance.

Figure1C) Genetic markers that can be studied in cfDNA. A wide range of tests can be can be performed on cfDNA for the detection of mutational signatures, DNA copy number aberrations, identification of chromosomal translocations, fragment length analysis, estimation of tumor mutational burden, assessment of tumor heterogeneity through allelic fraction determination, analysis of methylation patterns, Non-Invasive Prenatal Testing (NIPT) on circulatory fetal DNA and pathogen identification.

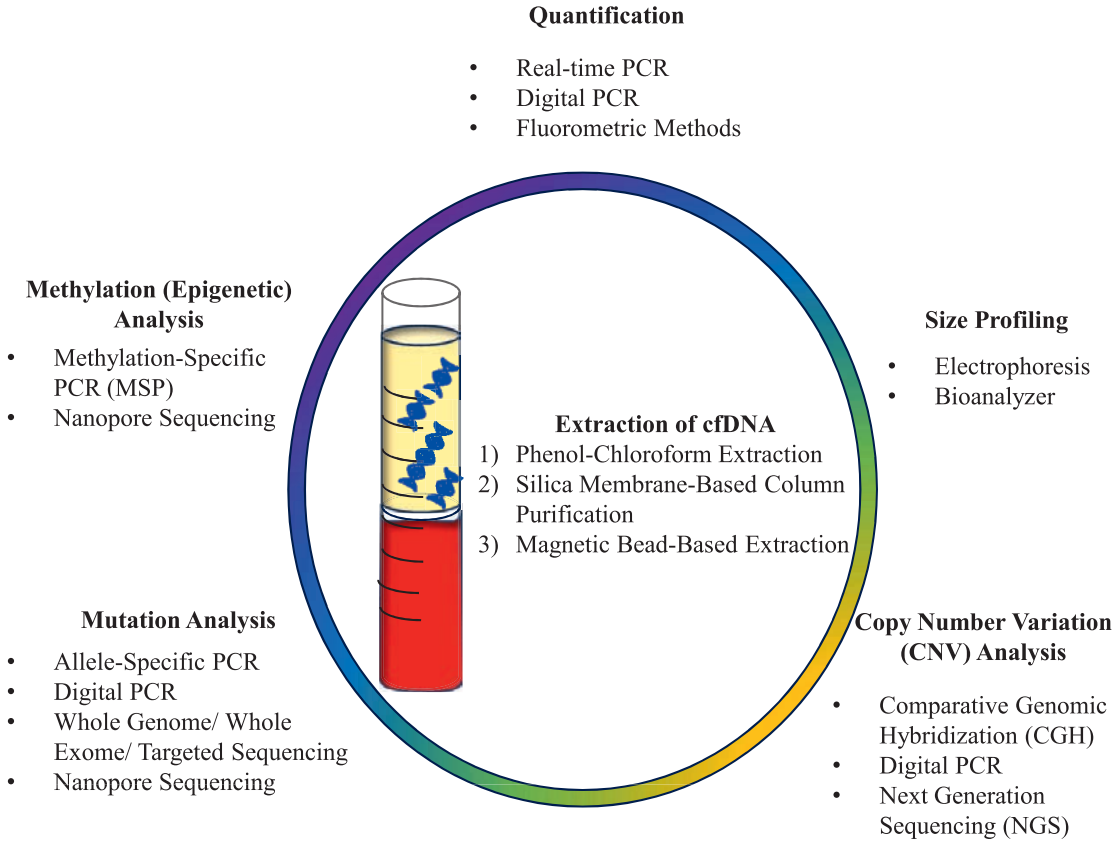

Figure 2) Methods and techniques for extracting, quantifying, and studying the cfDNA. The traditional approach for cfDNA extraction employs organic solvents such as Phenol-Chloroform. However, for enhanced cfDNA quality and yield, there are several commercial kits readily available. These kits offer a safer and more convenient alternative to Phenol-Chloroform-based extraction methods. Two commonly employed kit-based techniques for cfDNA isolation include Silica Membrane-Based Column Purification and Magnetic Bead-Based Extraction. There are various analytical techniques that can be used to study different molecular features of cfDNA. Polymerase Chain Reaction (PCR) based approaches can be used to quantify cfDNA (real-time PCR and digital PCR), identify mutations (Allele specific PCR), estimate copy number variation (digital PCR), and perform methylation analysis (methylation specific PCR). For profiling fragment length of cfDNA, electrophoresis-based approaches are utilized (like Bioanalyzer). Sequencing based approaches (next generation and third generation sequencing) are high throughput and sensitive for studying mutations, rearrangements, copy number variations and methylation status of cfDNA.

Table-1: Studies exploring cfDNA-based cancer liquid biopsy for clinical management of patients

Cancer Type	Sample	Marker	Method	References
Screening				
Pan Cancer (8 cancer types)	Plasma	61-amplicon panel (along with protein biomarkers)	PCR-based sequencing assay	*Cohen JD et al, 2018 (CancerSEEK)*
Pan-Cancer (7 cancer types)	Plasma	Genome-wide cfDNA fragmentation profiling (DELFI)	Low coverage Whole Genome Sequencing	*Cristiano S et al, 2019*
Non-small cell lung cancer	Plasma and sputum	Promoter methylation of 6 cancer specific gens (SOX17, TAC1, HOXA7, CDO1, HOXA9, and ZFP42)	Quantitative methylation-specific real-time PCR	*Hulbert A et al, 2017*
Colorectal cancer	Stool	methylation of NDRG4, BMP3, and seven mutation sites of KRAS (along with immunohisto chemical assay for hemoglobin)	Quantitative methylation-specific real-time PCR	*Imperiale TF et al, 2014 (Cologuard)*
Diagnosis				
Hepatocellular carcinomas (HCC)	Serum	p16, p15, and ras association domain family 1A (RASSF1A)	Methylation-specific PCR	*Zhang YJ et al, 2007*
Pan Cancer (4 cancer types)	Plasma	58 cancer-related genes	Targeted error correction sequencing	*Phallen J et al, 2019*
Central Nervous System Lymphoma	Cerebrosp inal fluid	MYD88, L265P, and V217F	Droplet digital PCR (ddPCR)	*Zorofchian S et al, 2018*

Thyroid carcinoma	Plasma	Integrity index 180/67	Quantitative real-time PCR (qPCR)	*Salvianti F et al, 2017*
Prognosis				
Non-small cell lung cancer	Plasma	cfDNA concentration	PicoGreen dsDNA Kit	*Tissot C et al, 2016*
Pancreatic cancer	Plasma	cfDNA fragment size	Bioanalyzer	*Lapin M et al, 2018*
Prostate cancer	Plasma	GSTP1, and APC	Methylation-specific PCR	*Hendriks RJ et al, 2018*
Non-small cell lung cancer	Serum	EGFR and KRAS	PNA-LNA PCR	*Kim ST et al, 2013*
Response to therapy				
Hepatocellular carcinoma (Radiotherapy)	Plasma	cfDNA concentration	UV-visible spectrophotometer	*Park S et al, 2018*
Rectal cancer (Chemo-radiotherapy)	Plasma	cfDNA concentration	Fluorescent assay	*Schou JV et al, 2018*
Rectal cancer (Chemo-radiotherapy)	Plasma	400-/100-bp DNA ratio, KRAS mutation, MGMT promoter methylation	Quantitative real-time PCR	*Sun W et al, 2014*
Cervical cancer (Chemo-radiotherapy)	Plasma	48 cancer driver genes panel	Targeted deep sequencing	*Tian J et al, 2019*

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