

Lung Cancer Diagnosis and Prognostic Monitoring Through Cell-Free RNA via Liquid Biopsy

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Abstract: Lung cancer remains a leading cause of cancer-related mortality worldwide, largely due to challenges in its early detection and effective management. Despite advances in treatment modalities, the complex nature of lung cancer, characterized by its molecular heterogeneity and resistance mechanisms, underscores the need for innovative approaches. Cell-free RNA (cfRNA) has emerged as a promising biomarker with significant clinical applications in lung cancer diagnosis, monitoring, and precision medicine. We explore key themes including the utility of cfRNA in early detection, differentiation between benign and malignant lung nodules, molecular subtyping, and real-time therapeutic monitoring. Advances in liquid biopsy technologies, particularly non-invasive cfRNA analysis, provide dynamic means of tracking tumor evolution. cfRNA biomarkers such as miRNA, long non-coding RNAs, and circular RNAs offer unique insights into tumor biology, paving the way for personalized treatment strategies. Further, we discuss the application of cutting-edge technologies such as AI-driven analytics, next-generation sequencing, and multi-omics integration, which are enhancing the clinical utility of cfRNA in identifying treatment resistance and improving outcomes in immunotherapy, targeted therapy, and chemotherapy. The review addresses significant challenges facing cfRNA applications, including pre-analytical variability, technical limitations in detection methods, economic constraints, and the lack of standardization in clinical protocols. Through multidisciplinary collaborations and standardized methodologies, significant progress can be made toward integrating cfRNA into routine clinical practice. Emphasis is placed on future research directions, which include validating cfRNA biomarkers across diverse populations, streamlining workflows, and addressing scalability issues for real-world applications. This comprehensive exploration positions cfRNA at the forefront of innovations in lung cancer management, offering a pathway for improved diagnostic accuracy and individualized care.

Keywords: cell-free RNA, lung cancer, liquid biopsy, early detection, precision medicine

Introduction

Overview of Lung Cancer Diagnosis and Treatment Challenges

Lung cancer remains a significant global health issue due to its high incidence and mortality rates.^{1,2} It is one of the most common malignant tumors.³ Despite advances in treatment, lung cancer continues to have a poor prognosis, especially when diagnosed at advanced stages.⁴ The challenges in lung cancer management stem from difficulties in early detection and the complex nature of the disease, which often leads to delayed diagnosis and suboptimal treatment outcomes.⁴ Traditional diagnostic methods for lung cancer include imaging techniques such as X-rays, CT scans, and PET scans, as well as invasive procedures like bronchoscopy and tissue biopsy⁵ (Figure 1). While these methods are essential for diagnosis, they have limitations. Imaging techniques may not detect small or early-stage tumors, and tissue biopsies are

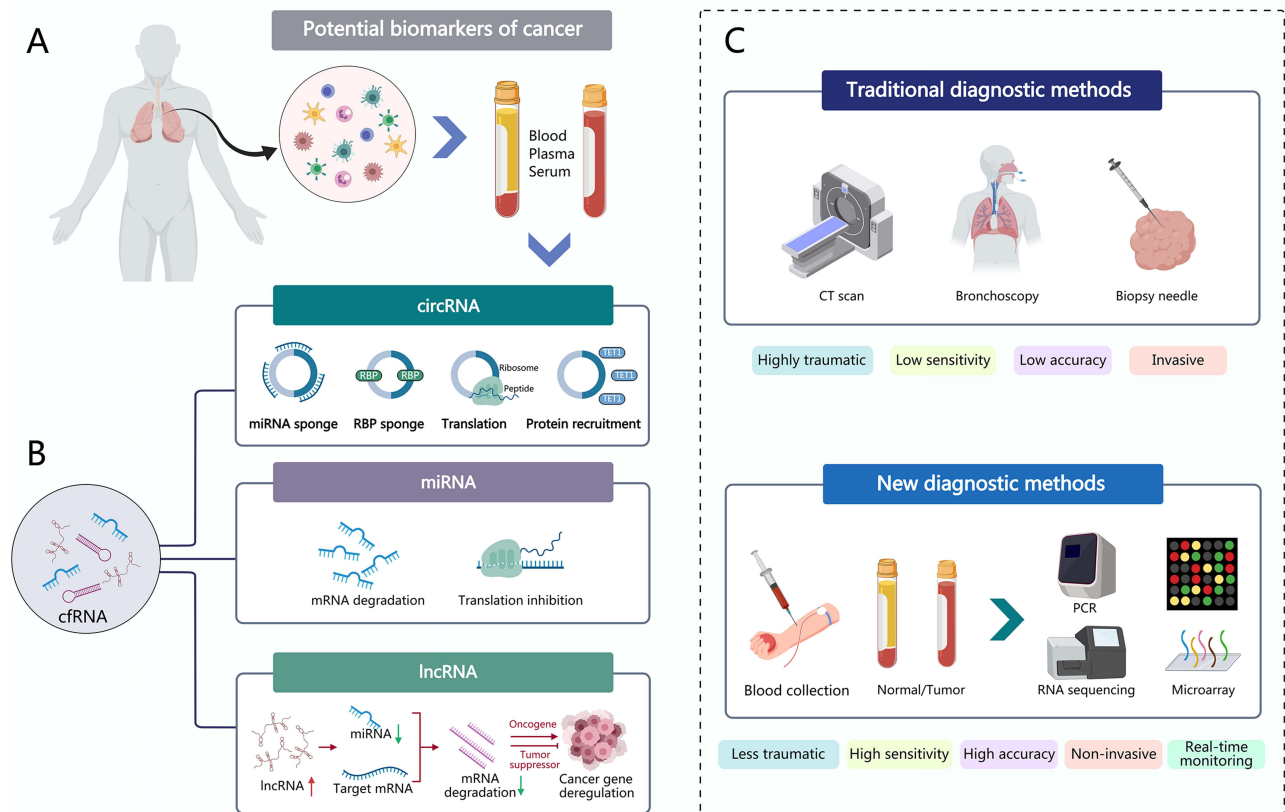


Figure 1 The sources, classification, and advantages of cfRNA. **(A)** cfRNA comes from human blood and contains tumor-derived cells and released substances, making it one of the biomarkers for cancer. **(B)** cfRNA includes three types: circRNA, miRNA, and lncRNA. CircRNA adsorbs miRNA, promoting transcription and translation; miRNA can degrade mRNA, inhibiting protein translation; lncRNA reduces miRNA content, increasing mRNA, thereby inhibiting tumor occurrence. **(C)** Compared to traditional detection methods such as CT scans, bronchoscopy, and tissue biopsy, cfRNA has the advantages of being non-invasive, highly sensitive, highly specific, non-intrusive, and capable of real-time monitoring.

invasive, carry risks, and may not always be feasible, particularly in patients with comorbidities or tumors located in difficult-to-reach areas.^{4,5}

Treatment options for lung cancer vary depending on the stage and type of cancer, as well as the patient's overall health. Common treatment modalities include surgery, radiation therapy, chemotherapy, targeted therapy, and immunotherapy.⁵ However, treatment selection is often complicated by factors such as tumor heterogeneity and the development of resistance to therapy.⁵ The increasing understanding of the genomic landscape of lung cancer has led to targeted therapeutic advances, especially in non-small cell lung cancer (NSCLC).⁵ Despite these advances, a substantial proportion of patients do not respond to these therapies, or they eventually develop resistance, highlighting the need for personalized treatment strategies.⁵ The 5-year disease-free survival rate after surgery for stage IB-IIIIB NSCLC patients has notably declined from 73% to 13%, further emphasizing the need for early detection and personalized treatment plans.¹

Importance of Early Diagnosis and Precision Medicine

Early diagnosis is crucial in improving survival rates and treatment outcomes for lung cancer patients. When lung cancer is detected at an early stage, treatment options such as surgery or radiation therapy are more likely to be effective, leading to better long-term survival.² However, a significant proportion of lung cancers are diagnosed at advanced stages, when the disease has already spread to other parts of the body, making curative treatment less likely.² Therefore, there is a critical need for effective screening and early detection methods that can identify lung cancer at its earliest stages, when it is most treatable.²

Precision medicine aims to tailor treatment strategies based on individual patient characteristics, including their genetic makeup, tumor biology, and other factors.⁶ This approach recognizes that lung cancer is not a single disease but

rather a collection of distinct molecular subtypes, each with its own unique characteristics and treatment response.⁵ By identifying the specific molecular drivers of a patient's cancer, clinicians can select the most appropriate targeted therapies or immunotherapies, maximizing the chances of treatment success while minimizing unnecessary toxicity.⁵ Precision diagnosis is essential to ensure the effectiveness of molecularly targeted drugs that are now available to around a quarter of NSCLC patients.⁷ Liquid biopsies have the potential to furnish comprehensive (epi-)genetic insights into the tumor, thereby aiding personalized treatment strategies.⁷

However, the widespread implementation of precision medicine in lung cancer treatment faces several challenges. One major obstacle is the need for comprehensive molecular profiling of tumors, which can be costly and time-consuming.⁵ Additionally, the interpretation of complex genomic data requires specialized expertise, and the development of new targeted therapies is an ongoing process.⁵ Despite these challenges, the potential benefits of precision medicine in improving outcomes for lung cancer patients are substantial, driving ongoing efforts to overcome these obstacles and integrate personalized approaches into routine clinical practice.⁶

Liquid Biopsy as a Game Changer: Concept and Advantages

Liquid biopsy is a minimally invasive or non-invasive method to analyze a range of tumor material in blood or other body fluids.⁸ It involves the collection and analysis of biological markers, such as circulating tumor cells (CTCs), cell-free DNA (cfDNA), messenger RNA (mRNA), microRNA (miRNA), and exosomes, from bodily fluids like blood or urine.^{3,8} This approach offers several advantages over traditional tissue biopsies, which are invasive and may not always be feasible or representative of the entire tumor.⁵

One of the key advantages of liquid biopsy is its non-invasive nature, which allows for repeated sampling and monitoring of the tumor over time.⁶ This is particularly important in lung cancer, where the disease can evolve and develop resistance to treatment.⁹ Liquid biopsies provide a real-time snapshot of the tumor's characteristics, allowing clinicians to track changes in the tumor's genetic makeup and response to therapy.⁹ Moreover, liquid biopsy can capture tumor heterogeneity more effectively than conventional tissue biopsies, ensuring a more precise depiction of the tumor's (epi-)genomic landscape.⁷ This capability facilitates the identification of (epi-)genetic alterations, enabling informed treatment decisions, disease progression monitoring, and early detection of resistance-causing mutations for timely therapeutic interventions.⁷

Liquid biopsies hold great promise for various clinical applications in lung cancer management. They can be used for early detection and screening, diagnosis and staging, treatment selection and monitoring, and detection of minimal residual diseases.⁴ By identifying tumor-specific markers in the blood or other bodily fluids, liquid biopsies can help detect lung cancer at an early stage, when it is most treatable.⁶ They can also be used to monitor the effectiveness of treatment and detect the emergence of resistance mutations, allowing clinicians to adjust treatment strategies accordingly.⁹ Furthermore, liquid biopsies can potentially identify minimal residual disease after surgery or other treatments, helping to predict recurrence and guide adjuvant therapy decisions.¹⁰

The advent of liquid biopsies has revolutionized oncology and non-invasive diagnostics, with cell-free DNA (cfDNA) and cell-free RNA (cfRNA) emerging as two pivotal analytes. While both are nucleic acids shed into the bloodstream, they possess distinct biological origins, offer complementary clinical information, and present unique technical challenges. Understanding their differences is crucial for leveraging their full potential in precision medicine.

The fundamental difference lies in their origin and inherent stability. CfDNA is predominantly a product of cell death. Its fragmentation pattern is non-random, reflecting cleavage between nucleosomes, which also contributes to its relative stability in the bloodstream. In contrast, cfRNA originates from multiple processes, including cell death and active cellular secretion via extracellular vesicles like exosomes, which offer a protective microenvironment against RNases.¹¹ This inherent lability of RNA is the single greatest factor differentiating its handling from cfDNA. Without this protection or immediate stabilization, cfRNA degrades rapidly, making pre-analytical procedures paramount.

The applications of cfDNA and cfRNA diverge based on the type of information they carry. CfDNA excels in interrogating the genome. It is the gold-standard analyte for detecting somatic mutations and calculating tumor mutational burden (TMB). Its use in monitoring minimal residual disease (MRD) is powerful because the DNA sequence itself is a highly specific clonal barcode for the tumor.¹² Furthermore, epigenetic modifications like methylation patterns

on cfDNA are emerging as a highly sensitive tool for cancer detection and tissue-of-origin identification. CfRNA provides a window into the transcriptome. While a DNA mutation tells you an alteration exists, RNA analysis can reveal if and how that mutant gene is being expressed. This is critical for detecting gene fusions, which are often poorly detected by DNA-seq due to their large intronic breakpoints but are easily identified from the fused mRNA transcript via RNA-seq.¹³ CfRNA analysis also allows for the assessment of gene expression profiles, which can reveal pathways driving resistance to therapy or confirm the tissue of origin in a way that DNA-based methods cannot.

CfDNA and cfRNA are not competitors but rather complementary partners in liquid biopsy. cfDNA is the established leader for genotyping and detecting genetic alterations, offering a stable readout of the tumor's DNA.^{14–19} CfRNA adds a crucial functional layer, revealing the dynamic transcriptional activity of the tumor and capturing biomarkers like fusions and expression changes that are invisible to DNA-based assays.^{20–23} The future of comprehensive liquid profiling lies in integrated multi-analyte approaches, simultaneously harnessing the genetic power of cfDNA and the functional insights of cfRNA to guide cancer diagnosis, treatment, and monitoring with unprecedented precision.²³ The choice between them depends entirely on the specific clinical question being asked.

Role of cfRNA in Lung Cancer Diagnostics and Monitoring

CfRNA is a type of RNA that is present in bodily fluids, including blood, plasma, and serum.³ It originates from various sources, including tumor cells, normal cells, and immune cells.³ In the context of cancer, cfRNA can provide valuable information about the tumor's characteristics, such as its genetic makeup, gene expression patterns, and response to therapy.⁸ CfDNA is the most widely used in clinical practice among cancer biomarkers⁸ (Figure 1A).

The detection of cfRNA in the plasma and serum of cancer patients has spurred significant interest in its potential as a diagnostic tool in oncology. Studies have demonstrated elevated levels of both particle-associated and non-particle-associated cfRNA in 16 individuals diagnosed with hepatocellular carcinoma.¹¹ Similarly, in lung cancer, cfRNA derived from serum and cell-free bronchial lavage fluid supernatant has shown promise as a biomarker for differentiating between patients with and without tumors.¹¹ In rectal cancer, plasma cfRNA has been identified as a potential indicator of tumor response following preoperative chemoradiotherapy.¹¹ Collectively, these findings highlight the considerable potential of cfRNAs in enabling sensitive, specific, and noninvasive approaches for repeated screening, monitoring of tumor progression, and evaluation of therapeutic efficacy in cancer patients. This growing relevance is evidenced by the increasing number of recent studies dedicated to exploring the role of cfRNAs in cancer.

CfRNA plays a significant role in lung cancer diagnostics and monitoring due to its potential to provide a comprehensive and dynamic view of the disease.³ Different types of cfRNA, such as miRNA, lncRNA, and circRNA, have been shown to be dysregulated in lung cancer and can serve as potential biomarkers for diagnosis, prognosis, and treatment response⁵ (Figure 1B). MicroRNAs, for example, have shown high accuracy in discerning cancerous versus benign nodules in high-risk, screened patients.⁵ The detection of cfDNA in the blood or other body fluids can predict response to treatment and relapse.⁹

In early detection, cfRNA can help identify individuals at high risk of developing lung cancer and distinguish between benign and malignant lung nodules.¹³ In diagnosis, cfRNA can provide molecular subtyping and personalized medicine insights, improving the accuracy and precision of diagnostic assessments.³ In treatment monitoring, cfRNA can track tumor evolution during treatment, predict resistance mechanisms, and adapt therapy accordingly, making it applicable in immunotherapy, targeted therapy, and chemotherapy.⁹ Overall, cfRNA holds great promise for improving the management of lung cancer by providing a non-invasive and dynamic means of assessing the disease's characteristics and response to therapy.²⁴ Further research and clinical validation are needed to fully realize the potential of cfRNA in lung cancer management and to establish standardized protocols and guidelines for clinical use.²⁴

Technological Platforms for Liquid Biopsy

Liquid biopsy is revolutionizing cancer diagnostics by offering a minimally invasive method to access tumor-derived material, such as cfRNA. This approach allows for repeated sampling, providing a real-time snapshot of the tumor's characteristics and its response to therapy.²⁵ Several technological platforms have been developed to extract, analyze, and

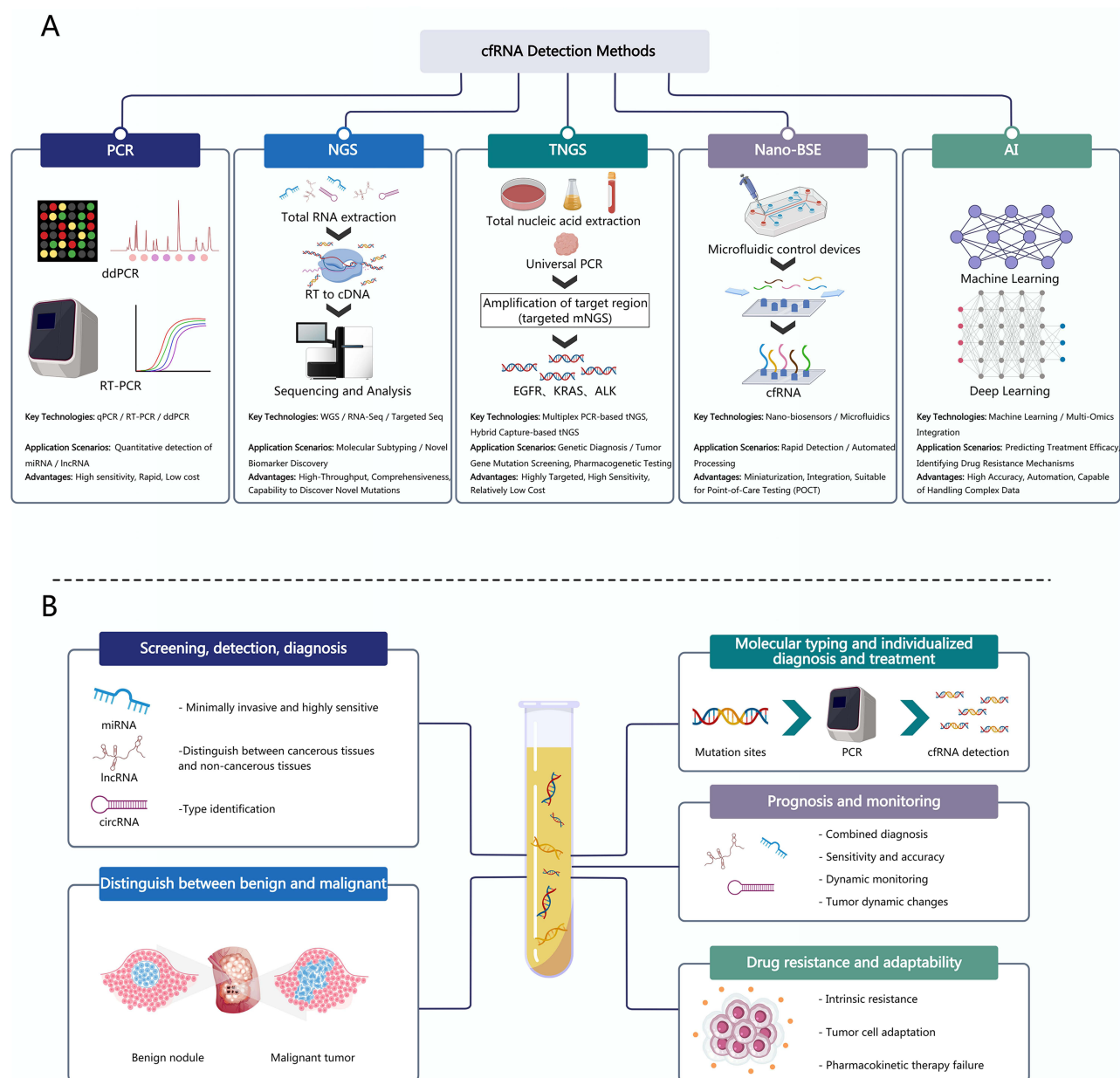


Figure 2 cfRNA detection technology and its relative roles. **(A)** cfRNA detection methods. There are five detection technologies for cfRNA: PCR, NGS, TNGS, Nano-BSE, and AI, which include key technologies, applicable scenarios, and advantages. **(B)** Application of cfRNA. Specific applications include early cancer diagnosis, distinguishing between benign and malignant tumors, molecular typing, efficacy monitoring, and resistance prediction.

interpret cfRNA from liquid biopsies, each with its own strengths and limitations. These platforms play a crucial role in lung cancer detection and monitoring in clinical settings (Figure 2A).

PCR-Based Techniques

PCR (Polymerase Chain Reaction) technology forms the bedrock of many liquid biopsy applications. PCR's primary function is to amplify small amounts of nucleic acid, making it possible to detect and quantify even trace amounts of cfRNA in liquid biopsies.⁶ This amplification is essential because cfRNA is often present in low concentrations in bodily fluids.

Quantitative PCR and Real-Time PCR: Sensitivity and Applications (Figure 2A)

Quantitative PCR (qPCR) and real-time PCR (RT-PCR) are advanced forms of PCR that not only amplify DNA but also quantify the amount of DNA present in the sample. Unlike conventional PCR, which only provides a qualitative assessment of the presence or absence of a target sequence, qPCR and RT-PCR provide precise quantification of the target cfRNA.⁶

qPCR monitors the amplification of DNA in real-time by using fluorescent dyes or probes that bind to the amplified DNA. The intensity of the fluorescence signal is directly proportional to the amount of DNA present, allowing for accurate quantification. RT-PCR is specifically designed to amplify RNA targets. It involves a preliminary step of reverse transcription, where RNA is converted into complementary DNA (cDNA) by reverse transcriptase. The resulting cDNA is then amplified using PCR, and the amplification is monitored in real-time.²⁶

The high sensitivity and specificity of qPCR and RT-PCR make them ideal for quantifying cfRNA levels in lung cancer patients. These techniques can detect subtle changes in cfRNA expression, which can be indicative of disease progression or response to treatment.⁵ Several lung cancer biomarkers can be detected using these techniques, including miRNAs, lncRNAs, and specific mRNA transcripts. For example, RT-PCR can quantify the expression levels of specific miRNA known to be associated with lung cancer, providing valuable information for monitoring disease progression or response to treatment.²⁶ The clinical significance of these findings lies in their ability to provide timely and actionable information for clinicians, enabling them to make informed decisions about patient management.

Droplet Digital PCR: Enhanced Resolution and Quantification (Figure 2A)

Droplet Digital PCR (ddPCR) represents a significant advancement over traditional PCR methods, offering enhanced resolution and quantification accuracy. ddPCR works by partitioning a sample into thousands or millions of individual reactions, each containing either zero or one copy of the target molecule. After PCR amplification, the number of positive reactions is counted, providing an absolute quantification of the target molecule without the need for standard curves.⁶

The key advantage of ddPCR is its ability to detect low-abundance cfRNA markers with high precision. This is particularly important in the early detection of lung cancer, where cfRNA markers may be present at very low levels. ddPCR's enhanced sensitivity allows for the identification of subtle changes in cfRNA expression that may be missed by other techniques. Studies have demonstrated the effectiveness of ddPCR in the context of lung cancer, showing its ability to accurately quantify rare mutations and gene expression changes in cfRNA samples.⁶

Next-Generation Sequencing (NGS) Platform (Figure 2A)

NGS has revolutionized genomic studies with its capacity for high-throughput sequencing. NGS enables the simultaneous sequencing of millions of DNA or RNA fragments, providing a comprehensive view of the genetic landscape of a sample.²⁷ In the context of liquid biopsy, NGS is used to analyze cfRNA, providing valuable information for biomarker discovery, molecular subtyping, and personalized medicine.

Whole Genome and Transcriptome Sequencing (Figure 2A)

Whole-genome sequencing (WGS) involves sequencing the entire genome of an organism, while transcriptome sequencing (RNA-Seq) focuses on sequencing all the RNA molecules in a sample. WGS can identify extensive genetic changes, including mutations, insertions, deletions, and copy number variations, while RNA-Seq provides a comprehensive view of gene expression profiles.²⁷

In lung cancer, WGS and RNA-Seq can be used to identify novel biomarkers and understand the complex molecular mechanisms driving tumor development and progression. The ability to detect novel biomarkers has significant implications for personalized medicine approaches for lung cancer patients. By identifying unique genetic alterations and gene expression patterns in individual patients, clinicians can tailor treatment strategies to maximize efficacy and minimize toxicity.

Targeted Sequencing for Biomarker Discovery

Targeted sequencing methods focus on specific genomic regions or transcripts of interest, offering a cost-effective and efficient approach for biomarker discovery. These methods use targeted capture techniques to enrich for specific DNA or RNA sequences before sequencing, allowing for deeper coverage of the regions of interest.²⁶

Targeted sequencing has successfully identified new actionable mutations and transcripts in lung cancer, impacting treatment decisions. For example, targeted sequencing can be used to detect mutations in genes such as EGFR, KRAS, and ALK, which are known to be associated with response to targeted therapies.⁵ These findings highlight the potential of targeted sequencing to guide treatment decisions and improve outcomes for lung cancer patients.

Emerging Technologies in Liquid Biopsy

Beyond PCR and NGS, several emerging technologies are poised to enhance liquid biopsy capabilities. These technologies offer new approaches for processing and analyzing cfRNA samples, with the potential to improve diagnostic accuracy, streamline workflows, and reduce costs.²⁵

Nano-Biosensors and Microfluidics (Figure 2A)

Nano-biosensors and microfluidics are innovative technologies that offer new possibilities for liquid biopsy analysis. Nano-biosensors are nanoscale devices that can detect specific molecules or biomarkers with high sensitivity and specificity. These sensors can be used to detect cfRNA directly in liquid biopsy samples, without the need for amplification or labeling.²⁵

Microfluidics involves the manipulation of small volumes of fluids in microchannels. This technology can be used to automate and miniaturize liquid biopsy workflows, enabling faster, more efficient, and cost-effective analysis. Microfluidic devices can perform a variety of functions, including sample preparation, nucleic acid extraction, and PCR amplification, all on a single chip.⁶ The combination of nano-biosensors and microfluidics has the potential to transform routine clinical practice in lung cancer management.

Integration of Artificial Intelligence in Detection (Figure 2A)

Artificial intelligence (AI) is playing an increasingly important role in analyzing large datasets derived from liquid biopsies. AI algorithms can be trained to recognize patterns and predict outcomes based on complex cfRNA profiles. AI can improve diagnostic accuracy, streamline workflows, and discover novel biomarkers from complex cfRNA profiles.²⁶

AI algorithms can be used to analyze sequencing data to identify subtle changes in gene expression or detect rare mutations that may be missed by traditional methods. AI can also be used to integrate data from multiple sources, such as imaging and clinical data, to provide a more comprehensive assessment of a patient's condition. The integration of AI into liquid biopsy workflows has the potential to revolutionize lung cancer diagnosis and treatment.

Role of Cell-Free RNA in Lung Cancer Diagnosis

CfRNA holds significant promise in revolutionizing early detection and treatment monitoring for lung cancer (Figure 2B). Liquid biopsies, which analyze substances derived from tumors in bodily fluids, are advantageous due to their non-traumatic nature, ease of accessibility, ability to reflect the overall tumor state, and capacity for real-time monitoring.³ Among the various components of liquid biopsies, cfRNA has emerged as a potential biomarker for cancer diagnosis.^{3,26,28}

The choice of analyte in liquid biopsy is critical and depends on the balance between sensitivity, stability, and clinical applicability. Circulating tumor cells (CTCs), exosomes, and cell-free RNA (cfRNA) each offer distinct advantages and limitations.

Sensitivity: CTCs are extremely rare in early-stage disease, making their detection challenging and limiting analytical sensitivity. Exosomes, being more numerous, offer better sensitivity as they are actively secreted by both tumors and healthy cells. However, isolating tumor-specific exosomes requires complex markers like CD63/CD81 or EGFRvIII.²⁹ cfRNA often provides the highest sensitivity for specific transcript detection. Techniques like RT-qPCR or ddPCR can amplify and detect minute quantities of tumor-derived RNA fragments that are abundantly released into the bloodstream through apoptosis and necrosis, making it highly suitable for early detection.

Stability: CTCs are fragile, intact cells that require specialized stabilization tubes to prevent degradation, making sample handling cumbersome. Exosomes are highly stable lipid bilayer vesicles that protect their cargo (including RNA) from RNases, allowing for robust storage and transport. cfRNA is inherently fragmented and vulnerable to degradation in

blood plasma if not processed quickly. However, when samples are properly collected and promptly processed, its profile remains consistent and analyzable, though its half-life is generally shorter than exosome-protected RNA.

Clinical Applications: CTC enumeration is FDA-cleared for prognostic monitoring in metastatic breast, prostate, and colorectal cancer. Exosomes are being explored for their rich cargo (proteins, miRNAs, mRNAs) for disease monitoring and understanding tumor heterogeneity. cfRNA shines in specific, actionable molecular diagnostics. Its most significant clinical application is the rapid, non-invasive detection of targetable genetic alterations, such as EGFR mutations in lung cancer, avoiding the need for invasive tissue biopsies for genotyping.

While CTCs and exosomes provide valuable cellular and vesicular information, cfRNA analysis is often simpler, more cost-effective, and directly translatable to existing molecular pathology workflows. This high clinical utility, combined with superior sensitivity for detecting these specific analytes and the ability to perform repeated longitudinal monitoring, makes cfRNA an indispensable tool in modern oncology.^{3,15,30}

Early Detection and Differentiation of Lung Cancer

Early detection is critically important for improving outcomes in lung cancer.^{2,4} The ability to identify lung cancer at an early stage significantly increases the chances of successful treatment and long-term survival. CfRNA offers a minimally invasive approach to achieving this goal.^{5,9} By analyzing cfRNA in blood or other body fluids, clinicians can potentially detect the presence of lung cancer before it is visible through traditional imaging techniques^{3,9} (Figure 1C).

MiRNA Expression Profiling

MiRNAs are small, non-coding RNA molecules that play a crucial role in regulating gene expression.^{26,28} These molecules, typically 19–25 nucleotides long, function by binding to messenger RNA (mRNA) molecules, leading to either the degradation of the mRNA or the repression of its translation into protein. This regulatory activity affects a wide range of cellular processes, including cell growth, differentiation, apoptosis, and stress response.

Altered miRNA expression profiles are associated with various types of lung cancer.^{26,28} Specific miRNAs have shown promise in distinguishing early-stage lung cancer from healthy controls or benign conditions.⁵ For example, some miRNAs are upregulated in lung cancer cells, promoting tumor growth and metastasis, while others are downregulated, losing their tumor-suppressing functions.

Several studies have highlighted the potential of specific miRNAs as diagnostic biomarkers for lung cancer. Circulating miRNAs can discern cancerous versus benign nodules in high-risk, screened patients.⁵ Despite these findings, miRNA is not yet used in clinical practice as biomarkers, as their clinical utility and validity have not been confirmed in prospective clinical studies, and there is no consensus on standardized (pre)analytical procedures.²⁸

While individual miRNA dysregulation offers diagnostic clues, their synergy with other RNAs is key. Specific miRNA panels can distinguish NSCLC from SCLC, yet their regulatory role is often intertwined with lncRNAs, which compete for shared miRNA targets, creating a complex ceRNA network that modulates oncogenic pathways.

lncRNA as Potential Biomarkers

lncRNAs are RNA molecules longer than 200 nucleotides that do not code for proteins.²⁶ Once considered transcriptional “noise,” lncRNAs are now recognized as critical regulators of gene expression, influencing a wide range of cellular processes, including development, differentiation, and disease.²⁶ lncRNAs can interact with DNA, RNA, and proteins, modulating gene transcription, RNA splicing, and protein activity.

Recent findings have implicated specific lncRNAs in lung cancer, highlighting their roles in tumorigenesis and their potential as diagnostic biomarkers.²⁶ Some lncRNAs promote cancer cell proliferation, migration, and invasion, while others act as tumor suppressors.

Several studies have demonstrated the efficacy of lncRNAs in differentiating lung cancer from non-cancerous conditions.²⁶ For instance, certain lncRNAs are significantly upregulated in lung cancer tissues and plasma compared to healthy controls, suggesting their potential as diagnostic markers.

LncRNAs often function as miRNA sponges in lung cancer, yet their variable stability limits diagnostic use; this highlights the need for more robust regulators, a role where circular RNAs (circRNAs) excel due to their unique closed-loop structure.

CircRNA: Emerging Applications

CircRNAs are a class of non-coding RNA molecules characterized by their unique circular structure, formed through a process called back-splicing.³¹ This circularization makes them more stable and resistant to degradation compared to linear RNAs, enhancing their potential as biomarkers. CircRNAs can act as microRNA sponges, binding to miRNAs and preventing them from regulating their target genes. They can also interact with RNA-binding proteins and even be translated into small peptides.

Current research explores the potential of circRNA in early diagnosis and its predictive value in lung cancer.³¹ Specific circRNAs have shown promise in distinguishing between various lung cancer types or stages. Their stability, abundance, and high specificity make them attractive liquid biopsy biomarkers for cancers.³²

Their functions are highly complementary. While miRNAs act as rapid, potent repressors, lncRNAs and circRNAs provide a counterbalancing layer of control.¹⁶ LncRNAs offer immense diversity and context-specificity; they can not only sponge miRNAs but also scaffold chromatin-modifying complexes to regulate transcription epigenetically.^{33,34} CircRNAs, due to their covalently closed loop structure, are exceptionally stable and often exhibit tissue-specific expression, making them ideal, persistent miRNA sponges and biomarkers.^{35,36} Furthermore, some lncRNAs can be processed to yield miRNAs, and certain circRNAs can also be translated, adding further complexity.

In essence, this trio operates as a complex communication network. miRNAs provide the targeted silencing signal, while lncRNAs and circRNAs integrate and modulate this signal, allowing for a robust and adaptable regulatory system that is essential for normal development, cellular homeostasis, and when dysregulated, contributes to diseases like cancer.³⁷⁻³⁹ Their interplay exemplifies the complexity of the genomic regulatory landscape beyond the central dogma.

Distinguishing Benign From Malignant Lung Nodules

Accurately distinguishing between benign and malignant lung nodules presents a significant clinical challenge.¹³ Many lung nodules detected on imaging are benign, but the possibility of malignancy requires further investigation. Traditional diagnostic approaches, such as biopsies, are invasive and carry risks (Figure 1C).

Cell-free RNA (cfRNA) profiling, including miRNA, lncRNA, and circRNA, can provide critical insights in this differentiation process.^{26,32} By analyzing the expression patterns of these RNA molecules in liquid biopsies, clinicians can potentially identify biomarkers that distinguish between benign and malignant nodules (Figure 2B).

Several studies and clinical trials have investigated cfRNA profiles in lung nodules, presenting findings on sensitivity and specificity in this context.^{5,26,32} These studies have shown that specific cfRNA signatures can accurately differentiate between benign and malignant nodules, with varying degrees of sensitivity and specificity.

Integrating cfRNA analysis into current diagnostic paradigms has the potential to reduce the number of unnecessary invasive procedures and improve the accuracy of lung cancer diagnosis.^{1,3,4} By providing additional information about the nature of lung nodules, cfRNA analysis can help clinicians make more informed decisions about patient management.

Applications in Molecular Subtyping and Personalized Medicine

CfRNA plays a vital role in the molecular subtyping of lung cancer, which contributes to personalized treatment approaches.^{4,7,9,26} Molecular subtyping involves classifying lung cancers into distinct subgroups based on their genetic and molecular characteristics. This classification is essential for tailoring treatment strategies to the individual patient, optimizing therapeutic outcomes.

Specific biomarker panels involving cfRNA have been developed for subclassifying lung cancer, along with their implications for treatment decisions.^{7,26,40} These panels often include a combination of miRNAs, lncRNAs, and circRNAs, each providing unique information about the tumor's biology.

cfRNA profiling can guide the selection of targeted therapies or inform prognosis.^{1,7,10} For example, the detection of specific miRNA signatures in cfRNA can predict a patient's response to EGFR tyrosine kinase inhibitors (TKIs).

Similarly, the presence of certain lncRNAs can indicate a higher risk of disease recurrence, prompting more aggressive treatment strategies.

Clinical studies and trials have demonstrated the utility of cfRNA profiling in personalizing lung cancer treatment.^{4,7,9,10} These studies have shown that patients whose treatment is guided by cfRNA profiling experience improved outcomes compared to those treated with standard approaches.

cfRNA in Prognosis and Therapeutic Monitoring

CfRNA plays a crucial role in both prognostication and therapeutic monitoring in lung cancer, reflecting the dynamic landscape of cancer treatment and holding significant implications for precision medicine. Liquid biopsies, including the analysis of cfRNA, offer a minimally invasive approach to assess tumor characteristics, treatment response, and potential resistance mechanisms in real-time^{1,3,6} (Figure 2B).

Prognostic Value of Single and Multiple cfRNA Biomarkers

Integration Into Multi-Marker Panels

Using multiple cfRNA biomarkers instead of single ones improves sensitivity and specificity.^{6,33} The rationale is that lung cancer is a heterogeneous disease, and different cfRNAs may reflect distinct aspects of tumor biology, such as proliferation, angiogenesis, or immune evasion. By combining multiple biomarkers, clinicians can obtain a more comprehensive and accurate assessment of a patient's prognosis.

Several studies have successfully utilized multi-marker panels in lung cancer prognosis. For instance, combinations of miRNAs, lncRNAs, and circRNAs have shown promise in predicting survival outcomes.^{5,9} Specific multi-marker combinations and their associated outcomes include:

- (1) miRNA panels: Certain miRNA signatures in cfRNA have been associated with overall survival (OS) and progression-free survival (PFS) in NSCLC patients.⁵ For example, a panel including miR-21, miR-155, and miR-210 may provide more robust prognostic information than any single miRNA alone.
- (2) lncRNA and miRNA combinations: Combining lncRNAs with miRNAs can enhance prognostic accuracy. For example, the ratio of specific lncRNAs to miRNAs in cfRNA might be indicative of disease progression or treatment response.
- (3) cfRNA combined with ctDNA: Integrating cfRNA markers, like mRNA or miRNA, with ctDNA analysis provides a more holistic view of the tumor's molecular status. This approach can improve risk stratification and identify patients who may benefit most from aggressive treatment strategies.⁷

Integrating multiple biomarkers also presents challenges. Data interpretation becomes more complex and requires robust analytical methodologies, including advanced bioinformatics and statistical modeling. Standardization of cfRNA isolation, quantification, and data analysis is essential to ensure reproducibility and comparability across different studies and clinical settings.⁵

Comparative Analysis with Traditional Biomarkers (Figure 1C)

cfRNA biomarkers offer several advantages over traditional biomarkers, such as tumor tissue biopsies and serum protein markers (eg, CEA, CA125, CYFRA21-1), regarding predictive capabilities for lung cancer prognosis.^{5,40}

- (1) Invasiveness: cfRNA analysis is performed on blood samples, making it minimally invasive compared to tissue biopsies, which carry risks of complications and are not always feasible due to tumor location or patient comorbidities.^{3,4}
- (2) Frequency of Monitoring: cfRNA can be easily collected at multiple time points, allowing for real-time monitoring of disease progression and treatment response. Traditional biomarkers, particularly tissue biopsies, are not suitable for frequent monitoring.^{9,31}

- (3) Turnaround Time: cfRNA results can be obtained more quickly than tissue biopsy results, enabling timely clinical decision-making. Modern liquid biopsy platforms often provide rapid turnaround times, facilitating prompt adjustments to treatment plans.⁵
- (4) Representation of Tumor Heterogeneity: cfRNA reflects the overall genomic landscape of the tumor, including multiple metastatic sites, thus overcoming the limitations of tissue biopsies, which only represent a single location and may not capture tumor heterogeneity.^{5,7}

However, cfRNA also has limitations compared to traditional methods:

- (1) Sensitivity: cfRNA concentrations in blood can be very low, especially in early-stage disease, which may limit the sensitivity of detection assays.
- (2) Standardization: Lack of standardized protocols for cfRNA isolation, quantification, and data analysis can lead to variability in results and limit comparability across studies.
- (3) Cost: Advanced cfRNA analysis techniques, such as next-generation sequencing (NGS), can be expensive, which may limit their widespread adoption in clinical practice.

Several studies have provided empirical data on the advantages of cfRNA over traditional methods. For example, ctDNA analysis, a type of cfRNA, has shown superior sensitivity and specificity in detecting EGFR mutations compared to traditional tissue biopsies in NSCLC patients.^{1,31} In cases where tissue is unavailable or insufficient, cfRNA analysis can provide valuable molecular information to guide treatment decisions.

Despite these advantages, cfRNA also has limitations. False positives and false negatives can occur, and the clinical utility of some cfRNA biomarkers remains uncertain. In some cases, traditional biomarkers may provide complementary information and should be used in conjunction with cfRNA analysis.

Real-Time Therapeutic Monitoring

cfRNA allows for dynamic monitoring of therapies in lung cancer treatment, reflecting changes in tumor dynamics during treatment, including tumor regression or progression.^{9,24}

Tracking Tumor Evolution During Treatment

cfRNA can reflect changes in tumor dynamics during treatment, including tumor regression or progression. The levels of specific cfRNAs can be tracked to assess treatment response and identify potential resistance mechanisms. Significant cfRNA metrics to track include:

- (1) Levels of specific miRNAs: Changes in miRNA levels in cfRNA can indicate treatment response or resistance. For example, a decrease in miR-21 levels may indicate tumor regression, while an increase in miR-155 levels may suggest the development of resistance.⁵
- (2) Levels of specific lncRNAs: Certain lncRNAs are associated with tumor growth and metastasis, and changes in their levels can reflect tumor evolution during treatment.
- (3) Mutational Status of ctDNA: Monitoring ctDNA for the emergence of resistance mutations can provide early warning of treatment failure and guide subsequent therapy decisions.⁷

Clinical studies focusing on real-time monitoring have provided insights into tumor heterogeneity and behavior. For instance, serial cfRNA analysis has revealed that tumors can evolve and adapt during treatment, with the emergence of new mutations and changes in gene expression profiles.⁷ These findings highlight the importance of dynamic monitoring to personalize treatment strategies.

Predicting Resistance Mechanisms and Adapting Therapy (Figure 2A)

cfRNA plays a crucial role in identifying emerging resistance mechanisms in response to therapeutic pressures.^{7,9} By analyzing cfRNA, clinicians can detect genetic and epigenetic changes that confer resistance to specific therapies. Profiles of cfRNA changes that may indicate potential resistance include:

- (1) Emergence of Resistance Mutations: ctDNA analysis can detect the emergence of resistance mutations in genes such as EGFR, ALK, and ROS1, which can guide the selection of alternative targeted therapies.^{1,7}
- (2) Changes in miRNA Expression: Alterations in miRNA expression profiles can indicate the activation of alternative signaling pathways that bypass the targeted therapy.
- (3) Changes in lncRNA Expression: Upregulation of certain lncRNAs has been associated with drug resistance in lung cancer cells (Figure 2B).

Recent research and case studies demonstrate the efficacy of cfRNA in predicting resistance and tailoring therapies accordingly. For example, in patients with EGFR-mutant NSCLC treated with EGFR tyrosine kinase inhibitors (TKIs), ctDNA analysis can detect the emergence of the T790M resistance mutation, which can be overcome with the use of third-generation EGFR TKIs such as osimertinib.^{1,31}

Applicability in Immunotherapy, Targeted Therapy and Chemotherapy (Figure 2B)

cfRNA monitoring has specific applications across different treatment modalities for lung cancer, including immunotherapy, targeted therapy, and chemotherapy.²⁴

- (1) Immunotherapy: cfRNA can be used to predict response to immune checkpoint inhibitors (ICIs) and monitor immune-related adverse events (irAEs). Changes in miRNA expression profiles in cfRNA have been associated with response to ICIs, and ctDNA analysis can track tumor burden and identify patients who are more likely to benefit from immunotherapy.²⁴
- (2) Targeted Therapy: cfRNA is particularly useful in monitoring response to targeted therapies, as it can detect the emergence of resistance mutations and guide the selection of alternative targeted agents.
- (3) Chemotherapy: cfRNA can be used to assess the effectiveness of chemotherapy and identify patients who are unlikely to respond. Changes in ctDNA levels during chemotherapy can predict prognosis and guide decisions about treatment intensification or de-escalation.

Current findings illustrate how cfRNA levels correlate with treatment responses or adverse effects in various therapeutic contexts. However, the potential benefits and limitations of cfRNA in these settings should be carefully considered. For example, while cfRNA analysis can provide valuable information about treatment response, it may not always accurately predict long-term outcomes.

In conclusion, cfRNA is a versatile tool in both prognostication and ongoing treatment monitoring. Integration of cfRNA strategies into clinical practice requires addressing ongoing challenges and pursuing future research directions in this evolving field. Standardized protocols, improved analytical methodologies, and large-scale clinical trials are needed to fully realize the potential of cfRNA in lung cancer management.

Challenges and Bottlenecks in Clinical Translation

Translating cfRNA technologies into clinical practice for lung cancer management faces numerous challenges and bottlenecks. These issues are complex and multifaceted, requiring careful consideration to ensure reliable and effective clinical application.

Pre-Analytical Variables: Sample Handling and Preservation

The integrity and quality of cfRNA are critically affected by sample collection and handling procedures. Several pre-analytical variables can significantly impact the accuracy and reliability of downstream analyses.⁴¹ These include:

- (1) Time-to-processing: Delaying the processing of blood samples after collection can lead to RNA degradation. cfRNA is susceptible to enzymatic degradation, and prolonged storage at room temperature can result in a decrease in RNA yield and altered RNA profiles.
- (2) Storage conditions: Temperature and duration of storage are crucial. Improper storage temperatures can accelerate RNA degradation. Generally, immediate processing or storage at -80°C is recommended to preserve cfRNA integrity.
- (3) Choice of anticoagulants: The type of anticoagulant used during blood collection can influence cfRNA yield and quality. EDTA is commonly used, but other anticoagulants like heparin can interfere with certain downstream enzymatic reactions.
- (4) Collection tubes: The type of blood collection tube can also influence cfRNA yield and stability. Some tubes contain additives that stabilize RNA, while others may not be suitable for cfRNA preservation.

These variables can lead to RNA degradation, impacting subsequent analyses and clinical outcomes. Improper handling can skew results and lead to inaccurate diagnoses or prognoses. For example, delays in processing can lead to the preferential degradation of certain RNA species, altering the apparent expression profile. To mitigate these risks, adherence to strict standard operating procedures (SOPs) is essential. Best practices include:

- (1) Immediate processing of blood samples or storage at -80°C .
- (2) Using appropriate blood collection tubes with RNA-stabilizing additives.
- (3) Standardizing the type of anticoagulant used.
- (4) Implementing rigorous quality control measures to assess RNA integrity before downstream analyses.

Technical Limitations of Detection Methods

Current detection methods for cfRNA have limitations that can hinder the reliability and accuracy of measurements in clinical settings. These limitations vary depending on the specific technique used, such as PCR-based methods or NGS.

Sensitivity Constraints

The sensitivity of detection techniques is a critical factor, especially when dealing with low-abundance cfRNA biomarkers. Techniques like qPCR and reverse transcription PCR (RT-PCR) have been widely used for cfRNA detection, but they may not be sensitive enough to detect very low levels of certain RNA species. NGS offers higher sensitivity but can be more expensive and complex to implement.

Low sensitivity can have significant implications for early diagnosis and monitoring of lung cancer. If the concentration of a specific cfRNA biomarker is below the detection limit of the assay, it can lead to false-negative results, delaying diagnosis or underestimating disease burden. In early-stage lung cancer or during minimal residual disease monitoring, the levels of cfRNA biomarkers may be particularly low, making sensitive detection methods essential.

To enhance sensitivity, researchers are exploring several approaches:

- (1) Optimization of RNA extraction methods: Improving RNA extraction protocols to maximize yield and minimize loss of RNA during the process.
- (2) Enrichment strategies: Employing techniques to selectively enrich for specific cfRNA species before amplification or sequencing.
- (3) Advanced amplification methods: Utilizing more efficient amplification methods to increase the signal-to-noise ratio.
- (4) Digital PCR (dPCR): Using dPCR can enhance resolution and quantification.⁴¹

False Positives and Reproducibility Issues

False-positive results in cfRNA detection pose a significant challenge, with potentially serious consequences for patient management. Sources of variability that can lead to false positives include:

- (1) Contamination: Contamination with exogenous RNA or DNA during sample collection, processing, or analysis.
- (2) Sample mishandling: Improper handling or storage of samples can introduce artifacts and affect the accuracy of results.
- (3) Inherent variability in detection methods: Technical noise or biases in the detection platform can lead to false signals.

Reproducibility across different laboratories and studies is another critical concern. Variations in protocols, reagents, and equipment can lead to inconsistent results, making it difficult to compare data across different settings. The lack of standardized protocols for cfRNA extraction, quantification, and data analysis contributes to this variability.

To address these issues, potential solutions include:

- (1) Rigorous quality control measures: Implementing strict quality control procedures to minimize contamination and ensure the integrity of samples and reagents.
- (2) Standardized protocols: Developing and adhering to standardized protocols for cfRNA extraction, quantification, and data analysis to reduce variability.
- (3) Proficiency testing: Participating in proficiency testing programs to assess and improve the accuracy and reliability of cfRNA assays.
- (4) Validation studies: Conducting thorough validation studies to evaluate the performance of cfRNA assays in different laboratories and clinical settings.

Economic and Logistical Challenges in Clinical Adoption

The widespread adoption of cfRNA-based liquid biopsy approaches in clinical settings faces significant economic and logistical challenges. These challenges include the costs of technology and reimbursement issues, the need for specialized infrastructure and trained personnel, and streamlined workflows for sample collection and processing.

Economic factors: The cost of cfRNA analysis can be a barrier to clinical adoption, especially for routine screening or monitoring. NGS-based approaches are generally more expensive than PCR-based methods, and the cost of reagents, equipment, and data analysis can be substantial. Reimbursement policies also play a crucial role. If cfRNA tests are not adequately reimbursed by insurance companies or healthcare providers, it can limit their accessibility to patients.

Logistical challenges: Implementing cfRNA testing in clinical settings requires specialized infrastructure and trained personnel. Laboratories need to have the necessary equipment for RNA extraction, quantification, and analysis, as well as personnel trained in molecular biology techniques and bioinformatics. Streamlined workflows for sample collection, processing, and reporting are also essential to ensure timely and accurate results.

Several institutions have successfully implemented cfRNA technologies in clinical practice. These institutions have overcome barriers by:

- (1) Investing in infrastructure and training: Allocating resources to acquire the necessary equipment and train personnel.
- (2) Developing streamlined workflows: Optimizing sample collection, processing, and reporting procedures to improve efficiency.
- (3) Establishing collaborations: Collaborating with researchers, clinicians, and industry partners to share expertise and resources.
- (4) Demonstrating clinical utility: Conducting studies to demonstrate the clinical utility of cfRNA testing in specific patient populations.

Gaps in Standardization and Process Validation

A significant bottleneck in the clinical translation of cfRNA technologies is the lack of standardized protocols for cfRNA extraction, quantification, and interpretation. This absence of standardization leads to variability in results across different laboratories and clinical practices, hindering the widespread adoption of cfRNA testing.

Process validation is essential for ensuring reliable and reproducible results. Validation involves demonstrating that a specific assay or procedure consistently produces accurate and precise results within defined parameters. Without proper validation, it is difficult to have confidence in the clinical utility of cfRNA testing.

Several efforts and initiatives are underway to promote standardization in cfRNA methodologies. These include:

- (1) Development of reference materials: Creating reference materials that can be used to calibrate and standardize cfRNA assays.
- (2) Establishment of proficiency testing programs: Implementing proficiency testing programs to assess and improve the accuracy of cfRNA testing.
- (3) Publication of guidelines and best practices: Developing and disseminating guidelines and best practices for cfRNA extraction, quantification, and data analysis.

To fill the gaps in standardization and process validation, collaborative efforts among clinicians, researchers, and regulatory bodies are needed to develop consensus guidelines. These guidelines should address all aspects of cfRNA testing, from sample collection to data interpretation. It is also important to establish regulatory frameworks for cfRNA testing to ensure that assays meet certain standards of quality and performance.

Future Perspectives in cfRNA Liquid Biopsy for Lung Cancer

Advancements in Detection Technologies

Innovative cfRNA detection technologies hold the potential to significantly improve lung cancer diagnostics and treatment monitoring. Staying at the forefront of these technological advancements is crucial for enhancing sensitivity, accuracy, and clinical utility.

AI-Driven Analytical Pipelines

AI-driven analytical pipelines refer to the integration of artificial intelligence (AI) technologies into the analysis of liquid biopsy data, particularly cfRNA data. These pipelines leverage machine learning algorithms to process large datasets, identify complex patterns, and extract meaningful insights that may not be readily apparent through traditional analysis methods.

AI enhances cfRNA analysis by improving the sensitivity and accuracy of lung cancer detection.⁴² Machine learning algorithms can be trained to recognize subtle changes in cfRNA profiles that are indicative of early-stage cancer or tumor progression. These algorithms can also help to reduce false positive rates by distinguishing between cancer-related cfRNA signatures and background noise.⁴³

Specific examples of AI integration in cfRNA analysis include (Figure 2A):

- (1) Improved Sensitivity and Accuracy: Studies have demonstrated that AI algorithms can improve the sensitivity and accuracy of lung cancer detection using cfRNA data.⁴³ For example, machine learning models can be trained to identify specific miRNA signatures that are indicative of lung cancer, even in patients with early-stage disease.
- (2) Real-time Data Analysis: AI can enable real-time analysis of cfRNA data, allowing clinicians to monitor treatment response and detect resistance mechanisms as they emerge. This can facilitate timely adjustments to treatment plans and improve patient outcomes.
- (3) Predictive Modeling: AI can be used to develop predictive models that forecast patient outcomes based on cfRNA profiles. These models can help clinicians to identify patients who are at high risk of relapse or progression and to tailor treatment strategies accordingly.

- (4) Automation of Workflows: AI can automate many of the manual and time-consuming tasks associated with cfRNA analysis, such as data preprocessing, normalization, and quality control. This can free up laboratory personnel to focus on more complex tasks and improve overall efficiency.

Future capabilities of AI in cfRNA analysis may include:

- (1) Real-time data analysis: Enabling immediate insights during patient care.
- (2) Predictive modeling: Accurately forecasting patient outcomes to inform personalized treatment strategies.
- (3) Automated workflows: Streamlining processes to enhance efficiency and reduce human error.

Multi-Omics Integration for Comprehensive Insights

Multi-omics refers to the integration of data from multiple “omics” disciplines, such as genomics, transcriptomics, proteomics, and metabolomics, to create a more comprehensive understanding of biological systems. In the context of cfRNA analysis, multi-omics integration involves combining cfRNA data with other types of molecular data to gain a more holistic view of lung cancer biology.

Integrating genomics, transcriptomics, proteomics, and metabolomics can significantly enhance cfRNA analysis, leading to improved biomarker discovery, enhanced disease characterization, and the ability to track tumor evolution.⁴⁴ By analyzing multiple layers of molecular information, researchers can identify complex relationships between genes, RNAs, proteins, and metabolites that drive cancer development and progression.

Benefits of employing multi-omics approaches include:

- (1) Improved Biomarker Discovery: Multi-omics integration can help to identify novel biomarkers that are more sensitive and specific than single-analyte biomarkers.
- (2) Enhanced Disease Characterization: Multi-omics data can provide a more detailed picture of the molecular subtypes of lung cancer, which can inform treatment decisions.
- (3) Tracking Tumor Evolution: Multi-omics analysis can be used to track changes in the molecular profiles of tumors over time, which can help to predict treatment response and identify resistance mechanisms.

Challenges associated with multi-omics integration include data management and interpretation complexities. The large datasets generated by multi-omics studies can be difficult to manage and analyze, requiring specialized bioinformatics tools and expertise. Additionally, interpreting the complex relationships between different types of molecular data can be challenging.

Future research directions that could facilitate smoother integration processes include:

- (1) Developing standardized data formats and analysis pipelines for multi-omics data.
- (2) Creating new bioinformatics tools that can integrate and analyze multi-omics data in a user-friendly manner.
- (3) Training researchers in the skills needed to analyze and interpret multi-omics data.

The Integration Potential of cfRNA and Single-Cell Sequencing

The integration of cell-free RNA (cfRNA) sequencing with single-cell RNA sequencing (scRNA-seq) presents a transformative potential for non-invasive disease monitoring, particularly in oncology and prenatal health.^{45–47} While scRNA-seq provides an unparalleled high-resolution atlas of cellular heterogeneity, gene expression states, and rare subpopulations within a tissue biopsy, it is inherently limited by its invasive nature, inability to capture dynamic temporal changes easily, and the challenge of accessing certain tissue sites repeatedly. cfRNA sequencing, which profiles RNA fragments shed by cells into the bloodstream, offers a complementary “liquid biopsy” approach that provides a real-time, global snapshot of the transcriptional activity occurring across the entire body. The true synergy lies in using scRNA-seq as a foundational reference map to deconvolve the complex mixture of cfRNA. By leveraging computational deconvolution algorithms, the relative contributions of specific cell types, such as tumor cells, immune cells, or placental trophoblasts, to the circulating transcriptome can be quantified. This allows researchers to not just detect the presence of

a disease but to monitor shifts in cellular population dynamics in response to therapy, identify the emergence of treatment-resistant clones, and track metastatic progression, all from a simple blood draw. For instance, in pregnancy, this integration could precisely pinpoint abnormal placental cell development by matching cfRNA signals to specific scRNA-seq-defined placental cell subtypes. Furthermore, longitudinal cfRNA sampling can capture the evolution of a disease or biological process at a frequency impossible with repeated tissue biopsies, while scRNA-seq from initial or key time points provides the critical cellular context to interpret these changes. Thus, the convergence of these technologies bridges the gap between detailed cellular snapshots and continuous, system-wide monitoring, paving the way for a new era of personalized, dynamic, and non-invasive diagnostic and therapeutic strategies.^{48,49}

Expanding Clinical Applications

Current applications of cfRNA liquid biopsies in clinical practice include cancer screening, diagnosis, prognosis, and monitoring treatment response.⁴³ However, there is significant potential to expand these uses to improve patient management.

Optimizing Early Screening Programs

Early detection of lung cancer is critical for improving patient outcomes. The earlier lung cancer is diagnosed, the more likely it is to be treated successfully. Current screening practices, such as low-dose computed tomography (LDCT), have limitations, including relatively high false positive rates and exposure to radiation.⁴⁴

cfRNA can be utilized to develop more effective early screening programs due to its non-invasive nature and ability to detect early-stage cancers. cfRNA analysis can potentially improve the specificity of lung cancer screening, reducing the number of false positive results and unnecessary follow-up procedures.

Examples of potential biomarkers that can signal the presence of early-stage lung cancer include:

- (1) miRNAs: Specific miRNAs are often dysregulated in lung cancer cells and can be detected in the circulation.
- (2) lncRNAs: lncRNAs have been shown to play a role in lung cancer development and progression and can be used as biomarkers for early detection.
- (3) circRNAs: circRNAs are stable and abundant in the circulation, making them attractive biomarkers for lung cancer detection.

Strategies for optimizing these screening programs include:

- (1) Combining cfRNA analysis with existing screening modalities: cfRNA analysis can be used in conjunction with LDCT to improve the accuracy of lung cancer screening.
- (2) Developing multi-marker panels: Combining multiple cfRNA biomarkers into a single panel can improve the sensitivity and specificity of lung cancer detection.
- (3) Implementing public health initiatives: Public health campaigns can be used to raise awareness of the importance of early lung cancer detection and to encourage people to participate in screening programs.

Guiding Individualized Treatment Protocols

Precision medicine in lung cancer treatment involves tailoring therapies to individual patients based on the unique molecular characteristics of their tumors. cfRNA plays a crucial role in precision medicine by providing a non-invasive way to assess these molecular characteristics and monitor treatment response.⁴³

cfRNA analyses have successfully guided treatment decisions in several cases:

- (1) Selecting targeted therapies: cfRNA analysis can be used to identify patients who are likely to respond to specific targeted therapies based on the presence of specific mutations or gene expression profiles.
- (2) Selecting immunotherapy options: cfRNA analysis can be used to assess the immune microenvironment of tumors and to predict which patients are most likely to benefit from immunotherapy.

Incorporating cfRNA analysis into clinical protocols could enable continuous monitoring and adjustments to treatment plans, allowing clinicians to detect resistance mechanisms early and to switch to alternative therapies as needed. Future perspectives include:

- (1) Real-time monitoring of treatment response: cfRNA analysis can be used to monitor treatment response in real-time, allowing clinicians to make timely adjustments to treatment plans.
- (2) Prediction of resistance mechanisms: cfRNA analysis can be used to identify the emergence of resistance mechanisms before they become clinically apparent.
- (3) Development of personalized treatment strategies: cfRNA analysis can be used to develop personalized treatment strategies that are tailored to the unique molecular characteristics of each patient's tumor.

Building Standardized Protocols and Guidelines for Clinical Use

Existing gaps in standardization of cfRNA protocols within clinical settings impact research reproducibility and clinical adoption. These inconsistencies affect the reliability and validity of results, hindering the widespread use of cfRNA in clinical practice.

Developing comprehensive guidelines for the collection, processing, and analysis of cfRNA is necessary to ensure the reliability and validity of results. Standardized protocols can improve the reproducibility of research findings and facilitate the clinical translation of cfRNA-based diagnostics and monitoring tools.

Multidisciplinary collaboration among clinicians, researchers, and regulatory bodies is essential in creating these standardized protocols. Stakeholders can contribute by:

- (1) Sharing best practices for cfRNA collection, processing, and analysis.
- (2) Developing consensus guidelines for data analysis and interpretation.
- (3) Working with regulatory bodies to establish standards for cfRNA-based diagnostics.

Standardized protocols could influence the future of cfRNA in clinical practice by leading to wider acceptance and integration into routine diagnostics for lung cancer. This could improve the accuracy of diagnosis, guide treatment decisions, and ultimately improve patient outcomes. Future directions include:

- (1) Developing standardized protocols for cfRNA extraction and quantification.
- (2) Establishing quality control metrics for cfRNA analysis.
- (3) Creating a central repository for cfRNA data.
- (4) Working with regulatory agencies to approve cfRNA-based diagnostics.

Conclusions

Summary of cfRNA's Potential in Lung Cancer Diagnostics and Treatment

This review has synthesized the critical insights regarding the potential of cfRNA as a biomarker in lung cancer, highlighting its multifaceted role in early diagnosis, differentiation of cancer types, prognostic assessment, and therapeutic monitoring. Compared to traditional diagnostic methods, cfRNA analysis offers the advantage of non-invasive access to tumor-specific information, enabling earlier and potentially more accurate detection of lung cancer.⁶ cfRNA's utility extends beyond simple detection, allowing for molecular subtyping that can inform personalized treatment strategies. By integrating cfRNA analysis into existing clinical workflows, we can foresee improvements in patient outcomes, more tailored treatment approaches, and potentially reduced healthcare costs.⁶

Importance of Multidisciplinary Collaboration for Advancement

Advancing the research and clinical application of cfRNA in lung cancer necessitates a collaborative approach involving oncologists, molecular biologists, bioinformaticians, and clinical pathologists. Pooling expertise and resources across

these disciplines can facilitate the refinement of cfRNA detection methods, enhance the interpretation of cfRNA data, and improve patient management strategies.⁶ Successful interdisciplinary collaborations in liquid biopsy and cancer research have already led to significant advancements in technology and patient care, underscoring the importance of continued collaboration to fully realize the potential of cfRNA in lung cancer.⁶

Key Directions for Future Research and Clinical Implementation

Future research should focus on addressing specific gaps in our understanding of cfRNA biology in lung cancer. This includes further studies on the biological mechanisms underlying cfRNA release and uptake, validation of promising cfRNA biomarkers across diverse patient populations, and the development of robust, standardized clinical protocols.⁶ A crucial step forward involves establishing standardized methodologies for cfRNA analysis and validating these protocols across different laboratories and clinical settings to ensure reproducibility and reliability of results.⁶ The integration of emerging technologies, such as AI-driven analytics and multi-omics approaches, holds immense potential for deepening our understanding and enhancing the accuracy of cfRNA applications in lung cancer.⁶ Ultimately, the design and execution of well-controlled clinical trials aimed at validating cfRNA biomarkers and improving therapeutic strategies based on cfRNA monitoring are essential. Disseminating these findings through publications and clinical guidelines will foster acceptance and integration of cfRNA analysis into routine clinical practice.⁶

Cell-free RNA (cfRNA) analysis, often referred to as liquid biopsy for transcriptomics, holds immense promise for revolutionizing clinical diagnostics and monitoring. By sequencing RNA fragments circulating in the bloodstream, which are shed by cells throughout the body, cfRNA profiling offers a non-invasive, real-time snapshot of gene expression across multiple tissues.^{50–52} This potential is particularly compelling in oncology, prenatal medicine, and transplant rejection monitoring. However, the translation of this promise into routine clinical practice is fraught with significant technical and biological challenges that must be rigorously addressed.

- (1) **Pre-Analytical Variability and Standardization:** The journey from blood draw to sequencing data is a minefield of variability. Factors such as the type of blood collection tube, the time interval between blood draw and plasma processing, centrifugation speed and duration, RNA extraction methods, and storage conditions can dramatically impact the yield, quality, and composition of the isolated cfRNA. The lack of universal, standardized protocols across laboratories makes it extremely difficult to compare results between studies and establish reliable reference ranges, which is a fundamental requirement for any clinical test.
- (2) **Biological Complexity and Background Noise:** Unlike analyzing a homogeneous tissue sample, cfRNA is a complex mixture of transcripts originating from every cell type in the body. This creates a high background of “biological noise.” An individual’s cfRNA profile is influenced by a multitude of factors including exercise, diet, circadian rhythm, underlying inflammatory conditions, and comorbidities. Distinguishing a disease-specific signal from this dynamic background noise is a major hurdle affecting the specificity and sensitivity of tests.
- (3) **Low Abundance and Transcript Fragmentation:** cfRNA molecules are present in plasma at very low concentrations and are highly fragmented. This poses a dual challenge. First, it requires deep sequencing to achieve sufficient coverage for robust quantification, increasing costs. Second, the fragmented nature of the transcripts complicates bioinformatic analysis, as mapping short reads to their gene of origin and identifying specific isoforms or fusion transcripts is inherently difficult compared to analyzing high-quality RNA from cells.
- (4) **Bioinformatic and Deconvolution Hurdles:** Interpreting cfRNA data is arguably its greatest challenge. Advanced computational methods are required to “deconvolute” the mixed signal—that is, to identify the tissue(s) of origin and quantify the proportional contributions of different cell types. This deconvolution is entirely dependent on high-quality reference datasets, often from single-cell RNA sequencing (scRNA-seq) atlases of healthy and diseased tissues. The accuracy of these algorithms directly determines the clinical utility of the test, and their validation is a complex task.
- (5) **Clinical Validation and Cost-Effectiveness:** Finally, for any cfRNA-based test to be adopted, it must undergo rigorous validation in large-scale, prospective clinical trials. It must demonstrate not only analytical validity (accuracy and reproducibility of the measurement) but also clinical validity (ability to accurately identify the

clinical condition) and, most importantly, clinical utility-proof that using the test leads to improved patient outcomes compared to the current standard of care. Furthermore, the test must be cost-effective for healthcare systems to reimburse it.

In summary, while cfRNA analysis represents a paradigm shift towards non-invasive, dynamic monitoring of health and disease, its path to the clinic is contingent upon overcoming substantial obstacles. The field must prioritize the establishment of standardized pre-analytical protocols to ensure reproducibility. Concurrently, advancements in sequencing sensitivity and, crucially, in bioinformatic tools for deconvolution and interpretation are essential to enhance the specificity of disease detection.

The most promising near-term applications likely lie not in replacing tissue biopsies for primary diagnosis, but in complementing them for longitudinal monitoring. This includes tracking treatment response in cancer patients, monitoring for minimal residual disease or emergence of resistance, and assessing organ health in transplant recipients. As these challenges are systematically addressed through collaborative efforts between researchers, clinicians, and regulatory bodies, cfRNA profiling is poised to become an indispensable tool in the era of precision medicine, transforming patient care from reactive to proactive and predictive.

Abbreviations

AI, Artificial intelligence; cDNA, complementary DNA; cfDNA, cell-free DNA; cfRNA, Cell-free RNA; circRNA, Circular RNAs; CTCs, Circulating tumor cells; dPCR, Digital PCR; ICIs, Immune checkpoint inhibitors; irAEs, Immune-related adverse events; LDCT, Low-dose computed tomography; LncRNA, Long non-coding RNAs; miRNA, MicroRNAs; mRNA, Messenger RNA; NGS, Next-generation sequencing; NSCLC, Non-small cell lung cancer; PCR, Polymerase Chain Reaction; qPCR, Quantitative PCR; RT-PCR, Real-time PCR; RT-PCR, Reverse transcription PCR; SOPs, Standard operating procedures; TKIs, Tyrosine kinase inhibitors; WGS, Whole-genome sequencing.

Data Sharing Statement

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Ethics Approval and Consent to Participate

This study did not involve human or animal subjects, and thus, no ethical approval was required. The study protocol adhered to the guidelines established by the journal.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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