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REVIEW

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Harnessing viral footprints in circulating free DNA (cfDNA) for early cancer detection: A focus on liquid-biopsy-based screening

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Abstract

Viral infections play a significant role in cancer development, making detecting viral signatures a promising approach for early cancer diagnosis. Circulating free DNA (cfDNA), released into the bloodstream by tumors and other cells, has emerged as a powerful biomarker for non-invasive cancer screening. This review explores the potential of cfDNA in detecting virus-associated cancers through the analysis of viral footprints. It provides a comprehensive overview of the biological mechanisms underlying cfDNA release, the role of oncogenic viruses in cancer progression, and the unique viral markers that can be traced within cfDNA. By leveraging viral genetic material, cfDNA analysis offers transformative potential for the early detection of virus-driven malignancies. Highlighting its clinical relevance, this review discusses how cfDNA-based viral biomarker screening could significantly enhance cancer monitoring, improve prognosis accuracy, and pave the way for innovative, non-invasive diagnostic tools and personalized cancer management strategies.

KEYWORDS

circulating free DNA (cfDNA), early cancer detection, liquid biopsy, oncogenic viruses, virus-associated cancers

1 | INTRODUCTION

Cancer affects approximately 1 in 5 people during their lifetime, according to gender-based analysis, indicating that 1 in 9 men and 1 in 12 women die from cancer.[1] Infections account for 15.4% of all cancers and 9.9% are linked to viruses.[2] Infectious agents are estimated to contribute to 17.8% of the global cancer burden, with *Helicobacter pylori*, human papillomaviruses (HPV), and hepatitis B and C viruses (HBV, HCV) accounting for the primary causes of over half of all infection-attributable cancer cases worldwide.[3] Additional contributors include Epstein–Barr virus (EBV), Kaposi sarcoma-associated herpesvirus (KSHV), and human T-cell lymphotropic virus (HTLV-1).[4]

Abbreviations: cfDNA, circulating free DNA; cHL, Hodgkin's lymphoma; ctDNA, circulating tumor DNA; ddPCR, droplet digital polymerase chain reaction; DNA, deoxyribonucleic acid; EBV, Epstein–Barr virus; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HPV, human papillomavirus; HTLV-1, human T-lymphotropic virus type 1; KSHV, Kaposi's sarcoma-associated herpesvirus; MCPyV, Merkel cell polyomavirus; NGS, next-generation sequencing; NPC, nasopharyngeal carcinoma; PCR, polymerase chain reaction; RNA, ribonucleic acid; Vh-DNA, virus-host chimera DNA; VirCAPP-Seq, viral cancer personalized profiling by deep sequencing.

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Understanding the role of oncogenic viruses in cancer development is complex. A single factor cannot explain it because viral infections alone are insufficient to cause cancer and typically require additional factors to contribute to tumorigenesis.[5] Many tumor viruses can remain dormant in the host through a latent state, allowing them to take advantage of the host cell's machinery during cell division.[5,6] These viruses are broadly categorized into those directly causing cancer by encoding oncogenes and those that indirectly promote cancer through chronic inflammation and mutations.[7,8] The diversity and complexity of these oncogenic viruses, such as their distinct replication strategies, host interactions, and carcinogenic mechanisms, highlight the critical need for personalized and disease-specific risk stratification methods to inform targeted prevention, surveillance, and treatment strategies.[9]

Virus-associated cancer rates will continue to rise over the next four decades,[10,11] highlighting the increasing public health concern with substantial societal and economic impacts of these cancers.[12] As such, biomarkers for early screening and prognosis are crucial. Advancements in screening, diagnosis, prevention, and treatment[11,13] contributed to a 27% decrease in cancer-related deaths[14] from 1991 to 2016. Although scientific advancements have provided several drugs to manage various cancers,[15] early detection of cancer remains strongly correlated with improved clinical outcomes, including 1-year survival rates across cancer types, except for lung and ovarian cancers.[16,17] This exception is likely due to their high genomic instability in the early stages of these cancers,[18,19] which complicates effective detection and intervention.

cfDNA has emerged as a promising non-invasive biomarker for early cancer detection and prognosis. Present in low concentrations in the blood of healthy individuals, cfDNA levels are significantly elevated in cancer patients.[20] A part of cfDNA, known as circulating tumor DNA (ctDNA), originates from tumor cells and carries tumor-specific genetic alterations.[21] Advancements in molecular biology techniques and next-generation sequencing (NGS) have heightened interest in ctDNA as a non-invasive biomarker for cancer detection and treatment monitoring.[21,22] Recent clinical trials have revealed the sensitivity and specificity of cfDNA as biomarkers for cancer detection, leading to the Food and Drug Authority's (FDA) approval of multigene assays for detecting genetic alterations in cfDNA.[23] Despite its great potential, cfDNA-based cancer tests require further research before achieving widespread clinical adoption.[22] This review explores the possibility of cfDNA as a non-invasive biomarker for early virus-associated cancer detection, focusing on the detection of viral footprints in cfDNA. We also provide a comprehensive overview of the biological basis of cfDNA release, the role of oncogenic viruses in cancer progression, and the unique viral markers traced in cfDNA while addressing technological advancements and existing limitations.

2 | ONCOGENIC VIRUSES AND THEIR ROLE IN CANCER DEVELOPMENT

Seven viruses are associated with human cancers: EBV, HPV, HCV, HBV, HTLV-1, Merkel Cell Polyomavirus (MCPyV), and KSHV.[24–27] Additionally, Human Immunodeficiency Virus 1 (HIV-1), while not directly oncogenic, is classified as an oncogenic virus due to its ability to cause immunosuppression, increasing susceptibility to carcinogenesis in the presence of other opportunistic infections.[28] Oncogenic viruses are commonly divided into RNA and DNA tumor viruses.[28,29] RNA tumor viruses are categorized based on their mode of transformation. The known RNA tumor viruses are often retroviruses,[29] though HCV is an exception. DNA tumor viruses are further classified by genome size and transformation mechanism. Large DNA viruses such as EBV and KSHV[29,30] transform cells through various mechanisms. In contrast, small DNA viruses such as HPV and polyomaviruses rely on viral oncogene expression to transform host cells. Small oncogenic viruses can escape apoptotic responses while keeping their host alive. This leads to the accumulation of cells with altered genomes.[29] The classification of tumor viruses and their associated cancers is summarized in Figure 1.

Viral-induced carcinogenesis involves multiple mechanisms, including viral integration into host genomes, oncogene activation, immune evasion, and inflammation. Oncogenic viruses can cause DNA damage and genomic instability by targeting tumor suppressors such as p53 and retinoblastoma protein (pRB).[31] They drive cell proliferation, inhibit apoptosis, and activate oncogenes, including mitogen-activated protein kinases, nuclear factor kappa-light-chain-enhancer of activated B cells, and signal transducer and activator of transcription 3 signaling.[32,33] Additionally, they can manipulate host epigenetic machinery, altering gene expression and contributing to tumor progression.[34] Chronic oxidative stress induced by viral infections further contributes to genomic instability and fosters neoplastic transformation.[35] Viral integration in HBV and HPV plays a crucial role in tumor development and is linked to DNA damage.[31] Chronic inflammation is primarily driven by the immune cell-mediated destruction of tissue, which occurs as a result of the immune response triggered by viral infection. However, this immune response is often ineffective in controlling the infection, leading to ongoing tissue damage. For example, in HBV and HPV infections, cytotoxic T cells and inflammatory cytokines induce oxidative stress and genomic instability, driving carcinogenesis despite incomplete viral clearance.[36] Viral immune evasion mechanisms, such as the major histocompatibility complex class 1 (MHC-1) downregulation, exacerbate this process by prolonging infection and inflammation.[37]

3 | BIOLOGICAL BASIS OF cfDNA IN CANCER

cfDNA is released as a single-stranded and/or double-stranded (ds) nuclear DNA and mitochondrial DNA[38,39] into the blood[40] of healthy and cancer patients (Figure 2). It is primarily released from cells through apoptosis, necrosis, and active secretion[41,42] and has a short half-life ranging from 16 min to 2.5 h.[43,44] In patients with advanced lung cancer, cfDNA exists as long fragments (approximately 5 kb in size) and short fragments (approximately 170 bp), with the total amount of cfDNA being higher in cancer patients than

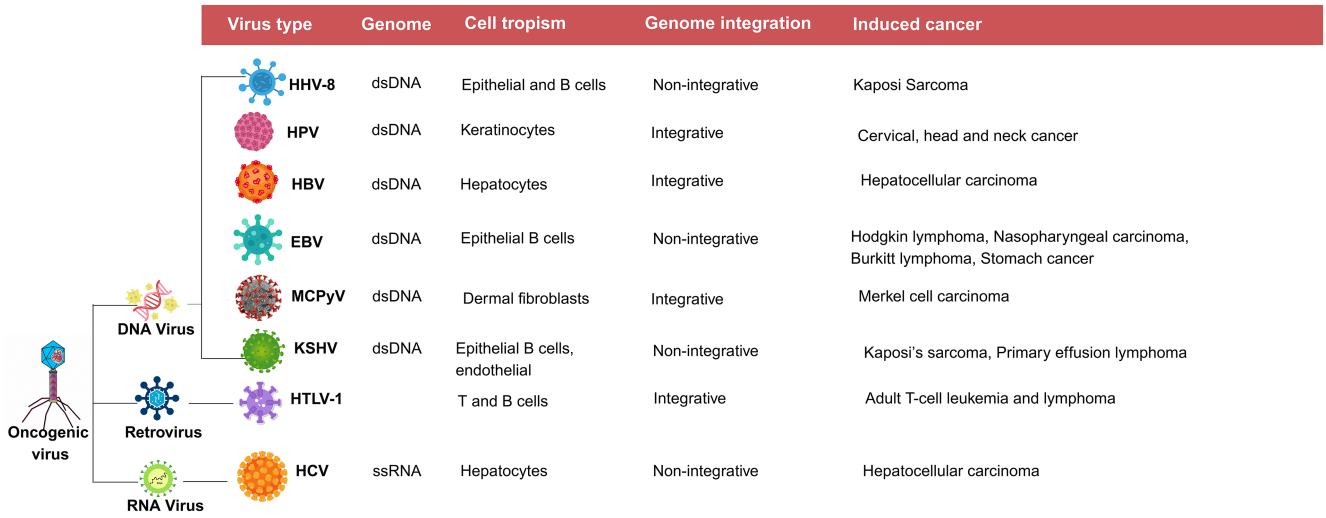
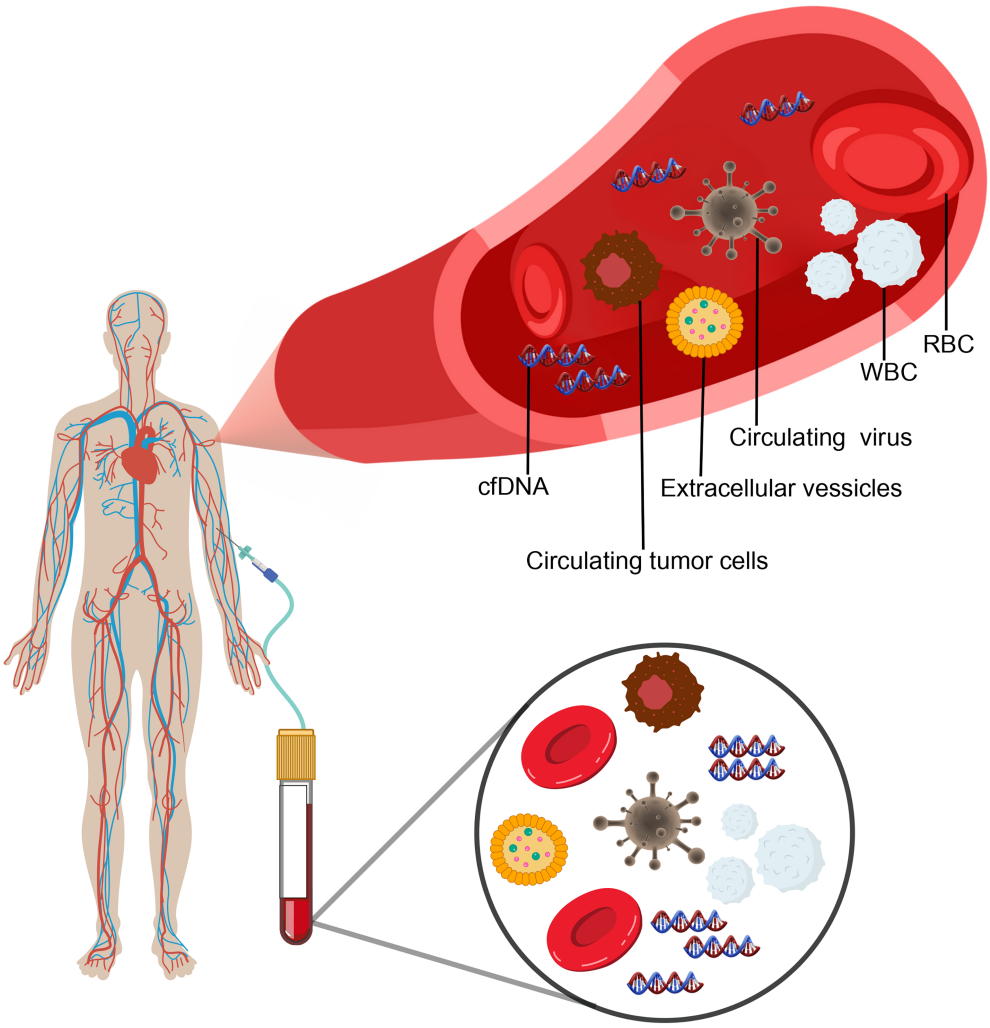


FIGURE 1 Tumor viruses and their cell tropism, host genome integration, and associated cancers. HHV-8, human herpesvirus 8; HPV, human papillomavirus; HBV, hepatitis B virus; EBV, Epstein–Barr virus; MCPyV, Merkel cell polyomavirus; HTLV-1, human T-cell lymphotropic virus type 1; HCV, hepatitis C virus; KSHV, Kaposi's sarcoma-associated herpesvirus. [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 2 Sources of circulating free DNA in the blood. It is primarily released from the cells through apoptosis and necrosis. It is composed of healthy, viral, and tumor DNA (ctDNA). [Color figure can be viewed at wileyonlinelibrary.com]



in healthy individuals.[45,46] It contains genetic and epigenetic information that reflects cellular and tissue status, making it a tool for disease assessment.[47,48] As such, researchers are now using cfDNA analysis to screen for tumors because it consists of healthy and tumor DNA.

ctDNA, a subset of cfDNA, is shed into the bloodstream by tumor cells (Figure 2).[49] It has gained widespread recognition as a noninvasive biomarker for cancer diagnosis and prognosis.[50,51] cfDNA holds great promise for real-time cancer monitoring through liquid biopsy. Elevated cfDNA levels are not only observed in advanced cancer cases but have also been proposed as a diagnostic tool for breast cancer.[52] Mead et al.[53] highlighted the potential combination of cfDNA with other markers to offer a simple blood test as a secondary screening tool for colorectal cancers and polyps. Moreover, cfDNA end motifs were identified by analyzing the first four-nucleotide (4-mer) sequences at each cfDNA fragment end.[54] These 4-mer motifs have been demonstrated to serve as valuable biomarkers, showing promising performance for multi-cancer detection and immunotherapy responses prediction.[47]

4 | ORIGINS AND MECHANISMS OF cfDNA RELEASE

Cell-free DNA (cfDNA) in the human body originates from both endogenous and exogenous sources. Endogenous contributions include DNA released during normal cellular activity, tissue damage, or programmed cell death,[54] while exogenous sources encompass microbial or dietary DNA introduced via infection, inhalation, or digestion.[55] A well-characterized example is fetal DNA released into maternal circulation during pregnancy,[56] which contributes to the cfDNA pool alongside DNA from cancerous and non-cancerous cells.[57] Rostami et al. demonstrated that necrosis, not apoptosis, is the primary mechanism of cfDNA release in certain cancers exposed to ionizing radiation.[58] Nevertheless, the full spectrum of cfDNA release mechanisms remains incompletely defined.[59]

Apoptotic cell death generates mononucleosomal DNA fragments that enter the bloodstream, where nucleosomes and chromatin subunits released during DNA damage or apoptosis are detectable in circulation.[59,60] Beyond apoptosis, specialized physiological processes contribute to the cfDNA pool. For example, during erythroblast enucleation, a critical step in red blood cell maturation, the expulsion of nuclei involves cytoskeletal reorganization (e.g., actin polymerization) and establishment of nuclear polarity, followed by macrophage-mediated engulfment of the extruded nucleus, which releases cfDNA fragments into the bloodstream.[57,61–63] Similarly, neutrophil extracellular traps (NETs)—a defense mechanism where neutrophils extrude chromatin webs to trap pathogens—release cfDNA during infections or inflammatory conditions through a process termed NETosis.[64] These pathways highlight the complexity of cfDNA sources and signify the importance of distinguishing cellular origins when interpreting cfDNA profiles in clinical or research settings.

5 | MECHANISTIC AND FUNCTIONAL INSIGHTS INTO cfDNA DYNAMICS

While apoptosis, necrosis, and active secretion are established sources of cfDNA,[41,42,55] the biochemical pathways governing its release and stability warrant deeper elaboration. During apoptosis, nuclear DNA is cleaved into ~180-bp fragments by caspases, which are transiently packaged into apoptotic bodies. However, subsequent nuclease degradation releases these fragments as free, unbound cfDNA into circulation, explaining why most circulating cfDNA is not vesicle-encapsulated.[56] In contrast, necrosis releases longer, irregular fragments (>1000 bp) due to uncontrolled membrane rupture. Active secretion via extracellular vesicles (e.g., exosomes) contributes minimally (<5% of total cfDNA) compared to apoptotic/necrotic sources.[57] The stability of cfDNA is influenced by its association with plasma proteins (e.g., histones, albumin) that protect against nucleases, prolonging its half-life to 16 min to 2.5 h.[38] Beyond passive release, cfDNA may exert biological functions: upon uptake by recipient cells via endocytosis, it can activate Toll-like receptor 9 (TLR9) mediated inflammation or integrate into genomes, potentially driving metastasis.[58,59] Tumor-derived cfDNA, for instance, triggers neutrophil extracellular traps, fostering pro-metastatic microenvironments.[60] These mechanistic insights highlight the dual role of cfDNA as both a biomarker and a potential mediator of disease progression.

6 | VIRAL FOOTPRINTS IN cfDNA: MECHANISMS AND DETECTION

When viral DNA genomes, or fragments thereof, get integrated into the host genome, they can cause cancer, which is observed in the cases of HPV in cervical and head-and-neck cancers, as well as with HBV in liver cancer.[61,64] This integration process is influenced by viral gene products that disrupt the DNA repair pathways.[62,63] cfDNA captures genetic material from viruses and indicates viral infection in diseases such as nasopharyngeal carcinoma (NPC).[65–67] Garofalo et al.[68] described how Viral Cancer Personalized Profiling by Deep Sequencing (VirCAPP-Seq), used for profiling viral cfDNA, detects and quantifies clinically relevant viral genetic material within cfDNA. This method identifies virome composition, fragment length distribution, host genome integration sites, and viral polymorphisms.

Viral genetic material can be packaged within extracellular vesicles, such as exosomes, and released by infected cells.[69] These vesicles carry diverse molecules, including DNA, RNA, and proteins.[70] Recent studies have explored the potential of viral genetic signatures in cfDNA as biomarkers for virus-related cancers. For example, HPV E7 cfDNA demonstrates high specificity for cervical cancer detection using droplet digital PCR and rapid isothermal detection methods.[71] Cheung et al.[71] reported the detection of HPV E7 and L1 sequences in cfDNA from cervical cancer patients with high viral load, indicating an increased risk of recurrence. In oropharyngeal squamous cell carcinoma patients, plasma HPV cfDNA levels correlated with tumor size and clinical outcome, suggesting its utility in monitoring treatment

response and disease progression.[72] Additionally, virus-host chimera DNA (vh-DNA) has been proposed as a novel biomarker for virus-related cancers. The integration of viral DNA into host genomes generates the unique vh-DNA fragments which can be enriched and utilized as a specific marker for tracking viral cancers in clinical settings, offering advantages such as reduced background interference and tumor localization.[73]

While cfDNA analysis offers a minimally invasive alternative to tissue biopsy, it has notable limitations compared to histopathology. Critically, cfDNA is tissue-agnostic and cannot localize the anatomical source of viral DNA (e.g., distinguishing EBV in nasopharyngeal carcinoma vs. latent infection in B-cells) or provide spatial context about disease progression (e.g., tumor-stroma interactions, inflammatory infiltrates).[74,75] Conversely, cfDNA outperforms serological detection for non-integrating viruses like EBV. Serology detects host antibodies (IgG/IgM), which may persist for years after resolved infections or fail to distinguish latent from active replication.[76] In contrast, cfDNA directly quantifies cell-free viral genomes released during lytic replication or tumor cell lysis, enabling real-time monitoring of viral load dynamics and treatment response.[77] For example, EBV cfDNA levels correlate with nasopharyngeal carcinoma tumor burden and predict relapses earlier than imaging or antibody titers.[78]

7 | cfDNA AS A BIOMARKER FOR VIRUS-ASSOCIATED CANCER DETECTION

cfDNA analysis has emerged as a promising non-invasive alternative to traditional tissue biopsies in cancer management (Table 1). This liquid biopsy approach captures tumor heterogeneity, enables real-time disease monitoring, and facilitates repeated sampling.[90,91] By carrying genomic, epigenomic, and fragmentomic signatures from tumor cells—including mutations, methylation patterns, and nuclease-mediated fragmentation profiles, cfDNA facilitates early cancer detection, prognosis assessment, and prediction of therapeutic response.[92] Advances in genomic technologies, such as NGS and digital PCR, have greatly improved the sensitivity of mutation detection in cfDNA, revolutionizing its role in clinical practice.[91]

The profiles of microbial cfDNA sequences indicate their prognostic value in cancer patients.[93] HPV cfDNA was successfully detected in 69% of patients before chemo-radiotherapy for locally advanced cervical cancer (LACC). According to Cabel et al.,[83] HPV-ctDNA levels are also associated with tumor HPV copy number ($r = 0.41$, $p < .001$). EBV serological antibody testing and plasma EBV-DNA analysis have emerged as tools in NPC screening and have contributed to early diagnosis and treatment.[94] Ji et al.[95] reported a sensitivity of 81.5% for EBV cfDNA in the initial screening of early NPC. Garofalo et al.[96] also demonstrated the effectiveness of cfDNA-based viral detection in early classical Hodgkin's Lymphoma (cHL). Using VirCAPP-Seq, they examined the circulating virome in cHL patients and identified correlations between EBV cfDNA levels and disease characteristics. The elevated plasma EBV loads also correlated with tumor EBV status and disease severity. The VirCAPP-Seq allows for the characterization of viral cfDNA, including virome composition, fragment length distribution, host genome integration sites, and viral polymorphisms.[68] Hanna et al.[82] found a relationship between total tumor burden and HPV cfDNA levels, which were detectable earlier than imaging scan changes. This suggests that monitoring HPV cfDNA levels could serve as an early indicator of cancer, allowing for earlier intervention.

Bønløkke et al.[97] analyzed pre-treated plasma samples from cervical cancer (CC) patients and detected HPV cfDNA in varying proportions across patient groups, with higher detection rates in patients undergoing oncological therapy. This study revealed an association between cfDNA levels, HPV16 integration status, and disease severity, suggesting that cfDNA-based viral detection could provide valuable insights into disease progression. This could guide treatment decisions in selecting patients for adjuvant oncological therapy. Similarly, Thangarajah et al.[98] also utilized droplet digital PCR (ddPCR) to quantify HPV cfDNA as a biomarker for monitoring molecular therapy in HPV-driven cervical and vulvar cancers. Their findings showed that HPV cfDNA levels increase with the disease stage, highlighting its potential utility in advanced cancer management. In another study, peripheral blood samples were obtained from HBV-related HCC patients, and the total plasma viral cfDNA was quantified using a fluorometric double-stranded DNA assay to predict early recurrence of HBV-related HCC.[99] In 2019, a study found cell-free chimeric DNA, made up of both human and HBV DNA, in the blood of 20 patients with chronic HBV infection. This chimeric DNA came from 87 sites where HBV had integrated into the human genome. Interestingly, these integration sites were often located in genes related to tumors. This suggests that the chimeric DNA in blood is a potential biomarker for diagnosing liver cancers.[100] Zheng et al.[101] also found that the most recurrent integration events detected in blood cfDNA had originated from tumor tissues. This concludes the potential utility of non-invasive detection of HBV-DNA integration as a circulating tumor marker for HBV-related HCC.

8 | CURRENT TECHNOLOGIES FOR DETECTING VIRAL FOOTPRINTS IN cfDNA

Liquid biopsy offers a non-invasive alternative to tissue biopsy, addressing issues such as sampling bias and tissue heterogeneity.[102,103] cfDNA has emerged as a valuable tool, particularly in oncology, to enable minimally invasive diagnosis, treatment, and disease monitoring. However, isolating cfDNA from plasma is challenging due to its low concentration, with only a range from 0.1% to ~1% of total cfDNA contributed by tumor cells.[104] The tumor fraction and concentration of cfDNA have a strong correlation with the tumor burden in tumors. Besides, the ratio of ctDNA in cfDNA has been associated with tumor size, stage, and metastasis in multiple studies. Effective extraction methods are crucial for achieving high yields and purities.[50,103,105]

Various pre-analytical factors influence the quality and quantity of cfDNA, including matrix type, sample collection tubes, centrifugation

TABLE 1 Summary of studies showcasing virus-associated cancer detection and prognosis using cell-free DNA.

Authors	Patient population	Study design	Sample size	Cancer type	CfDNA type	Detection method	Key findings	Inference
Kang et al., 2017[79]	HPV16- or HPV18-positive metastatic cervical cancer patients	Retrospective study	19	Metastatic cervical cancer	HPV cfDNA	Duplex digital droplet PCR	Sensitivity: 100% Specificity: not reported	HPV cfDNA is a biomarker for detecting, monitoring, and genotyping metastatic cervical cancer for targeted therapies
Chan et al., 2017[66]	Chinese men who were 40 to 62 years of age	Prospective study	20,174	Nasopharyngeal carcinoma (NPC)	EBV cfDNA	Real-time PCR	Sensitivity: 97.1%, Specificity: 98.6% for EBV DNA in detecting nasopharyngeal carcinoma	EBV DNA analysis in plasma samples effectively screens for early asymptomatic nasopharyngeal carcinoma
Lv et al., 2024[80]	NPC Patients Undergoing Sequential Chemo-Radiotherapy	Prospective study	1000	Nasopharyngeal carcinoma (NPC)	EBV cfDNA	Quantitative PCR-based assay	cfEBV DNA demonstrated sensitivity to treatment changes	On-treatment cfEBV DNA is a strong prognostic biomarker for cancer assessment
Adrian et al., 2023[81]	Patients with p16- positive Oropharyngeal carcinoma (OPSCC)	Prospective phase III multicenter study	136	Oropharyngeal carcinoma (OPSCC)	HPV16-ctDNA	Real-time quantitative PCR	Sensitivity: 79.4% pre-treatment; post- treatment clearance: 74%	ctHPV16-DNA is an independent prognostic factor in HPV-related OPSCC
Hanna et al., 2018[82]	Patients with recurrent, metastatic HPV+ head and neck cancer	Pilot observational cohort study	22	Oropharyngeal cancer (OPC)	HPV cfDNA	Droplet digital (dd) PCR	HPV cfDNA levels strongly correlate with total tumor burden, disease site, and prognosis	cfHPV DNA levels are a promising biomarker for assessing tumor burden in metastatic HPV- positive oropharyngeal cancer
Reder et al., 2020[72]	Patients with HPV16-driven Oropharyngeal carcinoma (OPSCC)	Pilot observational study	30	Oropharyngeal carcinoma (OPSCC)	HPV cfDNA	Real-time quantitative PCR (qPCR)	Plasma cfHPV- DNA detection correlates with disease progression in HPV-driven OPSCC	cfHPV-DNA in liquid biopsies is a promising biomarker in HPV- driven OPSCC
Cabel et al., 2021[83]	Patients with HPV-positive Locally advanced cervical cancer (LACC)	Retrospective cohort and prospective cohort study	55	Locally advanced cervical cancer (LACC)	HPV cfDNA	Digital droplet PCR.	Sensitivity: 69% in detecting LACC before chemoradiotherapy (CRT)	HPV-ctDNA detection was associated with tumor stage and lymph node status
Galati et al., 2022[84]	Women with HPV16-positive cervical cancer	Retrospective study	180	Cervical squamous cell carcinoma	HPV ctDNA	Bead-based HPV genotyping assay	Sensitivity: 74.7% Specificity: 97.8% for detecting HPV16- positive cervical cancer	Combined HPV markers (HPV16 ctDNA and E6 antibodies) may enhance sensitivity for early-stage cervical cancer detection
Jeannot et al., 2021[85]	Patients with HPV16- or HPV18-related cervical cancers	Prospective cohort study	94	Cervical cancers	HPV ctDNA	Digital droplet PCR	Sensitivity: 63% in detecting cancer before treatment	Patients with residual HPV ctDNA at the end of treatment have a high risk of cancer recurrence
Sivars et al., 2024[86]	Patients with locally advanced cervical cancer	Prospective observational cohort study	74	Locally advanced cervical cancer (LACC)	HPV ctDNA	Droplet digital PCR assays	ctHPV DNA was found in plasma before relapse was diagnosed	ctHPV DNA in follow-up plasma is a promising biomarker for prognosis and early relapse detection in patients with locally advanced cervical cancer (LACC)

TABLE 1 (Continued)

Authors	Patient population	Study design	Sample size	Cancer type	CfDNA type	Detection method	Key findings	Inference
Shoda et al., 2017[87]	Gastric cancer patients who underwent surgery	Prospective cohort study	153	Gastric cancer	EBV cfDNA	Quantitative real-time PCR	Sensitivity: 71.4% Specificity: 97.1% in detecting gastric cancer (GC)	Plasma EBV in EBVaGC reflects tumor burden and is a sensitive, cost- effective marker for monitoring tumor progression and treatment response
He et al., 2023[88]	Patients with pretreatment plasma EBV DNA	Retrospective study	1405	Nasopharyngeal carcinoma (NPC)	EBV cfDNA	Real-time quantitative PCR assay	Sensitivity: 41.5% Specificity: 99.3% in detecting nasopharyngeal carcinoma (NPC)	Pretreatment plasma EBV DNA is useful for NPC screening and patient management
Chen et al., 2021[89]	Participants seropositive for EBV VCA-IgA and EBNA1-IgA in a community- based NPC screening	Community-based screening cohort	1363	Nasopharyngeal carcinoma (NPC)	EBV cfDNA	Real-time quantitative PCR	Elevated plasma EBV DNA levels correlate with increased nasopharynge al carcinoma (NPC) incidence	Plasma EBV DNA monitoring complements EBV serological screening for nasopharyngeal carcinoma (NPC)

protocols, thawing procedures, DNA isolation methods, storage of isolated DNA, quantification methods, and downstream analysis.[102] Commercially available extraction kits utilize different binding methods, such as ethanol precipitation, spin columns, magnetic beads, and polymer-mediated enrichment technology. The state of automation of these kits depends solely on the protocol used; however, magnetic bead-based methods are often automated.[106] Among these, the QIAamp kit by QIAGEN is widely considered the gold standard, although selecting the appropriate kit depends on multiple factors such as sample type, efficiency rate, and downstream applications.[102,107]

Numerous methods have been developed to detect viral DNA within cfDNA, including RT-qPCR, digital PCR, and NGS-based approaches[108] (Table 2), single-molecule real-time (SMRT),[138] Nanopore sequencing,[139] and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) based diagnostics methods.[140] These techniques allow the analysis of mutational signatures, evaluation of copy number aberrations, and assessment of tumor heterogeneity.[141] Reverse transcription quantitative polymerase chain reaction (RT-qPCR) demonstrated great potential for detecting viral cfDNA for cancer screening.[142] It revealed significant differences in cfDNA concentration between breast cancer patients and healthy individuals. It also showed potential as a sensitive and specific method for viral DNA detection in cfDNA analysis. Crucitta et al.[143] compared Droplet digital polymerase chain reaction (ddPCR) and solid digital PCR (sdPCR) for detecting epidermal growth factor receptor and rat sarcoma (RAS) mutations in cfDNA from lung and colorectal cancer patients. Although both platforms showed similar specificity, sdPCR demonstrated higher sensitivity and detected mutations in a large number of patients, even with low cfDNA abundance. NGS-based assays, including Tagged-amplicon Deep Sequencing (TAm-Seq), Cancer Personalized Profiling by Deep Sequencing (CAPP-Seq), Whole Genome Bisulfite Sequencing (WGBS-Seq), Whole Exome Sequencing (WES), and Whole Genome Sequencing (WGS), offer a comprehensive approach to analyzing viral cfDNA fragments.[100,144] Sorbini et al.[144] suggested that NGS allows the detection of low-abundance viral targets within cfDNA, which is important for liquid biopsy applications. While NGS provides a broad evaluation of tumor mutations and oncogenes, its cost-effectiveness and efficiency are influenced by assay design and coverage depth.[145] Third-generation sequencing technologies, such as SMRT and Nanopore sequencing, detect DNA through optical or electrical signal detection methods,[146] revealing a substantial population of analyzable long cfDNA in plasma. Yu et al.[139] reported that single-molecule tissue-of-origin analysis using Nanopore and PacBio (SMRT) sequencing showed significantly higher liver-derived cfDNA percentages and elevated HCC methylation scores in patients with hepatocellular carcinoma (HCC) compared to HBV carriers. By setting a threshold of three standard deviations above the mean HCC methylation scores in non-HCC subjects, both sequencing methods achieved a sensitivity of 75% and a specificity of 100%.[139]

In addition to NGS and digital PCR methodologies, CRISPR-based diagnostic methods have recently emerged as innovative approaches aimed at enabling rapid, accurate testing at home. CRISPR-based diagnostic systems leverage Cas proteins, primarily Cas12 and Cas13, for

TABLE 2 Comparing technologies for detecting cfDNA.

Technology	Platform	Sensitivity	Specificity	cfDNA input	No. of targets	Type of alteration	Limitations	Clinical utility
NGS	Deep sequencing (>10,000×)	0.02%	80%–90%	2 ng	Panel	Genome-wide copy number changes	Unable to detect rearrangements without assay customization	Non-invasive tumor DNA detection, mutation screening, and targeted therapy selection[109–111]
	TAm-Seq	0.02%	99.99%	0.9–20 ng	Panel	Known point mutations	Detects only known mutations	Detection of low-frequency cancer mutations in circulating DNA[112]
	Safe-SeqS	0.10%	98.90%	3 ng	Panel	Known point mutations and copy number variations	Less comprehensive than WES	Accurate detection of rare mutations in circulating DNA[113,114]
	FASTSeqS	>10%	80%	5–10 ng	Panel	Genome-wide copy number changes	Low sensitivity and specificity	Estimation of circulating DNA levels[115,116]
	CAPP-Seq	0.004%	>99.99%	32 ng	Panel	Known point mutations, copy number variations, and rearrangements	High cfDNA input; detects only known mutations	High sensitive detection of circulating DNA[117–119]
	Multiplex-PCR NGS	>0.1%	99.6%	2–50 ng	Panel	Known point mutations	Detects only known mutations	Detection of circulating DNA in early-stage lung cancer[120]
Digital-PCR	ddPCR	0.10%	100%	25 ng	1–3	Known point mutations	Detects specific genomic loci; limited in multiplexing	Accurate detection of mutations in circulating DNA[121–123]
	BEAMing	0.01%	100%	1 ng	1–20	Known point mutations	Detects only known mutations	Detection of mutations (e.g., EGFR, PIK3CA) in circulating DNA and monitoring resistance in mutations (e.g., T790M)[124–127]
Real-Time PCR	AS-PCR	1.00%	98%	3–50 ng	1	Known point mutations	Low sensitivity; detects known mutations	Detection of single-base mutations or small deletions in circulating DNA[128–130]
	AS-NEPB-PCR	0.10%	100%	20 ng	1	Known point mutations	Detects only known point mutations	Detection of low-frequency mutations in cfDNA[131]
	(PNA-LNA) PCR clamp	0.1%–1%	79%	30 ng	1	Known point mutations	Low specificity; detects only known point mutations	Detection of mutations in plasma cfDNA[132–134]
	(COLD-PCR)	0.10%	94.9%	1–10 ng	45,294	Known point mutations	Detect limited genomic loci; limited in multiplexing	Detection of mutation in cfDNA[135]
	MS-PCR	0.62%	100%	20–100 ng	1	Known methylation sites	Detects only specific CpG islands	Detection of methylation in liquid biopsies[136]

Abbreviations: (PNA-LNA) PCR clamp, peptide nuclei acid-locked nucleic acid; AS-NEPB-PCR, allele-specific, non-extendable primer blocker PCR; AS-PCR, allele-specific amplification; BEAMing, beads, emulsion, amplification and magnetics; CAPP-Seq, Cancer Personalized Profiling by Deep Sequencing; COLD-PCR, co-amplification at lower denaturation temperature; ddPCR, droplet digital polymerase chain reaction; MS-PCR, methylation- specific PCR; Safe-SeqS, safe-sequencing system; TAm-Seq, tagged-amplicon deep sequencing; WES, whole exome sequencing.[137]

highly sensitive molecular detection. When the CRISPR-associated protein (Cas) or single guide RNA (sgRNA) complex binds to a specific target sequence, it becomes activated, triggering nonspecific cleavage of single-stranded DNA or RNA probes in solution. This process facilitates nucleic acid sensing through signal amplification and enables various detection methods by incorporating functionalized reporter nucleic acids, which are cleaved as a result of collateral activity.[147] Freije et al.[148] demonstrated the broad applicability of

Cas13-based diagnostics by identifying thousands of potential Cas13 crRNA target sites across various single-stranded RNA (ssRNA) viruses capable of infecting humans, highlighting its potential in sensitive viral diagnostics.

9 | RECENT ADVANCEMENTS IN cfDNA ANALYSIS AND THEIR IMPACT ON CLINICAL PRACTICE

New advances in analyzing cfDNA have enhanced its application in cancer screening and monitoring.[149] In 2023, researchers from the David Geffen School of Medicine, University of California, and Stanford University published a comprehensive map of DNA methylation patterns using 521 healthy tissue samples from 29 organs.[150] This map assists in pinpointing the sources of cfDNA in the blood, revealing organ-specific damage caused by diseases. They created the deep neural network (DNN)-based model, cfSort, which utilizes this data to accurately trace cfDNA origins, surpassing older methods. While traditional methods focus on single features like DNA methylation, a new method called Multimodal epigenetic sequencing analysis (MESA) developed by Li et al.[151] captures four epigenetic features at once—methylation, DNA packaging, variations in DNA packaging, and protection patterns near gene regions—using a single, cost-effective test. In tests on 690 blood samples from colorectal cancer patients, MESA outperformed older methods by combining these features, especially using new signals like DNA packaging variability and gene end-region patterns.

Furthermore, Martin-Alonso et al.[152] have explored a new strategy to further enhance cfDNA detection by transiently modulating cfDNA clearance in vivo. Their approach involved using intravenous priming agents administered shortly before a blood draw. One strategy employs succinyl phosphoethanolamine–based liposomal nanoparticles that reduce the uptake of cfDNA by liver macrophages, while a complementary approach uses engineered DNA-binding monoclonal antibodies to protect cfDNA from degradation. These priming agents were shown to increase ctDNA recovery by more than tenfold in preclinical cancer models, enabling more comprehensive tumor profiling and dramatically improving sensitivity in detecting small tumors. Adding to these developments, Esfahani et al.[153] have introduced a novel method based on promoter fragmentation entropy (PFE) to measure the variability in the length of cfDNA fragments. Their findings revealed a positive correlation between PFE and gene expression in peripheral blood cells, and their method was effective in detecting lung cancer with an AUC of 0.83. This approach was able to evaluate responses to chemotherapy in patients with various B-cell lymphomas.

10 | CURRENT CHALLENGES IN cfDNA IN CANCER DETECTION

When an individual is infected, genetic material from the invading virus can be found in their blood plasma as part of cfDNA. This has proven valuable for diagnosing and monitoring virus-associated cancer patients. However, studies have shown that viral cfDNA in plasma may not always be cancer-associated, as cfDNA can also originate from tissue injury, inflammation, and infection unrelated to cancer.[6] Generally, the analysis of cfDNA in blood for cancer screening faces several challenges. These include low concentrations of cfDNA, short fragment length, susceptibility to chemical damage, high sequence similarity between cancer-derived and healthy DNA, and the need for simultaneous analysis of multiple markers.[38,154] Additionally, circulating tumor cells (CTCs) could be considered as a complementary liquid biopsy material for viral detection. CTCs are shed from primary or metastatic tumors into the bloodstream and carry genomic material from their tumor of origin.[155] This makes them a potential source for detecting virus-integrated tumor genomes, such as HPV-driven cervical cancer,[156] EBV-associated nasopharyngeal carcinoma,[157] and HBV-driven hepatocellular carcinoma.[158] CTC analysis could provide insights into viral integration events or the presence of episomal viral DNA at the single-cell level. However, CTCs are exceedingly rare in circulation, and not all tumor cells harbor detectable viral sequences.[159] Moreover, viral DNA within the CTC genome may exist at extremely low copy numbers, posing significant challenges for detection. Moving forward, integrating cfDNA and CTC analyses could enhance the sensitivity and specificity of viral detection in liquid biopsy, offering a more comprehensive understanding of virus-associated cancers.

Despite significant advancements in sequencing technologies, library preparation methods, and bioinformatics tools over the past 15 years, pre-analytical factors also limit clinical sensitivity. No dominant technology has yet addressed these challenges.[154] For instance, Huang et al.[93] reported that viral cfDNA detected in early-onset breast cancer patients was not necessarily present or was present at very low levels when the disease was fully established, complicating the use of cfDNA-based detection methods for accurate cancer diagnosis and screening. The high degree of fragmentation in viral-derived cfDNA, as noted by Phung et al.,[160] further complicates the enrichment and differentiation of viral sequences from the predominant human-derived cfDNA. While plasma EBV cfDNA is readily detected in EBV-associated malignancies, its specificity is insufficient to diagnose lymphoma, as individuals with HIV or other immunosuppression can show elevated levels of cfDNA, leading to false positives or negatives.[161,162] Moreover, interpreting results from cfDNA analysis requires careful consideration of factors such as tumor heterogeneity, variations in virus integration sites, and the potential for false positives.[163] Conventional PCR methods, although widely used, exhibit low sensitivity for serum HPV DNA, limiting their utility as biomarkers for early detection of HPV-related cancers.[84,163] While biological and technical challenges persist, careful optimization of viral cfDNA analysis holds promise for offering valuable information for early cancer detection.[164]

Clinical and ethical concerns regarding implementing the cfDNA-based assay in clinical settings have been raised. One important consideration is the timing of sampling, as various factors can influence ctDNA levels, including treatments being received, concurrent

inflammatory processes, and recent surgeries. For instance, when detecting minimal residual disease (MRD) post-surgery, blood sampling should ideally occur at least 1 week after surgery, or longer for major surgeries, to allow for proper healing and to minimize background interference.[165]

Multi-cancer early detection (MCED) tests using ctDNA are under development, with reported sensitivities ranging from 50% to 70% at high specificities.[166] However, differences in ctDNA release and detectability across cancer types and stages present significant challenges, as tumors of different histologies and progression levels shed varying amounts of ctDNA into the bloodstream.[167] Distinguishing between tumor-derived and non-tumor-derived ctDNA can also pose significant challenges in using ctDNA in cancer detection. This can result from clonal hematopoiesis of indeterminate potential (CHIP), a phenomenon in which age-related somatic mutations arise in non-malignant hematopoietic cells and may be misclassified as tumor-derived alterations, leading to false-positive results if not properly accounted for during analysis.[168] These tests also face challenges related to clinical utility and the potential harms of false positives.[169] Key ethical considerations include defining screening objectives, minimizing risks, and ensuring benefits outweigh harm for participants.[169] The non-invasive nature of cfDNA testing may lead to broader uptake and potential direct consumer access, highlighting the need for careful implementation strategies and robust informed consent processes.[170] While ctDNA has valuable applications in prognosis and therapy monitoring, its application in early cancer detection remains controversial. Further validation through large-scale studies involving non-patient populations is essential to establish its clinical relevance and address concerns about accuracy and effectiveness.[166,169]

Economic considerations also play a key role in the widespread implementation of cfDNA-based assays. HPV DNA testing for cervical cancer, beginning at age 30 and repeated at 5-year intervals, has been identified as the most cost-effective strategy.[171] In cancer immunotherapy monitoring, tumor cfDNA testing can lead to significant cost savings by enabling the early evaluation of treatment efficacy.[172] However, early cancer detection using ctDNA faces challenges such as the requirement for large blood volumes and high sequencing costs. The application of cfDNA analysis in viral-associated cancers is evolving rapidly, offering the potential to improve diagnostic and monitoring capabilities.[6] To achieve cost-effective, large-scale implementation in public health systems, integrating cfDNA testing with other blood-based biomarkers and employing longitudinal monitoring may be necessary to overcome current limitations.

11 | FUTURE DIRECTION

Multi-omics integration—combining methylation, fragmentomics, and protein biomarkers—offers improved specificity. Methylation signatures at viral integration sites (e.g., HPV in cervical cancer) may distinguish malignancies from transient infections.[173] Long-read sequencing and AI-driven fragmentomics could achieve >95% accuracy in discerning cancer-derived cfDNA via end motifs and nucleosome patterns.[174] Longitudinal monitoring through serial HPV cfDNA testing demonstrated promising results for detecting cervical cancer recurrence.[175]

Through non-invasive blood-based testing, ctDNA shows further promise for early cancer detection and tumor site identification. By analyzing molecular markers such as somatic mutations, DNA methylation patterns, and fragmentomic profiles, it is feasible to diagnose cancers early on[176] and determine their tissue of origin.[177] However, for cfDNA-based screening to become clinically practical, additional breakthroughs are required to improve sensitivity in early-stage disease, improve tissue-of-origin prediction accuracy, and minimize false-positive rates. Integrating multi-omics techniques, fine-tuning machine learning algorithms, and standardizing pre-analytical and analytical workflows will be critical. Large-scale prospective validation studies in different, asymptomatic groups, as well as cost-effectiveness assessments and clear therapeutic guidelines, will be required to ensure widespread uptake and responsible clinical application.

12 | CONCLUSION

Detection of viral footprints in circulating free DNA (cfDNA) represents a significant advancement in cancer screening, particularly for virus-associated malignancies. By identifying viral genetic material within cfDNA, this approach not only enhances the early diagnosis of cancers linked to oncogenic viruses but also provides a non-invasive method for monitoring disease progression and treatment response. Given that viral infections contribute to approximately 15.4% of all cancers, integrating cfDNA analysis into routine clinical practice has the potential to revolutionize cancer screening and management, paving the way for personalized medicine.

Looking ahead, the future of cfDNA research is poised for remarkable breakthroughs. Innovations in sequencing technologies, such as NGS and digital PCR, are expected to enhance the sensitivity and specificity of cfDNA detection, enabling the discovery of novel biomarkers and improving our understanding of tumor heterogeneity. Additionally, combining cfDNA analysis with other biomarkers could facilitate comprehensive cancer profiling and more effective therapeutic strategies. As the field evolves, cfDNA is anticipated to play a central role in clinical oncology, driving earlier interventions and better patient outcomes.

However, to fully realize the potential of cfDNA as a biomarker for virus-associated cancers, further rigorous research and clinical validation remain essential. Key existing challenges include pre-analytical factors, assay validation, low concentration of cfDNA in blood samples, and the complexities of distinguishing between viral and non-viral DNA fragments. Addressing these hurdles through focused studies will be critical to ensuring the effective clinical application of cfDNA analysis. The call to action is clear: continued investment in cfDNA research is imperative to unlock its transformative potential in cancer diagnostics and management, ultimately enhancing cancer detection and treatment strategies.

AUTHOR CONTRIBUTIONS

Richard Donkor Amponsah: Conceptualization; writing – original draft; writing – review and editing; methodology; visualization; software. **Emmanuel Addai Gyabaah:** Writing – original draft; writing – review and editing. **Moro Amidu:** Writing – original draft; writing – review and editing. **Zhao Cheng:** Supervision; writing – review and editing; methodology; visualization; validation; software. **Fazlur Rahman Talukdar:** Conceptualization; supervision; writing – review and editing; methodology; visualization; validation; software.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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