Circulating cell-free DNA as a diagnostic and prognostic marker for cervical cancer

Preetiparna Parida,[1] Gayathri Baburaj,[2] Mahadev Rao,[2] Shirley Lewis,[3] Rama Rao Damerla [1]

For numbered affiliations see end of article.

Correspondence to
Rama Rao Damerla, Department of Medical Genetics, Kasturba Medical College Manipal, Manipal, Karnataka 576104, India; rama.damerla@manipal.edu and Shirley Lewis, Department of Radiotherapy and Oncology, Kasturba Medical College Manipal, Manipal Academy of Higher Education, Manipal, Karnataka 576104, India; shirley.salins@manipal.edu

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ABSTRACT

Circulating cell-free DNA (cfDNA) is a promising tool for liquid biopsy-based tests. cfDNA has been reported to help in the diagnosis, quantification of minimal residual disease, prognosis, and identification of mutations conferring resistance in various types of cancers. Cervical cancer is the fourth most common cancer among women worldwide. High-risk human papillomavirus (hr-HPV) infections have been associated with almost all cervical cancers. Lack of HPV vaccines in national vaccination programs and irregular screening strategies in nations with low or moderate levels of human development index have led to cervical cancer becoming the second leading cause of cancer mortality in women. As HPV integration and overexpression of E6/E7 oncoprotein are crucial steps in the development of cancer, HPV cfDNA could potentially be used as a specific biomarker for the detection of cervical cancer. Many studies have used HPV cfDNA and other gene mutations or mRNA expression profiles for diagnosis and disease surveillance in patients with cervical cancer at various stages of disease progression. In this review we present an overview of different studies discussing the utility of cfDNA in cervical cancer and summarize the evidence supporting its potential use in diagnosis and treatment monitoring.

INTRODUCTION

Cervical cancer continues to be the second most common cause of death among women in developing nations and the fourth most common cause of cancer in women worldwide.[1 2] According to estimates, it caused 342 000 deaths and 604 000 new cases worldwide in 2020. Up to 90% of the estimated cervical cancer deaths in 2020 were reported in low- and middle-income nations.[1] Ninety-five percent of cervical cancer cases are caused by persistent infection with carcinogenic human papillomavirus (HPV).[3] The dysregulation of the tumor suppressor proteins p53 and pRB by HPV oncoproteins E6 and E7, respectively, contribute significantly to the development of cancer.[4] National vaccination programs in several countries have resulted in a rapid decrease in the prevalence of high-risk HPV (hr-HPV) types, translating into a decrease in the incidence of cervical cancer.[5] However, vaccination programs for hr-HPV are not commonplace in developing and underdeveloped countries, resulting in cervical cancer being a major public health problem.[6]

The commonly used methods for screening of cervical cancer include Papanicolaou (Pap) smear, liquid-based cytology, HPV DNA testing, visual inspection with acetic acid or Lugol's iodine (VIA/VILI).[7] These screening methods can suggest the presence of cancer, but only tissue biopsy provides a definitive diagnosis.[8] Nevertheless, these methods have some limitations, including false negativity[9 10] or false positivity.[11] The major problem with cytology-based methods is the inter-observer and intra-observer variations observed among pathologists, which makes diagnosis of cervical cancer more complicated.[12] Despite the fact that tissue biopsies are the gold standard for tumor diagnosis and screening, they are invasive, painful, and often associated with a risk of complications like local infections, bleeding, and damage to nearby tissue. Furthermore, tissue biopsy has limited benefits and cannot accurately reflect the dynamics of the tumor or the response to treatment.[13] Additionally, taboos in some communities across the world regarding cervical and sexually transmitted disease testing further complicates the effectiveness of mass screening programs.

Liquid biopsy-based tests offer a less invasive alternative to traditional biopsy or imaging-based diagnostic methods. Recently, progress in the detection and analysis of blood-based circulating tumor cells or circulating tumor DNA has provided a potential for cancer detection and treatment monitoring. Similar studies have been conducted in cervical cancer, with the focus on cell-free DNA (cfDNA) for early diagnosis and treatment monitoring.[14]

Liquid Biopsy

Most cancer diagnostic tools currently rely on using tissue samples from surgical resection or biopsies as the source material for testing. Tissue biopsies are invasive surgical procedures that carry the chance of potential complications, low repeatability, and they cannot be performed in cases of tumor inaccessibility or worsening clinical condition of the patient.[13] Genomic profiling using tissue biopsies provides a picture of the tumor at a single point in time, while tumors have a heterogenous genetic profile due to the numerous tumor sub-clones. Moreover, genomic landscapes of tumors and metastases significantly change over time in response to therapeutic interventions, and these landscapes cannot be captured by tissue biopsies.[15]

Liquid biopsy is a non-invasive approach for the profiling of tumors without invasively collecting tumor tissue. In the past decade, significant progress has been made in investigating the presence of tumor-derived material in body fluids including plasma, serum, cerebrospinal fluid, saliva, urine, and stool. The analytes used for liquid biopsy include circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), mRNA, proteins, miRNA, exosomes, and metabolites.[16] Liquid biopsy is currently not regarded as a standard test in clinical practice, but its potential applications are expanding rapidly in the field of diagnostics, monitoring of therapies, evaluation of response and resistance to treatments, and quantification of minimal residual disease.[13]

Circulating Cell-Free DNA

cfDNA consists of short fragments of DNA that are found circulating in body fluids such as plasma, serum, saliva, bile, lymph, breast milk, cerebrospinal fluid, and amniotic fluid.[17] Cell-free nucleic acids were first reported by Mandel and Metais in the late 1940s in the blood samples of healthy participants.[18] cfDNA is derived from almost all the types of cells present in the body and is of nuclear as well as mitochondrial origin.[19] The DNA is released into the circulation via two processes, either by cellular breakdown processes like necrosis and apoptosis or through active secretion. Chromosomal DNA is broken up into fragments that are 160–180 base pairs (bp) in size or their multiples during apoptosis.[20] These represent the DNA strands that form a nucleosome by wrapping around a histone protein complex. However, in necrosis the fragments of DNA are typically around 10 000 bp[20] whereas, in active secretion, cfDNA is either enveloped in vesicles or associated with protein complexes.[21] Under normal physiological conditions, cfDNA from the circulation is often cleared by nuclease activity by degradation by macrophages or by renal excretion.[18 22] However, this does not usually occur in the case of tumor mass which causes the proliferation of cellular debris and its release into the bloodstream.[22] In healthy individuals, around 60–90% of the cfDNA is contributed by the hematopoietic cells whereas the liver contributes around 2–18% of the cfDNA fraction.[18 23] The concentration of cfDNA in healthy individuals is reported to be in the range of 1–10 ng/mL whereas, in pregnant women, it ranges up to 200 ng/mL.[18] Cancer patients are reported to have a higher level of cfDNA than healthy individuals, with levels of >1000 ng/mL in plasma.[18 24 25] These observations can be used to conclude that cfDNA is contributed significantly by tumor cells into the circulation of patients with cancer. The term ctDNA denotes the fraction of cfDNA derived from tumor cells.[18] This term was coined during a study on pancreatic cancer that reported identical mutations in tumors and plasma DNA fragments.[18 26] The size of cfDNA derived from tumors is comparatively shorter than the physiologically originating background cfDNA.[27] This factor helps in the sorting of the smaller fragments of cfDNA, which might help to increase the sensitivity of cfDNA as a biomarker.[18 27] This review will primarily focus on the most recent advances in the field of liquid biopsies and their relevance in the early diagnosis and surveillance of cervical cancer. These technologies are based on plasma cfDNA and how they might impact clinical decision-making in the treatment of cervical cancer.

CELL-FREE DNA AS A DIAGNOSTIC BIOMARKER IN CERVICAL CANCER

HPV Circulating Tumor DNA as a Biomarker for Detection

The majority of cervical cancers are associated with hr-HPV infections, which usually result in the integration of the HPV genome into the host genome and are considered to be an important factor in tumorigenesis.[28] Numerous research groups have studied the dynamics of HPV integration into the human genome in cancers, but the process is yet to be completely understood. The integrated HPV sequences offer an excellent model for studying ctDNA in HPV-associated cancers.[29 30] Due to the non-human origin of HPV DNA, single-site assays and small panel tests for HPV detection have been used as a biomarker of liquid biopsy. One of the first studies to report cfDNA in cervical cancer was by Pornthanakasem et al in 2001.[31] This study targeted the E6 gene of HPV using plasma samples from patients with cervical cancer. It was reported that approximately three times as much HPV DNA was detected in metastatic patients than in patients without metastasis.[31] They also reported that the presence of HPV DNA in patient plasma was associated with a tendency to develop distant metastases within 1 year of treatment compared with patients with HPV+ cervical cancer of the same stage where plasma HPV DNA was undetected.[31] Similar studies in the early 2000s used the quantitative polymerase chain reaction (qPCR) to study HPV ctDNA in patient-derived plasma cfDNA samples.[31–33] It has numerous inherent limitations, such as poor sensitivity in detecting viral DNA in low copy numbers and inadequate accuracy in predicting minor variations in DNA copy number across different samples.[33] To overcome these deficiencies there has been an increasing demand for new analytical techniques, specifically to quantify HPV DNA in ctDNA of patients with cervical cancer. Droplet digital PCR (ddPCR) has emerged as a cutting-edge technology that offers an alternative approach for quantifying nucleic acids and offers various potential advantages over qPCR.[34] With enhanced precision, sensitivity at low template concentrations and a higher detection rate than qPCR, ddPCR makes it possible to quantify nucleic acids in an absolute manner without the use of standard curves.[35]

Recent years have seen a tremendous increase in research studies employing ddPCR technologies for HPV DNA detection/quantification in cervical cancer. In a retrospective study by Kang et al, cfDNA was detected in serum samples from 19 patients with metastatic cervical cancer using ddPCR. The specificity of the assay was 100% and sensitivity was around 90%.[36] In another study, Jeannot et al detected HPV ctDNA in 61 out of 70 serum samples using ddPCR.[37] This study compared ddPCR and qPCR methods for the detection of HPV DNA in various cancers including head and neck cancer, cervical cancer, and anal cancer. The results showed that ddPCR assays were able to detect 61/70 samples positive for HPV ctDNA in patients with cervical cancer, 14/15 patients with anal cancer, and all eight cases of head and neck cancer, whereas qPCR assays were less sensitive and identified only 48/70 positive samples of cervical cancer, 12/15 samples of anal cancer, but all eight cases of head and neck cancer.[37] Cheung et al used ddPCR to target HPV E7 and L1 genes in cfDNA extracted from pre-treatment plasma samples from 138 patients with cervical cancer across different stages. They observed that patients with an elevated viral load (arbitrarily chosen as ≥20 E7 or L1 copies per 20 µL reaction

volume) had a higher risk of recurrence and a reduced 5-year survival rate.[38] In a similar study, Cabel et al also detected HPV ctDNA in 69% of patients with locally advanced cervical cancer before undergoing chemoradiotherapy.[39]

Recent studies have used digital PCR (dPCR) technology for detecting HPV cfDNA as a marker for HPV-associated cancer, but the clinical utility of this method remains limited in patients with a low disease burden.[40] Leung et al compared the accuracy of HPV sequencing (HPV-seq) and dPCR for detection of HPV cfDNA in patients with locally advanced cervical cancer. This next generation sequencing (NGS)-based test was able to outperform dPCR and could detect ctDNA at levels as low as 0.03 copies/mL plasma in dPCR-negative samples.[40]

Integration of the HPV genome into the host plays a major role in cervical carcinogenesis and could be directly correlated to malignancy. Therefore, the detection of these integration sites might also represent a suitable marker of identification of cervical lesions that are at high risk of becoming malignant.[41] Based on known integration sites, HPV–human genome junctions were tested as a biomarker for the diagnosis of cervical cancer using serum samples from patients with invasive cervical cancer.[42] This study also reported the usefulness of detection of integrated HPV sequences in predicting the disease progression and treatment monitoring for two patients where sequential samples were available.[42]

Circulating Tumor DNA Mutations as a Biomarker for Diagnosis

The Cancer Genome Atlas (TCGA) Research Network published one of the most extensive studies in cervical cancer in 2017, which identified previously unreported significantly mutated genes and other factors that make these tumors good targets for immunotherapy and targeted therapy based on their genetic profiles.[43] These data, along with emerging data from large clinical and genomics studies, make a good argument for using ctDNA-based technologies to identify these mutations as companion diagnostic tests.[44]

PCR-based technologies are usually used for the detection of common mutations in cancer predisposition genes. Genes other than HPV DNA have also been used as a target for liquid biopsy-based diagnosis of cervical cancer. B cell-specific Moloney murine leukemia virus integration site 1 (*BMI1*) gene has been reported to cause senescence due to inhibition of apoptosis induced by pRB and p53 pathways.[45] A similar research study was conducted by Zhang et al using reverse transcription quantitative PCR (RT-qPCR) to detect circulating *BMI1* mRNA in plasma samples of 138 patients with uterine cervical cancer and 80 normal participants. They showed that the level of *BMI1* mRNA was inversely proportional to disease-free survival as well as overall survival.[45] Using ddPCR, Chung et al recently evaluated the efficacy of *PIK3CA* mutation testing in patients with cervical cancer. Two *PIK3CA* mutations (p.E545K and p.E542K) were detected in the pre-treatment plasma cfDNA samples from 177 patients with invasive cervical cancer. It was found that at least one of the two *PIK3CA* mutations was present in the plasma cfDNA of 22.2% of the patients.[46] They also observed that *PIK3CA* mutation status in plasma cfDNA significantly correlated with median tumor size along with decreased disease-free survival and overall survival.[46]

Although PCR methods are efficient in detecting known mutations, NGS techniques cover a wider spectrum of mutations and also allow for discovery of novel mutations.[47] Using a bioinformatics approach, Lee et al designed an NGS panel consisting of 24 cervical cancer-associated genes to detect and characterize copy number variation (CNV) and single-nucleotide variation (SNV) patterns in cfDNA from 24 patients with cervical cancer. All of the 24 patients were found to have mutations in 18 of the 24 genes in the NGS panel. More than 75% of the samples included *ZFHX3, KMT2C*, and *KMT2D* mutations.[48] Charo *et al* presented the ctDNA results of 105 patients with gynecologic cancer, which also included 13 cervical malignancies (PREDICT trial, NCT02478931). There were two distinct panels used, with 54 or 73 genes, respectively. *PIK3CA* (n=8), *TP53* (n=5), *FBXW7* (n=3), *ERBB2* and *PTEN* (both n=2) were the most frequently detected mutations among patients with cervical cancer. The concordance rate between plasma and tissue in the overall research cohort ranged from 75% to 8%.[49] Studies that have used cfDNA as a biomarker are summarized in Table 1.

CIRCULATING CELL-FREE DNA IN TREATMENT MONITORING OF CERVICAL CANCER

The traditional treatment of cervical cancer mainly relies on surgery and a combination of radiotherapy and systemic chemotherapy.[50] HPV vaccination, cervical cancer screening, and early-stage advanced treatment have been crucial in reducing the incidence and mortality rate of cervical cancer, especially in developed countries.[51] Additionally, recent experimental approaches in adoptive T-cell therapies targeting HPV oncoproteins seem promising as viable strategies for the management of metastatic cervical cancer.[52 53] The non-invasiveness of liquid biopsy compared with tissue biopsies and other imaging techniques makes cfDNA a suitable biomarker for treatment surveillance in various cancers, or quantification of minimal disease after the surgery. Various studies have shown that the lower levels of cfDNA in patients is correlated with a positive response to the ongoing treatment in different types of cancers.[54] Similarly, a higher cfDNA level indicates a poor response to treatment and a poorer progression-free survival (PFS) rate.[55] In many studies it has been shown that treatment monitoring through cfDNA has proved to be a sensitive approach compared with the traditional methods of tissue biopsy or imaging.[54]

Jeannot et al correlated HPV cfDNA data with stages of cervical cancer of patients and found that the difference in levels of HPV cfDNA between stage I and stage II was around 20-fold.[38] The comparative rise in the levels of cfDNA was more between stages I and II than between stages II and III or stages III and IV. They also reported that the levels of cfDNA were comparatively higher for tumors >45 mm in size by CT imaging compared with tumors <45 mm. This analysis also showed that ddPCR allowed the detection of cfDNA in two cases of microinvasive carcinomas of the cervix which had a tumor mass of around 1 mm. Their study also showed that ddPCR was efficient enough to detect the levels of cfDNA for minimal tumor masses.[37] In a subsequent study they evaluated the significance of HPV ctDNA targeting E7 gene as a diagnostic marker for residual tumor, in comparison with the detection of HPV integration site and *PIK3CA* mutations which are considered to be the most common events for cervical cancer detection. The most accurate tumor marker was found to be the HPV E7 gene, which outperformed both HPV integration sites and serum *PIK3CA* mutations. A

Table 1 Studies using cell-free DNA as a biomarker for the detection of cervical cancer

No	Study	Year	Method	Target gene	Sample	Sensitivity (%)	Specificity (%)	Patient population	Metastatic vs non-metastatic
1	Pornthanakasem et al[31]	2001	qPCR	HPV DNA	Plasma	12.00	100	63	Non-metastatic (stage I–IV)
2	Dong et al[33]	2002	qPCR	HPV DNA	Plasma	64.30	98.33	232 patients and 60 normal controls	Non-metastatic patients (carcinoma in situ and advanced) and normal controls
3	Hsu et al[32]	2003	qPCR	HPV DNA	Serum	24.10	100	112 patients and 40 controls including patients with cervical carcinoma in situ or benign disease	Stage 1B and IIA
4	Campitelli et al[42]	2012	DIPS- PCR	HPV DNA	Serum	85.00	No controls	16	IB–IVA and one case was a pelvic relapse of cervical SCC
5	Zhang et al[45]	2016	RT-qPCR	*BMI1* mRNA	Plasma	69.70	95.90	109 patients with UCC, 138 patients with CIN and 80 healthy volunteers	Stage I–IV
6	Jeannot et al[37]	2016	ddPCR	HPV DNA	Plasma	83.00	100	47 cases of cervical cancer and 18 cases of CIN	Stage I–IV
7	Kang et al[36]	2017	ddPCR	HPV DNA	Serum	100	100	19 patients and 45 healthy controls	Metastatic
8	Chung et al[46]	2017	ddPCR	*PIK3CA*	Plasma	22.2	No controls	170	Stage I–IV
9	Cheung et al[38]	2019	ddPCR	HPV DNA	Plasma	61.6	No controls	138	Non-metastatic (mostly stage IB–II)
10	Cabel et al[39]	2021	ddPCR	HPV DNA	Serum/ plasma	69	No controls	55	Locally advanced cervical cancer
11	Leung et al[40]	2021	NGS	HPV DNA	Plasma	100	88	17 patients with cervical cancer, 13 with HPV positive oropharynx cancer, 50 controls (21 female, 29 male)	Non-metastatic

CIN, cervical intra-epithelial neoplasia; ddPCR, droplet digital PCR; HPV, human papillomavirus; NGS, next generation sequencing; PCR, polymerase chain reaction; qPCR, quantitative PCR; RT-qPCR, reverse transcription quantitative PCR; SCC, squamous cell carcinoma; UCC, uterine cervical cancer.

high International Federation of Gynecology and Obstetrics stage and involvement of para-aortic lymph nodes were associated with HPV ctDNA detection in serum samples. Additionally, there was a significant correlation between HPV ctDNA levels in serum and the number of HPV copies in tumor samples. A significantly longer PFS was associated with complete clearance of HPV ctDNA by the end of treatment.[28]

Cabel et al analyzed blood and tumor samples using ddPCR in a retrospective and prospective cohort of patients with locally advanced cervical cancer. This assay was able to detect two cases in the retrospective cohort with residual HPV ctDNA after curative therapy and both of the patients had a relapse.[39] In the prospective cohort, two more patients were positive for HPV ctDNA following chemoradiation therapy but only one of them experienced a metastatic relapse after 2 months. The low copy number of HPV ctDNA at baseline was thought to be the cause of the other patient's absence of relapse. These data suggest that the detection of HPV ctDNA in blood samples could be correlated with lower recurrence-free survival. Also, a higher HPV ctDNA copy number at baseline could be considered to be a risk factor for relapse in patients undergoing chemoradiation therapy.[39]

Imaging modalities for cervical cancers including MRI, CT, positron emission tomography-CT (PET-CT) provide information on important prognostic factors such as parametrial invasion, tumor size, endocervical extension, lymph node status, and pelvic side wall or adjacent/distal organ involvement. Additionally, imaging techniques aid in the monitoring of cervical cancer, the assessment of the tumor response to treatment, and the selection of patients for less invasive procedures like radical trachelectomy for fertility preservation.[56] In a recent study, Han et al compared the accuracy of fluorodeoxyglucose positron emission tomography (FDG-PET) in predicting locally advanced cervical cancer against plasma HPV ctDNA by using ddPCR. Through serial sampling, the levels of post-treatment plasma HPV ctDNA in patients could predict metastases. The study reported that undetectable levels of HPV ctDNA in the plasma of patients after treatment was correlated with a higher PFS compared with patients with detectable plasma levels of HPV DNA. Moreover, they reported that 3-month plasma HPV DNA levels were more accurate than 3-month FDG-PET in detecting residual disease. This study could prove that the measurement of HPV DNA in plasma using ddPCR may have better accuracy in detecting residual disease, avoidance of exposure to radiation, lower cost, and less dependence on experts for interpretation of the result.[57] Kang et al studied the clearance of HPV cfDNA in patients undergoing tumor infiltrating lymphocyte immunotherapy. Of the nine patients undergoing treatment, two had a complete response, one had a partial response, and the remaining six patients were reported to have progressive disease at the end of the treatment. In the patient who showed a partial response for a short interval of time, it was observed that the cfDNA levels fell below detection limits during the first month after treatment but were again detectable at day 70 and day 98, which correlated with the signs of disease progression seen on CT. The two patients who showed a complete response to the treatment were also followed up by collecting samples at 4.2 and 10.6 months after treatment. All the blood samples showed that there was a significant clearance of HPV cfDNA in the serum samples, which occurred from day 8 and day 32, respectively.[36]

Similarly, Kim et al used NGS for monitoring the treatment response in 25 patients with cervical cancer after radical radiotherapy using target capture libraries for HPV 16/18 and 67 oncogenes. They compared the efficacy of HPV cfDNA with the conventional treatment monitoring methods like tumor markers (squamous cell carcinoma antigen (SCC) for squamous cell carcinoma and carcinoembryonic antigen (CEA) for adenocarcinoma) and MRI in patients with cervical cancer. HPV cfDNA was quantified by determining the ratio of reads aligning to only HPV and total aligned reads (human aligned+HPV aligned). In most cases the tumor markers as well as the HPV ratio appeared to decline with a reduction in the size of the tumor. The HPV ratio had better sensitivity in detecting temporal changes in patients between visits 2 and 3. Two patients with distant metastasis showed a much higher increase in HPV ratios compared with tumor markers. Also, the HPV ratios were reduced in patients with no evidence of disease group whereas the tumor markers were increased in this case, indicating that the HPV cfDNA ratio calculated using targeted NGS may be a valuable tool for therapeutic monitoring and prediction of treatment outcomes.[58]

A recent study by Lalondrelle et al used a NGS assay called 'panHPV detect' for detection of HPV cfDNA to evaluate treatment response in patients with locally advanced cervical cancer. The test was able to detect relapse in three patients who showed complete clearance on imaging. HPV cfDNA was detected in these three patients 3 months after treatment. Also, another four patients who showed partial clearance on imaging and had undetectable HPV cfDNA at the 3-month follow-up did not show any signs of relapse, indicating the potential of the panHPV cfDNA detect test in assessing treatment response and predicting relapse in cervical patients after chemoradiotherapy.[59] Another study by Mittelstadt et al used a custom-made enrichment panel for a NGS assay targeting HPV E6 and E7 regions of 13 high-risk HPV. This test was able to detect cfDNA in the pre-treatment samples in all 17 patients with advanced disease and in 55.5% (5/9) of patients with early-stage cervical cancer. Patients with undetectable HPV cfDNA levels after 1 month of treatment remained disease-free even after 1.5 years, whereas two patients with persistently high levels of HPV cfDNA after treatment developed a relapse, which can indicate the utility of HPV cfDNA as a treatment monitoring biomarker in patients with cervical cancer.[60]

Chemotherapeutic drugs that exploit the genetic susceptibility of tumors have enormous potential for the treatment of cancers. However, the efficacy of these drugs seems to be diminishing due to the development of resistance to these therapies in various cancers. cfDNA can be used to monitor the emergence of resistance to the most widely used therapeutics by identifying mutations that confer resistance. Moreover, serial sampling and characterization of cfDNA would provide insights into novel mutations during the process of treatment.[54] Tian et al evaluated the role of cfDNA in blood by characterizing the mutations in 48 cancer driver genes in patients with cervical cancer. They performed targeted deep sequencing, developed an algorithm, allele fraction deviation (AFD), and used it to monitor the dynamic changes in genomic aberrations. They determined the mutant fraction of each single position in the target region and from this they compared the overall distance of mutation fraction between the control DNA from white blood cells (WBC) and cfDNA by empirical cumulative distribution function (ECDF) and AFD.

The level of deviation between the mutant allele fraction (MAF) found in the cfDNA and the MAF found in WBC in each patient was determined using AFD. Three germline variant statuses could be present in healthy cells of diploid human samples—that is, no germline variations whose allele fraction (AF) is 0%, homozygous variant whose AF is 100%, and heterozygous variant whose AF is 50%. Most of the cfDNA samples followed the same pattern and

Table 2 Selected studies validating cfDNA as a prognostic marker in cervical cancer

Study	Patient population	No of patients	Time of blood sample collection	Key findings
Kang et al (2017)[36]	Metastatic cervical cancer	19	Pre- and post-treatment time points	HPV cfDNA represents a promising tumor marker for non-invasive HPV genotyping and may be used in selecting patients for HPV type-specific T cell-based immunotherapies
Han et al (2018)[57]	Stage IB–IVA cervical cancer	23	At baseline, end of CRT, 3 months after CRT, and at recurrence	3-month plasma HPV DNA level is more accurate than 3-month FDG-PET imaging in detecting residual disease
Tian et al (2019)[61]	Different stages of cervical cancer (stages I–IV)	57	Blood samples available at various time points (once, twice or thrice randomly)	The decrease in values of cfDNA AFD was directly associated with reduction of tumor mass. Targeted deep sequencing of cfDNA along with genomic DNA may help in prediction of treatment response and relapse in cervical cancer
Lee et al (2020)[48]	Different stages of cervical cancer (stages I–IV)	4 for treatment monitoring	1 week prior to primary treatment and three times during the treatment	RNF213 mutation could be potentially used as a monitoring marker for response to chemo- and radiotherapy
Jeannot et al (2021)[28]	HPV16- or HPV18-positive cervical cancer patients	94	At baseline, at the end of treatment and during follow-up visits at 6, 12, and 18 months	HPV ctDNA detection in serum sample was associated with high FIGO stage and para-aortic lymph node involvement
Cabel et al (2021)[39]	Cervical cancer at any stage	55	At baseline (before treatment), days 7, 21 and 35 during CRT and then at 2, 6, 12, 18 and 24 months	Residual HPV ctDNA at the end of CRT or during follow-up could help to identify patients more likely to experience subsequent relapse
Tian et al (2021)[62]	Locally advanced or metastatic relapsed cervical cancer	82	Before and, when possible, during therapy	Five genes which are significantly associated with metastasis were identified. Reduction in mutations in these genes post therapy was associated with stable disease or partial remission
Kim et al (2022)[58]	Patients with pathologically proven uterine cervical cancer who had completed planned radical RT and 4 patients without distant metastasis	25	Before RT (visit 1), during RT (especially before brachytherapy, visit 2), and 3 months after RT (visit 3)	HPV cfDNA ratio outperforms tumor markers in treatment monitoring and may be considered as a valuable tool for monitoring and predicting treatment responses
Mittelstadt et al (2023)[60]	Advanced-stage disease (n=17, FIGO IB3–IVB) and patients with early-stage disease (n=9, FIGO IA–IB2)	26	Before and after therapy at different time points (8 patients followed for therapy monitoring)	HPV-cfDNA is a potential marker for treatment response monitoring in cervical cancer patients

Continued

Table 2 Continued

Study	Patient population	No of patients	Time of blood sample collection	Key findings
Lalondrelle et al (2023)[59]	Stage I–IV	22	Baseline (before therapy), at week 6–7 (during brachytherapy) and 3 months following completion of treatment	panHPV-detect test detects HPV cfDNA with higher sensitivity and specificity making it instrumental in assessment of the treatment response and in monitoring for relapse

AFD, allele fraction deviation; cfDNA, cell-free DNA; CRT, chemoradiotherapy; FIGO, International Federation of Gynecology and Obstetrics; RT, radiotherapy.

fell into these three points, but the presence of unanticipated points was observed whose AF did not follow the typical 0–50–100 pattern. These outlying points were somatic mutations whose AFs reflected the proportion of ctDNA that is derived from tumors in the total cfDNA. An ECDF curve was used for the analysis of AF for the WBC sample and cfDNA in plasma. This study also reported that the decrease in cfDNA AFD values were correlated with the reduction in the size of tumor. Furthermore, a lower value of AFD at the time of diagnosis followed by an increase in the AFD value was successfully able to predict relapse. This study indicated that plasma cfDNA together with deep sequencing may act as a potential method for predicting the treatment response and disease prognosis in cervical cancer.[61]

A study involving deep sequencing analysis of plasma samples from 82 patients with locally advanced or metastatic cervical cancer targeted 322 cancer-predisposition genes. This study identified five significantly mutated genes (*PIK3CA*, *BRAF, GNA11, FBXW7*, and *CDH1*) and mutations in these genes were correlated to a significantly shorter overall survival as well as PFS. Furthermore, a reduction in the number of mutations in these genes following chemotherapy was strongly associated with partial remission and stable disease in matched pre-chemotherapy and post-chemotherapy plasma samples. An increase in the mutation status of these five genes was observed much earlier in response to the disease progression compared with radiological imaging in the individuals included in the longitudinal tracking ctDNA analysis.[62] Lee et al analyzed cfDNA from patients with cervical cancer by NGS using a customized panel of 24 cancer-related genes using the Ion Torrent system. The study showed that the *RNF213* mutation could potentially be used as a monitoring marker for response to chemotherapy and radiation therapy.[48] Studies that have used cfDNA for treatment monitoring are shown in Table 2.

FUTURE PERSPECTIVE

The aim of this review was to describe different aspects of cfDNA dynamics in cervical cancer and to objectively analyze the utility of liquid biopsy-based technologies in early diagnosis and disease monitoring in cervical cancer. Figure 1 shows the various methods of

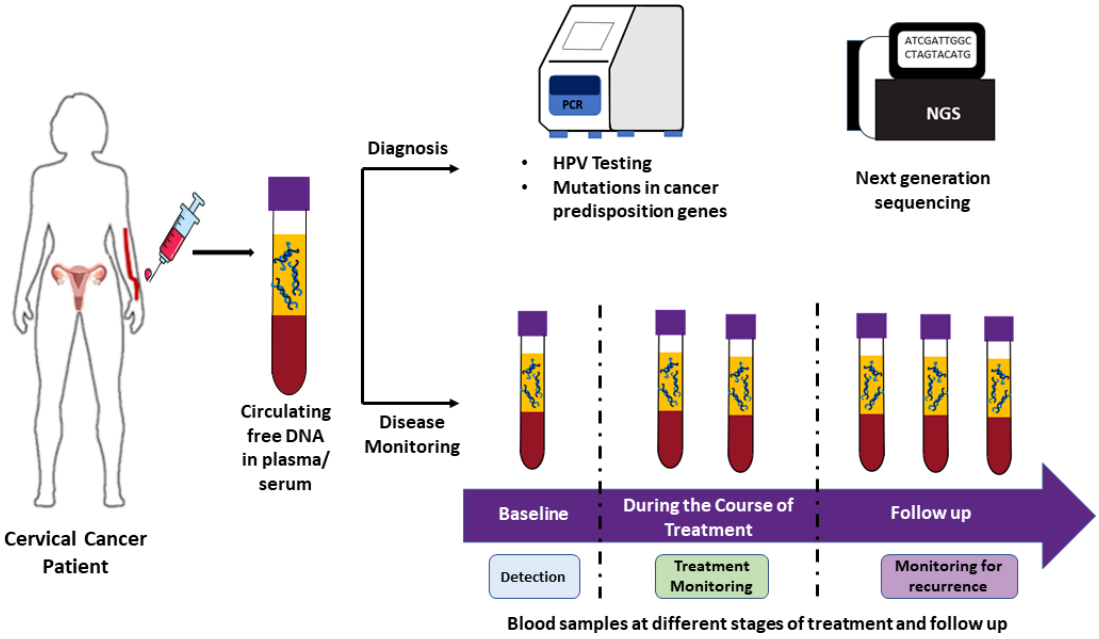

Figure 1 Diagrammatic representation of liquid biopsy for cervical cancer. Circulating cell-free DNA (cfDNA) can be used in the detection of cervical cancer by various modes including HPV testing, gene-specific PCR or next generation sequencing (NGS). cfDNA can also be used for treatment monitoring and detection of minimal residual disease.

using cfDNA for the detection and treatment monitoring of cervical cancer. Studies prior to the advent of dPCR and NGS used qPCR for detecting HPV ctDNA in the blood of patients with cervical cancer, targeting the E7 gene of HPV in serum or plasma samples.[32 33] However, due to the several limitations of qPCR including low sensitivity, lack of precision, and dependence on standard curves, there has been an increasing demand for more analytical assays for HPV cfDNA quantification.[34] With its excellent sensitivity and specificity, ddPCR has subsequently transformed liquid biopsies. It functions by dividing cfDNA into distinct droplets for accurate mutation detection. This technology has been used successfully to detect HPV DNA in cfDNA from patients with cervical cancer.[28 36 39]

NGS is another technology that has had a significant impact on cfDNA liquid biopsy for cervical cancer. NGS allows for the simultaneous detection of multiple genetic mutations in cfDNA, which can provide a more comprehensive view of the genetic alterations associated with cancer. NGS has been used to detect HPV DNA and other mutations in cfDNA from patients with cervical cancer.[48]

Recently, the use of artificial intelligence (AI) and machine learning (ML) algorithms has also had an impact on cervical cancer.[63] These technologies can be used to analyze large datasets of cfDNA sequencing data and identify patterns that are indicative of cancer. By training algorithms on large datasets of patient data, it is possible to develop predictive models that can accurately detect cervical cancer at an early stage.[63–65] AI-based medical diagnostic applications are on the rise, and they have excellent applicability in the screening and diagnosis of cervical cancer. Their advantages include reduction in time consumption along with lesser demand of professional and technical personnel.[63]

New technologies such as ddPCR, NGS, and AI/ML algorithms have had a significant impact on cfDNA liquid biopsy for cervical cancer. These technologies will provide clinicians with a non-invasive and efficient means of detecting the disease. As technology continues to evolve, it is likely that liquid biopsy will become an increasingly important tool for the diagnosis and management of cervical cancer.

Liquid biopsies can also influence novel therapies like CAR-T cell therapies and immunotherapy by providing information on the patients' immune status and presence of targetable biomarkers. Liquid biopsy has been useful in immunotherapy by providing information on the tumor mutational burden, microsatellite instability, and other molecular biomarkers.[66] Plasma-based cfDNA liquid biopsies have shown both prognostic and predictive value in detection of these biomarkers in patients treated with immune checkpoint inhibitors such as anti-PD-1. It also allows the detection of early on-therapy responses along with real-time monitoring of disease burden.[66] In cervical cancer, along with being a tumor marker for HPV genotyping, HPV ctDNA has also been helpful in selecting patients for T cell-based immunotherapies specific for HPV sub-types.[36] These initiatives demonstrate how liquid biopsies are playing an increasingly important role in guiding treatment selection and sequencing, monitoring therapeutic efficacy, and selecting patients for cancer immunotherapy.

In the future there is a need for designing personalized screening assays to identify precise screening markers applicable to population sub-sets and individual patients. These cfDNA-based assays will aid in the personalization of treatment by identifying patients with poor prognoses, such as those with high pre-treatment ctDNA, for treatment intensification. NGS-based assays to identify HPV integration sites followed by ultra-sensitive dPCR methods could personalize HPV ctDNA detection in patients to monitor disease progression. Analysis of circulating biomarkers as liquid biopsy tests in cervical cancer shows immense potential as a risk stratification tool with the ability to provide informed decisions for precision medicine ultimately improving patient outcomes.

Several obstacles, including early risk prediction, real-time tracking of tumor progression, and mutation detection of treatment resistance have been solved by using ctDNA-based assays. Larger prospective clinical trials with cfDNA are needed to prove its clinical utility in cervical cancer. cfDNA holds a promising future as a potential tool for clinical practice. Although there are still certain aspects of liquid biopsy technologies that need to be developed, with the rapid progress of science and technology, liquid biopsies will undoubtedly play a crucial part in the detection and management of cervical cancers.

CONCLUSION

There have been significant advances in the field of liquid biopsy for cervical cancer, including ddPCR, NGS, and AI/ML algorithms revolutionizing early detection, disease monitoring, and treatment selection. These developments provide healthcare providers with non-invasive, precise, and efficient techniques for detection and management of cervical cancer. Liquid biopsies also have the potential to guide innovative therapies such as CAR-T cell therapies and immunotherapy by providing crucial information on immune status and the presence of biomarkers. Despite the challenges, such as higher cost, the need for reliable biomarkers and larger clinical trials for validation, the future of liquid biopsies in cervical cancer appears bright. With ongoing scientific and technological progress, liquid biopsies are poised to play a pivotal role in improving treatment outcomes in patients with cervical cancer.

Author affiliations
[1]Department of Medical Genetics, Kasturba Medical College Manipal, Manipal Academy of Higher Education, Manipal, Karnataka, India
[2]Department of Pharmacy Practice, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal, Karnataka, India
[3]Department of Radiotherapy and Oncology, Kasturba Medical College Manipal, Manipal Academy of Higher Education, Manipal, Karnataka, India

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ORCID iD
Rama Rao Damerla http://orcid.org/0000-0001-6815-0097

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