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Prognostic value of circulating HPV cell-free DNA in cervical cancer using liquid biopsy

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Liquid biopsies, which analyze circulating tumor cells or cell-free circulating tumor DNA (ctDNA) from blood, have emerged as promising cancer detection and monitoring tools. Specifically, human papillomavirus (HPV) cell-free (cf) DNA is gaining recognition as a prognostic marker in high-risk HPV-related cancers. However, detecting circulating markers for cervical cancer (CC) requires highly sensitive techniques to quantify circulating HPV DNA. This study aimed to evaluate the use of droplet digital PCR (ddPCR), a highly sensitive technique, for detecting and quantifying circulating HPV DNA in cervical cancer patients, both at baseline (before chemo- or radiotherapy) and during follow-up, to assess its utility as a prognostic marker. Blood samples were collected from 60 cervical cancer patients (Stages I–IV) at AIIMS, New Delhi, at baseline and three months post-treatment. Samples from 10 healthy controls were also included. Plasma was separated and stored at − 80 °C, and cfDNA was extracted from 1 ml of plasma. The presence of high-risk HPV types, HPV16 and HPV18, in cfDNA from 35 patients was assessed using ddPCR. The median concentration of cfDNA in cervical cancer patients was 9.35 ng/µL at baseline, which decreased to 7 ng/µL after three months of treatment. In healthy controls, the median cfDNA concentration was 6.95 ng/µL. ddPCR screening showed that detection rates for HPV18 and HPV16 detection were 45.71% and 82.86%, respectively. A significant correlation was observed between cf HPV16 DNA levels and tumor size, suggesting its potential as biomarker for disease burden.

Keywords Cervical cancer, Digital droplet PCR, Human papillomavirus, Circulating tumor DNA, Circulating cell-free DNA

Abbreviations

cfDNA	Cell-free DNA
ccfDNA	Circulating cell-free DNA
ctDNA	Circulating tumor DNA
ddPCR	Digital droplet polymerase chain reaction
HPV	Human papillomavirus
ccfHPV DNA	Circulating cell-free HPV DNA
rHPVs	High-risk human papillomaviruses
HPV18	Human papillomavirus 18
HPV16	Human papillomavirus 16
SCC	Squamous cell carcinoma
NKSCC	Non-squamous cell carcinoma
CAA	Clear cell adenocarcinomas
AUC	Area under the curve

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Cervical cancer (CC) is the fourth most common malignancy among women worldwide, posing a significant threat to women's health. Despite widespread Pap smear screening programs, cervical cancer remains a major concern, with an estimated 662,301 cases and 348,874 deaths reported globally in 2022 (IARC, 2022). In India, there were 127,526 cases and 79,906 related deaths, underscoring the need for more effective diagnostic and monitoring strategies (WCRF, 2022). Common symptoms of cervical cancer include inter-menstrual bleeding, postcoital bleeding, and vaginal discharge[1]. According to the International Federation of Gynecology and Obstetrics (FIGO), over 55% of CC patients are diagnosed at advanced stages (II-IV), significantly affecting survival rates[2]. In India, the overall survival rate for cervical cancer is 46%, with a marked difference between early and late-stage diagnoses (George Institute, 2022).

While Pap smear-based screening programs offer a diagnostic method, their sensitivity is limited, especially in low-prevalence settings, with a mean sensitivity of approximately 58%, highlighting the need for more sensitive and specific biomarkers to detect asymptomatic recurrences[3,4].

Recent advancements in liquid biopsy techniques, which analyze circulating tumor cells or cell-free DNA (cfDNA) from blood samples, provide a promising non-invasive alternative to traditional tissue biopsies. Liquid biopsies offer real-time insights into tumor dynamics and the genetic landscape of cancers, with circulating cell-free DNA (ccfDNA) being explored as a potential marker for cancer detection and monitoring[5]. Circulating tumor DNA (ctDNA), a component of ccfDNA, contains tumor-specific genetic alterations, making it a valuable biomarker for detecting minimal residual disease and relapse, particularly in subclinical stages[6–8]. Recent studies have identified circulating cell-free HPV (ccfHPV) DNA as a promising biomarker for the early detection and prognosis of cervical cancer[9]. High-risk HPV genotypes, particularly HPV16 and HPV18, are known to drive oncogenesis by integrating their DNA into the host genome, promoting the expression of oncogenic genes such as E6 and E7[10]. However, detecting circulating HPV DNA presents challenges due to its low abundance, necessitating highly sensitive analytical methods. Traditional methods like quantitative Polymerase Chain Reaction (qPCR) often lack the sensitivity needed to detect low levels of circulating DNA in the blood. For example, Lv et al. reported that qPCR detected HPV DNA in only 8% (4/50) of clinical samples, indicating its limitations for clinical use[11]. This emphasizes the need for more advanced molecular techniques, such as droplet digital PCR (ddPCR), which offers exceptional sensitivity and precision, even at low DNA concentrations[12]. ddPCR enables the partitioning of DNA into thousands of nanolitre-sized droplets for individual amplification, allowing for the detection of DNA concentrations as low as 0.001%.

Recent investigations using ddPCR to quantify ccfHPV DNA have reported detection rates ranging from 31 to 100%, demonstrating variability and the need for further research[14–16]. Previous studies have focused on predicting tumor relapse using ccfHPV DNA, but a lack of follow-up data has limited their conclusions. Moreover, there is a gap in research specifically targeting Indian patients, presenting an opportunity to explore the role of ccfHPV DNA in predicting treatment response in this population.

This study conducted a retrospective analysis of circulating cell-free HPV DNA in Indian cervical cancer patients across various disease stages, comparing therapy-naive patients to those post-therapy, and included healthy controls for reference. We evaluated the diagnostic performance of ccfHPV16 and ccfHPV18 DNA and explored correlations with clinical parameters such as age, histology, disease stage, and tumor size. Our findings underscore the significance of ccfHPV DNA in cervical cancer management and demonstrate the broader applicability of ddPCR in oncology, particularly for virus-associated cancers.

Materials and methods

Patients and samples

This study included 60 patients diagnosed with cervical cancer who were treated between 2019 and 2022 at the Radiation Oncology Unit of Dr. B.R.A Institute-Rotary Cancer Hospital (BRAIRCH) at the All India Institute of Medical Sciences (AIIMS), Delhi. Inclusion criteria for the study cohort were: histopathologically confirmed cases of cervical cancer (stages IB2 to IV, according to the International Federation of Gynecology and Obstetrics, FIGO 2018), patients who were chemotherapy/radiotherapy-naive at the time of biopsy, and the availability of comprehensive clinical and follow-up data. Exclusion criteria included a history of any previous malignancy or prior chemotherapy/radiotherapy treatment. All patients provided informed consent for the use of their biological specimens in accordance with ethical guidelines and regulations. Blood samples were collected from patients at both pre-treatment (chemo/radiotherapy-naive) and post-treatment (after three months of therapy) time points. Additionally, plasma samples from 10 healthy donors were included as controls. Blood samples from both patients and healthy donors were collected in EDTA vials and processed on the same day. To obtain plasma, samples were centrifuged at 2000 rpm for 10 min. The plasma was then carefully separated and stored at -80 °C until further analysis.

Methodology for isolation of HPV circulating cell-free DNA

Circulating cell-free DNA (cfDNA) was isolated from 1,000 µL of plasma using the Maxwell RSC® ccfDNA Plasma Kit (AS1480; Promega, Madison, WI, USA), a magnetic bead-based automated system. For each patient sample, 1 mL of plasma was transferred to the first well of the Maxwell cartridge, with plungers placed in the eighth well. An empty elution tube was set on the deck tray, and 40 µL of elution buffer was added to each tube. The final elution volume was approximately 30 µL after processing. Extracted cfDNA was then stored at -80 °C. The quantity and quality of cfDNA were assessed using the NanoDrop® 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Droplet digital (dd) PCR analysis

The extracted cfDNA was analyzed for circulating HPV16 (ccfHPV16) and HPV18 (ccfHPV18) DNA using the QX200 Droplet Digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Each ddPCR reaction was

conducted in a total volume of 22 µL, consisting of a 2× ddPCR Supermix (Bio-Rad), primers, and probes at final concentrations of 450 nM and 250 nM, respectively, and a variable volume of template DNA, all in a final volume of 20 µL. The prepared ddPCR reaction mixtures were loaded into the sample wells of an eight-channel droplet generator cartridge (Bio-Rad). Droplet generation oil (70 µL per channel) was added, and the cartridge was processed through the droplet generator (Bio-Rad). The generated droplets were transferred to a 96-well PCR plate, heat-sealed with a foil seal, and subjected to thermal cycling for endpoint amplification (45 cycles). The cycling conditions were: 95 °C for 10 min, 39 cycles of 94 °C for 30 s, 55 °C for 1 min, followed by 98 °C for 10 min, and a final hold at 4 °C. Post-PCR, the plate was loaded onto the droplet reader (Bio-Rad) for droplet reading.

Primers and probes specific to the E7 oncogene of HPV16 and HPV18, targeting highly amplified regions in the tumor genome, were custom-designed (Supplementary Table S1). To validate assay specificity, purified DNA from SiHa (HPV16-positive) and HeLa (HPV18-positive) cell lines was used as positive controls. Each run included non-template controls and an internal control for the human reference gene beta-actin. The primers and probe sequences for beta-actin were designed using the BLAST tool, with detailed sequences provided in Supplementary Table S1.

Statistical analysis

All statistical analyses were performed using Stata 18.0 (StataCorp LLC, College Station, TX, USA). Descriptive statistics were reported as means and standard deviations for continuous variables and as frequencies and percentages for categorical variables. The differences in cfDNA and circulating HPV DNA (ccfHPV DNA) levels between pre-and post-treatment samples were assessed using paired t-tests or Mann-Whitney U tests, depending on data normality. Correlations between cfDNA levels and clinical parameters were evaluated using Pearson's or Spearman's correlation coefficients as appropriate for the data distribution. The correlation between two continuous outcomes were presented using scatter plot. Box-and-whisker plots were obtained to compare the distribution of a continuous outcome between two groups. The figure highlights the difference in circulating cell free DNA concentrations between the two groups, underscoring the potential utility of cell free DNA in differentiating between cancerous and non-cancerous states. The statistical significance of the differences in cell free DNA levels is indicated. The middle lines in the box plot are medians, the bottom of each box represents 25th percentile, and top of each box represents 75th percentile. The difference between 25th percentile and 75th percentile is inter-quartile range. The bottom of the whisker is called as lower adjacent value, it is calculated 25th percentile-1.5*Inter-quartile range. Whereas, the upper whisker plot is called as "upper adjacent value" and it is calculated as 75th percentile + 1.5*Interquartile range.

Results

Characteristics of study patients

The study included a total of 60 patients diagnosed with cervical cancer, treated between 2019 and 2022 at the Radiation Oncology Unit of Dr. B.R.A Institute-Rotary Cancer Hospital (BRAIRCH) at AIIMS, Delhi. A detailed overview of the clinical and histopathological characteristics of these patients is presented in Table 1 To establish a baseline for comparison, 10 healthy female volunteers were included as a control group. The average age of the study participants, including both cervical cancer patients and healthy controls, was 54 years.

Histopathological analysis revealed that the cervical cancer cases comprised three main types: Non-Keratinizing Squamous Cell Carcinomas (NKSCC), Squamous Cell Carcinomas (SCC), and Clear Cell Adenocarcinomas (CCA). Among the patients, 41 out of 60 (68.3%) were diagnosed with NKSCC, 26 cases (43.33%) with SCC, and 5 cases (8.33%) with CCA. Regarding disease staging, 5 patients (8.4%) were at stage I (early-stage disease), 27 patients (45%) at stage II, and another 27 patients (45.0%) at stage III, indicating advanced disease progression. Only 1 patient (1.6%) was diagnosed at stage IV, the most advanced stage of the disease. These distributions provide a comprehensive snapshot of the clinical and histopathological characteristics of cervical cancer within the study population, offering insights into the prevalent cancer types and typical stages at diagnosis.

Comparison of plasma cell-free DNA levels at baseline and post-treatment

The primary objective of our study was to assess and compare the median concentrations of circulating cell-free DNA (cfDNA) in cervical cancer patients before the initiation of therapy and after a follow-up period of three months. This analysis aimed to evaluate the potential of cfDNA as a biomarker for monitoring treatment efficacy and disease progression.

At baseline, prior to the commencement of any treatment, the median level of cfDNA in treatment-naïve cervical cancer patients was found to be 9.35 ng/µL. This elevated cfDNA concentration may indicate a significant presence of tumor-derived DNA in the bloodstream, reflecting the disease burden. Following a three-month course of therapy, a statistically significant reduction in cfDNA levels was observed, with the median concentration decreasing to 7 ng/µL ($p = 0.0308$). This reduction suggests a decrease in tumor burden as a result of the treatment, highlighting the potential of cfDNA levels as an indicator of therapeutic response in cervical cancer patients (Fig. 1A).

To further validate the utility of cfDNA as a diagnostic and monitoring tool, we compared the cfDNA levels in cervical cancer patients with those in the healthy control group. This comparison was essential to establish a baseline cfDNA level in individuals without cancer and to confirm the elevated cfDNA concentrations associated with cervical cancer. Healthy controls exhibited a significantly lower median cfDNA concentration of 6.95 ng/µL, markedly lower than the levels observed in cervical cancer patients both before and after treatment ($p < 0.001$)

Variables	Categories	Mean ± SD/ Median (P^{25}, P^{75})/Frequency (%) $N=60$
Age (in years)	–	52.76 ± 11.98[$]
Tumor types	NKSCC	26(43.33)[^]
	SCC	29(48.33)[^]
	CCA	5(8.34)[^]
Tumor Stages	IB3, IIA, IIB	32(53.33)[^]
	IIIB, IIIC1, IIIC2, IVA	28(46.67)[^]
Tumor Size (cm)	–	4.37 ± 1.88[$]
HPV18 Copies at Baseline *	–	0(0, 0.47)[&]
		[1.20 ± 4.15][$]
HPV18 Copies at three months*		0(0, 0.19)[&]
		[0.29 ± 0.93][$]
HPV16 Copies at Baseline *	–	0.38(0.08, 2.50)[&]
		[5.50 ± 12.44][$]
HPV18 Copies at three months *		0.23 (0.045, 1)[&]
		[3.59 ± 10.38][$]
HPV types: HPV18*	Present	16(45.72)
	Absent	19 (54.28)
HPV types: HPV16*	Present	29 (82.86)
	Absent	6 (17.14)

Table 1. Description of variables in cervical Cancer patients. Footnote: *: $n=35$; [$]: Mean ± Standard Deviation (SD); [&]: Median (P^{25}, P^{75}); [^]: Frequency (Percentage). *NKSCC* non-keratinizing squamous cell carcinomas, *SCC* squamous cell carcinoma, *CCA* clear cell carcinoma.

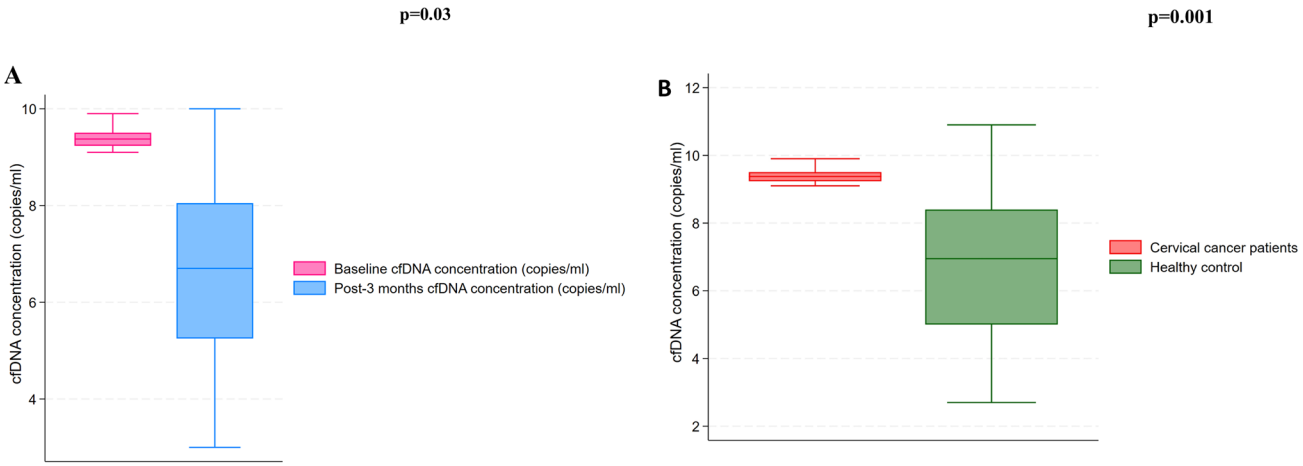

Fig. 1. Detection of Circulating Free DNA in Plasma Samples of Cervical Cancer Patients and Healthy Controls. (**A**) Quantification of circulating free DNA in plasma samples from cervical cancer patients ($n=60$) at two different time points: baseline (prior to any treatment) and three months post-therapy. The results are compared with plasma samples from healthy controls ($n=10$). (**B**) Comparative analysis of plasma cell free DNA levels between cervical cancer patients and healthy controls.

(Fig. 1B). The consistently elevated cfDNA levels in patients compared to healthy individuals reinforce the potential of cfDNA as a non-invasive biomarker for detecting and monitoring cervical cancer.

Detection and quantification of ccfHPV18 and ccfHPV16 DNA using ddPCR

After determining cfDNA levels in the cervical cancer cohort, we specifically detected and quantified the viral load of two high-risk human papillomavirus (HPV) types, HPV18 and HPV16, using droplet digital PCR (ddPCR). Of the initial 60 patient samples, 35 were available for ccfHPV DNA analysis; the remaining 25 samples were excluded due to patient dropout or insufficient sample quality during follow-up. The ddPCR screening analysis demonstrated that the detection rate for HPV18 was 45.71%, indicating a moderate prevalence of this subtype among the samples tested. In comparison, HPV16 showed a significantly higher detection rate of 82.86%, highlighting its predominance as a more frequently identified subtype in the study cohort. The prevalence of

HPV16 among patients aligns with epidemiological data indicating its higher oncogenic potential in cervical cancer. These findings provide a foundation for further analysis of HPV subtype prevalence, highlighting the significant differences in detection rates and their association with clinical and demographic parameters. The detection rates of HPV18 (45.71%) and HPV16 (82.86%) demonstrated a statistically significant difference ($P = 0.0012$) in a cohort of 35 patients. Stratification by tumor stage revealed that HPV18 detection was lower in early-stage tumors (36.84%) compared to later stages (56.25%), while HPV16 detection remained consistently high in both groups (84.21% and 81.25%, respectively). Among tumor subtypes, HPV18 was less prevalent in NKSCC (29.47%) compared to SCC (56.25%), whereas HPV16 detection was uniformly high in both (82.35% and 81.25%, respectively). Notably, both HPV18 and HPV16 were detected in both cases (100%, $n = 2$). Age-wise analysis showed the highest HPV18 detection rate in patients aged 46–59 years (64.71%) and the lowest in those 60 years or more (14.29%), while HPV16 detection was highest in patients 45 years or less (90.91%) with statistical significance ($P = 0.0078$). In tumors larger than 2 cm, HPV18 was detected in 45.71% of cases, while HPV16 was detected in 84.85%, with a statistically significant difference ($P = 0.0016$). In contrast, tumors smaller than 2 cm showed equal detection rates for both HPV18 and HPV16 at 50% ($P = 0.9999$). These findings underscore the predominance of HPV16 across clinical and demographic categories (Table 2). Furthermore we have checked the detection rate in subgroups in HPV18 and HPV16.

Correlation of ccfHPV DNA levels to clinical parameters

We also explored correlations between ccfHPV DNA levels and various clinical parameters, including histological type, patient age, FIGO stage, and tumor size. Patients were categorized into three groups based on their FIGO stage: Group 1 (Stages IB3, IIA, IIB), Group 2 (Stages IIIC1, IIIC2), and Group 3 (Stage IVA) (Fig. 2A).

Our analysis revealed no significant correlations between ccfHPV DNA levels (specifically ccfHPV16 and ccfHPV 18) and patient age ($p = 0.34$ and 0.49, respectively) or histological subtype ($p = 0.08$ for HPV16 and $p = 0.06$ for HPV18) (Fig. 2B,C). These findings suggest that viral DNA levels in circulation are not significantly influenced by demographic or histopathological factors. However, a notable correlation was observed between ccfHPV16 levels and tumor size ($p = 0.336$) (Fig. 2D), suggesting that higher ccfHPV16 DNA levels may be associated with larger tumors, reflecting a greater viral load in more advanced disease stages. No correlation was found between ccfHPV DNA levels and patient age, indicating that age does not confound the relationship between viral load and disease characteristics.

Detailed statistical analyses of these correlations are presented in Table 3, which provide comprehensive insights into the relationships between ccfHPV DNA levels and the examined clinical parameters.

Discussion

In this study, we focused on the utility of circulating HPV DNA (ccfHPV DNA) as a prognostic marker in cervical cancer, particularly in the Indian population. The growing interest in liquid biopsies for cancer detection and monitoring has highlighted the need for highly sensitive and specific analytical techniques, especially for conditions like cervical cancer, which is predominantly driven by high-risk HPV strains. The ability to accurately detect and quantify ccfHPV DNA offers a non-invasive means to monitor disease progression and treatment response and potentially predict outcomes in patients undergoing therapy[17]. This is due to ddPCR's capability to partition DNA into thousands of nano-litre-sized droplet, each undergoing individual end-point amplification.

Our findings underscore the challenges associated with the detection of circulating HPV DNA due to its typically low abundance in the bloodstream[18]. The use of droplet digital PCR (ddPCR) in this study provided the necessary sensitivity and precision, allowing us to detect and quantify ccfHPV DNA in plasma samples from Indian cervical cancer patients. The ddPCR approach demonstrated its superiority over traditional qPCR

	ccfHPV18	ccfHPV16	
	Detection rate Freq (%)	Detection rate Freq (%)	*P* value
Total ($n = 35$)	16 (45.71%)	19 (82.86%)	0.001
Tumor stage			
Early stage (IB3, IIA, IIB) ($n = 19$)	7(36.84%)	16 (84.21%)	0.003
Later stage (IIIB, IIIC1, IIIC2, IVA) ($n = 16$)	9 (56.25%)	13 (81.25%)	0.127
Tumor types			
NKSCC ($n = 17$)	5 (29.47%)	14 (82.35%)	0.002
SCC ($n = 16$)	9 (56.25%)	13 (81.25%)	0.127
CCA ($n = 2$)	2 (100%)	2 (100%)	0.999
Age (in years)			
≤ 45 ($n = 11$)	4 (36.36%)	10 (90.91%)	0.008
46–59 ($n = 17$)	11 (64.71%)	15 (88.24%)	0.106
≥ 60 ($n = 7$)	1 (14.29%)	4 (57.14%)	0.094
Tumor size (in cm)			
≤ 2 ($n = 2$)	1 (50.00%)	1 (50.00%)	0.999
> 2 ($n = 33$)	15 (45.71%)	28 (84.85%)	0.002

Table 2. Detection of ccfHPV16 and ccfHPV18 using the ddPCR.

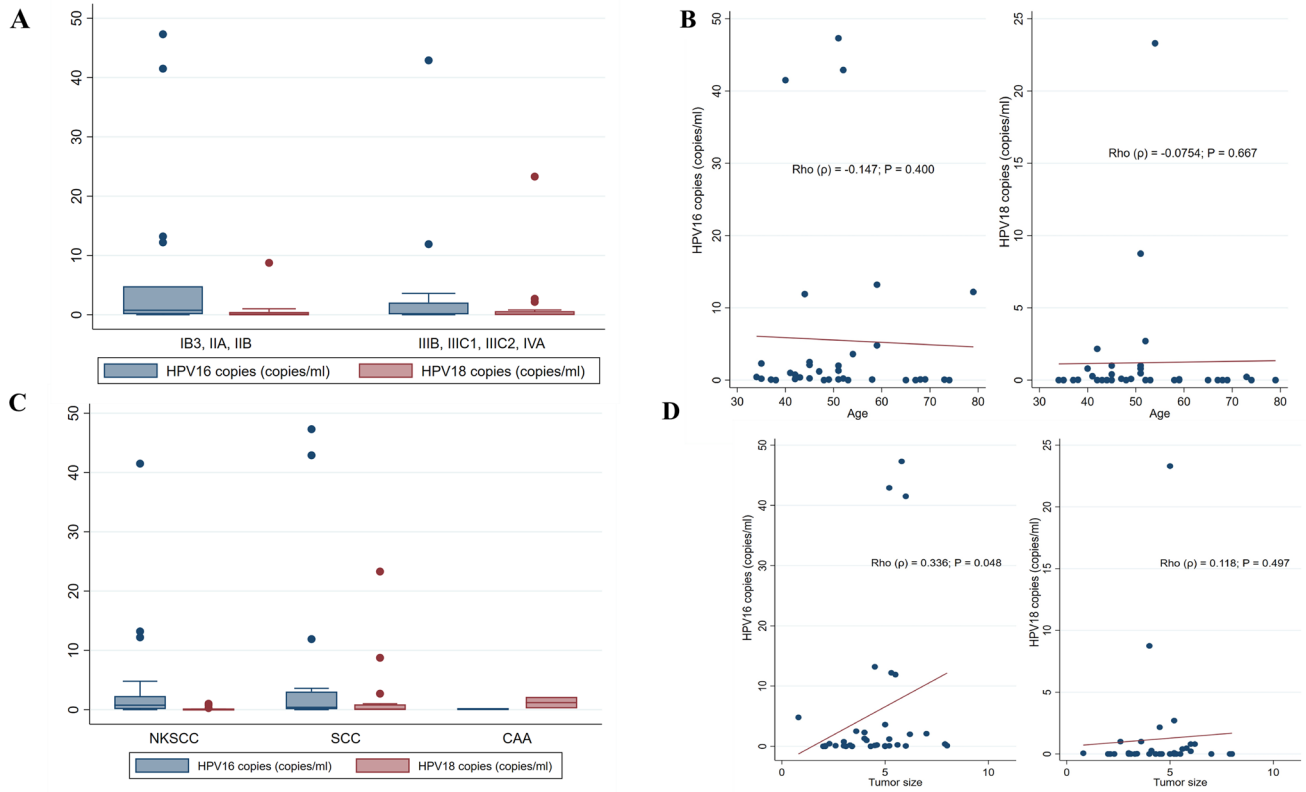

Fig. 2. Concentration of Circulating Cell Free HPV DNA (ccfHPV DNA) in Cervical Cancer Patients by Clinical Criteria (**A**) Concentration of circulating HPV DNA (copies mL, log scale) detected by ddPCR in cervical cancer patients, stratified by FIGO 2018 staging criteria. The stages are grouped into 2 categories: IB3, IIA, IIB patients and IIIB, IIIC1, IIIC2, IVA patients. This panel illustrates how the concentration of c-HPV DNA varies with disease progression, suggesting a correlation between higher c-HPV DNA levels and advanced disease stages. Statistical significance between groups was assessed using the t-test or Mann-Whitney U test, as appropriate. (**B**) Concentration of ccfHPV DNA (copies /mL, log scale) in cervical cancer patients categorized by age groups (in years). The figure explores whether patient age influences ccfHPV DNA levels, providing insights into the demographic factors that may affect viral load in cervical cancer patients. Statistical analysis was conducted to determine any significant differences between age groups. (**C**) Concentration of ccfHPV DNA (copies mL, log scale) based on histological classification of cervical cancer: Non-Keratinizing Squamous Cell Carcinoma (NKSCC), Squamous Cell Carcinoma (SCC), and Clear Cell Adenocarcinoma (CAA). This panel evaluates the variation in ccfHPV DNA levels across different histological subtypes of cervical cancer, highlighting the potential differences in viral DNA presence related to tumor type. Statistical comparisons were made to assess significance among these groups. (**D**) Concentration of ccfHPV DNA (copies/mL, log scale) correlated with tumor size (measured in centimeters). This figure demonstrates the relationship between tumor size and ccf HPV DNA levels, suggesting that larger tumors may release more viral DNA into the bloodstream. The data support the use of ccfHPV DNA quantification as a proxy for tumor burden, which could aid in monitoring disease progression and treatment efficacy. Statistical significance was determined using appropriate tests. These dots outside the box represent the outliers.

	ccfHPV18		ccfHPV16	
	Median (P^{25}, P^{75})	*P* value	Median (P^{25}, P^{75})	*P* value
Tumor type				
NKSCC (*n* = 17)	0.00 (0.00,0.06)	0.063	0.76 (0.10, 2.30)	0.565
SSC (*n* = 16)	0.05 (0, 0.90)		0.41 (0.08, 3.05)	
CCA (*n* = 2)	1.19 (0.22, 2.16)		0.10 (0.06. 0.14)	
Tumor stages				
1B3, IIA, IIB (*n* = 19)	0.00 (0.00, 0.47)	0.357	0.76 (0.09, 4.80)	0.335
IIIC1.IIIC2, IVA (*n* = 16)	0.05 (0.00, 0.6)		0.38 (0.08, 2.50)	

Table 3. Association of ccfHPV16 and ccfHPV18 viral load with tumor stages in various Cancer types (*n* = 35).

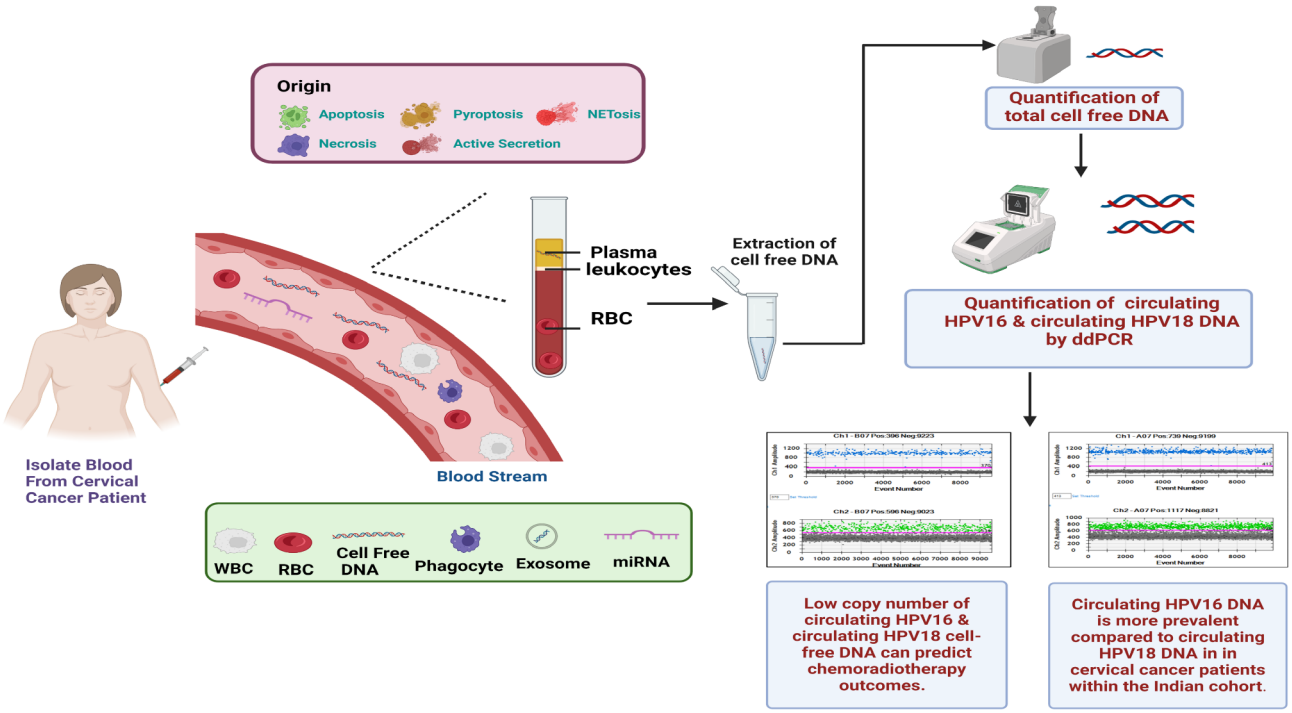

Fig. 3. Schematic representation of the steps involved in extraction, quantification and analysis of cfDNA from the whole blood of cervical cancer patients.

methods, which often lack the sensitivity required for detecting low levels of circulating DNA. Earlier studies conducted by Jeannot et al. suggested that a positive ccfHPV DNA test could precede tumor relapse; however, there is a lack of concrete data or follow-up studies to confirm this[19]. Similarly, a study done by Zhang et al. suggested that ccfHPV DNA has the potential to serve as an early indicator of tumor relapse, however, there is insufficient concrete data and follow-up studies to confirm this possibility[20]. Therefore, long-term follow-up studies are necessary to validate the utility of ccfHPV DNA levels as early indicators of relapse. Furthermore, the Indian population was not included in any of these studies. In light of this, we concentrated our research on Indian patients with cervical cancer, collecting blood at baseline blood and following up three months after treatment. Additionally, ccfHPV16 and ccfHPV18 DNAs were quantified using ddPCR in cervical cancer patients before and after therapy as shown in Table 1. This study builds upon these earlier works by offering population-specific insights into the clinical utility of ccfHPV DNA in an underserved demographic.

However in our analysis of ccfHPV DNA in both pre-and post-treatment plasma samples we did not find significant differences with p value of 0.27. The ability to track tumor dynamics in real-time through liquid biopsies presents a promising avenue for personalized cancer care, particularly in resource-limited settings like India, where late-stage diagnosis remains prevalent.

Our results indicate a significant decrease in median cell-free DNA levels in cervical cancer patients post-therapy, suggesting a reduction in tumor burden. Additionally, cell-free DNA levels were significantly higher in cervical cancer patients compared to healthy controls, reinforcing the potential of ccfDNA as a cancer biomarker. Additionally, the significant difference in HPV16 and HPV18 detection rates highlights the predominance of HPV16 in this cohort, reinforcing its higher oncogenic potential. Furthermore, we explored the correlation between ccfHPV DNA levels and clinical parameters such as patient age, histology, FIGO stage, and tumor size. While no significant association was found between ccfHPV DNA levels and patient age or histology, a correlation was observed with tumor size, particularly for ccfHPV16 DNA. This suggests that low copy number ccfHPV16 DNA levels may reflect tumor burden and serve as a useful indicator for monitoring disease progression.

Moreover, the study provides insights into the potential application of ccfHPV DNA as a diagnostic tool for early-stage cervical cancer. Its non-invasive nature offers a significant advantage over existing screening methods, such as Pap smears, which have shown limited sensitivity in low-prevalence population[21]. The schematic figure shows the steps involved in the extraction of cfDNA to quantification of ccfHPV DNA and further analysis, which are represented in Fig. 3. The integration of ccfHPV DNA analysis into routine clinical practice could enhance the early detection of cervical cancer, thereby improving survival rates, especially when combined with traditional cytological methods.

Conclusion

This study highlights the potential of circulating cell-free HPV DNA (ccfHPV DNA) as a valuable prognostic biomarker in cervical cancer. The application of droplet ddPCR in detecting low-abundance ccfHPV16 and ccfHPV18 DNA, establishing its superiority over traditional traditional diagnostic methods such as Pap smear

testing. High detection rates of HPV16 compared to HPV18 align with epidemiological trends and underscore the oncogenic predominance of HPV16. The correlation between ccfHPV16 levels and tumor size further supports its role as a biomarker for tumor burden. These results advocate for the integration of ccfHPV DNA analysis into clinical practice, potentially aiding in early detection, monitoring, and personalized treatment strategies. As we advance towards more individualized approaches to cancer care, liquid biopsies and the analysis of circulating tumor DNA (ctDNA) are poised to play an increasingly pivotal role. The non-invasive nature of liquid biopsies, combined with their ability to offer real-time insights into tumor dynamics, presents a significant advantage over traditional tissue biopsies. This is especially relevant in cervical cancer, where early detection and continuous monitoring are key to improving patient outcomes and survival rates.

However, while our study provides compelling evidence for the use of ccfHPV DNA as a biomarker, further research is essential to optimize detection methodologies and confirm the clinical utility of ccfHPV DNA in larger and more diverse patient populations. Future studies should focus on refining pre-analytical conditions, enhancing assay sensitivity, and establishing standardized protocols to ensure the reproducibility and accuracy of results across different clinical settings.

Moreover, longitudinal studies with extended follow-up periods are needed to validate ccfHPV DNA as an early indicator of relapse and to explore its potential in predicting long-term patient outcomes. These efforts will be crucial in integrating ccfHPV DNA analysis into routine clinical practice, where it could complement existing screening methods and provide a more comprehensive approach to managing cervical cancer.

Ultimately, incorporating ccfHPV DNA analysis into standard care protocols could revolutionize how we monitor and treat cervical cancer, leading to more personalized, effective, and timely interventions. By enhancing our ability to detect and respond to disease changes, this approach has the potential to significantly improve outcomes for cervical cancer patients, contributing to better survival rates and quality of life.

Data availability

Data is provided within the manuscript or supplementary information files.

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Author contributions

M.S. and A.A.B. conceived the study and designed the experiments. A.G., G.D., S.K.D., and R.C. collected the samples and performed experiments. A.G. and G.D. created the figures. M.S., A.A.B., A.G., and G.D. wrote the manuscript. D.N.S. and A.S. helped collect the samples. M.A.K. helped in the statistical analysis. M.S. and A.A.B. supervised the study. V.S., M.A.M., I.A., and A.S.A.A. critically revised the manuscript. All authors reviewed the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

Ethical approval for the current study was obtained from the AIIMS Institute ethics committee (AIIMS IEC), bearing no. IEC-208/05.05.2017.

Consent for publication

All authors consent to publication.

Additional information

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