RESEARCH ARTICLE

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Quantification of Circulating HPV DNA as a Biomarker for Cervical Pre-Cancer and Cancer: A Pilot Study

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Abstract

Background: Tumor cells release fragments of their DNA into the bloodstream, called cell-free tumor DNA (ctDNA) or liquid biopsy. In this study, we investigate whether human papillomavirus cell-free tumor DNA (ctHPV DNA) can be detected in patients with cervical cancer or premalignant lesions before and after treatment. We are also investigating whether ctHPV DNA levels correlate with patient or tumor characteristics and outcomes. **Methods:** A total of 67 cases were included, including 42 with locally advanced cervical cancer (LACC) and 11 with early-stage cervical cancer (ESCC), as well as 14 with premalignant lesions. Pre- and post-treatment plasma samples were tested for ctHPV DNA levels using digital droplet PCR. **Results:** The pretreatment ctHPV DNA was detected in 21.42%(n=09/42) cases with LACC and post treatment ctHPV DNA was detected in 16.66% (07/42) LACC cases. While circulating ctHPV DNA was not detected in ESCC and premalignant lesion cases. Higher levels of ctHPV DNA were correlated to the higher FIGO2018 stage. **Conclusion:** ctHPV DNA is a promising biomarker in locally advanced cervical cancer and should be further investigated for clinical use. In ESCC patients, the detection rate of ctHPV DNA is not sufficient for clinical benefit even using ddPCR, the most sensitive technologies available.

Keywords: Cervical cancer- Human Papillomavirus- Cell free tumor DNA- Liquid Biopsy- Biomarker

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Introduction

Cervical cancer is the most common cancer among middle-aged women worldwide, causing 6,000,000 cases annually [1]. Most cervical cancers are caused by persistent infection with high-risk human papillomavirus (HR-HPV) [2]. Early-stage cervical cancer (ESCC), classified as FIGO 2018 stages IB2 to IIA1, is generally treated through surgical procedures. In contrast, locally advanced cervical cancer (LACC), which falls under FIGO 2018 stages IB3 to IVB, is predominantly treated with definitive chemoradiotherapy (CRT). Given the different treatment modalities, outcomes and follow-up protocols associated with early-stage and locally advanced cervical cancer, these two categories will be examined independently in the following sections [3].

Cells release fragments of their DNA into the bloodstream, called cell-free DNA (cfDNA), through mechanisms such as active transport or during the process of apoptosis. The majority of cfDNA present in the circulation comes from immune cells, although significant contributions also come from vascular endothelial cells and the liver. Tumor cells also contribute to the cfDNA pool, particularly in the form of cell-free tumor DNA (ctDNA), although in relatively small amounts. Recent advances in highly sensitive methods such as digital PCR have enabled the analysis of ctDNA through simple blood tests, commonly known as liquid biopsies. Because the half-life of cfDNA is particularly short, approximately two hours, ctDNA provides a real-time representation of the tumor at the time of sampling while simultaneously including all malignant clones [4]. Total cfDNA concentration tends to increase in both localized and metastatic cancers, with increased concentrations typically observed in metastatic cases [5, 6]. Nevertheless, it is important to note that some cancer patients may not release ctDNA and total cfDNA levels may be influenced by various factors, including age, inflammation, and liver and kidney disease, which may affect the reliability of total cfDNA as a biomarker [4].

The high-risk human papillomavirus (HR-HPV) DNA is known to integrate into the host genome, resulting in

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the release of the HPV DNA as circulating tumor DNA (ctDNA). In addition, HR-HPV DNA is often present in multiple copies in individual cells. This property makes ctHPV DNA a compelling biomarker for cervical cancer [7-10]. Initial investigations into the presence of HPV DNA in blood samples showed inadequate sensitivity; However, recent studies using digital droplet PCR (ddPCR) have shown encouraging results in cervical cancer as well as other HPV-associated malignancies such as oropharyngeal and anal cancers [11–16]. Despite these advances, further research is essential to clarify the dynamics of ctHPV DNA during treatment, identify relevant patient populations, and determine the clinical applicability of ctHPV DNA. Furthermore, there are conflicting results regarding the presence of ctHPV DNA in the blood of patients with premalignant lesions that may potentially progress to cervical cancer [17-19].

The aim of this research was to evaluate the feasibility of detecting circulating tumor HPV DNA (ctHPV) in plasma using digital droplet polymerase chain reaction (ddPCR). In addition, the study aimed to analyse the relationship between ctHPV DNA levels and total circulating free DNA (cfDNA) load at different stages: before, during and after treatment. This analysis was intended to correlate these measurements with tumor and clinical characteristics while providing a preliminary assessment of ctHPV DNA as a potential biomarker of prognosis and treatment response in different stages of cervical cancer (CC) and in premalignant lesions.

Materials and Methods

Patients and samples

The study included patients diagnosed with locally advanced cervical cancer who were treated at the Department of Obstetrics and Gynaecology, King George Medical University, Lucknow, from December 2021 to June 2024. The study also included patients with premalignant lesions of the cervix who had previously tested positive for HPV types 16, 18 and/or 45, as documented in their medical records. A total of 4.00 mL of peripheral blood samples were collected from these patients both before and after treatment using EDTA vials. After collection, the plasma was isolated by centrifugation and then stored at -80 °C for DNA extraction. Patients were monitored for an average of 12 months, ranging from 7 to 28 months. Ethical approval for the study was granted by the institutional ethics committee of KGMU, Lucknow and written informed consent was obtained from all participants before their inclusion in the study.

Chemo radio therapy Treatment

The majority of patients underwent chemoradiotherapy (CRT), which generally included 6 weeks of external beam radiation therapy (EBRT), 6 cycles of weekly cisplatin, and 3 to 4 sessions of brachytherapy. Biopsy samples were collected from each patient for HPV typing. In addition, consecutive patients diagnosed with early-stage cervical cancer (FIGO 2018 stages IB1, IB2 and selected cases of IIA1) who were referred for treatment were invited to participate in the study. Of these, six patients had already undergone a diagnostic cone biopsy before their referral. All patients diagnosed with early-stage disease received radical surgical treatment.

HPV Testing

HPV testing was performed using PGMY09/11 primers as described by P.E. described. Gravitt et al. in 2000, which were specifically designed to amplify a 450 base pair fragment of the HPV L1 gene. Cervical cancer cases in which the presence of HPV had previously been confirmed served as positive controls. PCR amplification was performed in a total volume of 20 L containing 50 ng/µl genomic DNA, 10µl of Hot Start PCR Master Mix (Invitrogen, USA). The concentration of PGMY09/11 primers was reduced to 5pmol from previously used 10pmol and volume was makeup to 20 µl using nuclease free water. The amplification was carried out in a thermal cycler (S100TM, Bio-Rad, USA) under the following conditions: a first denaturation step at 95 °C for 9 minutes, followed by 35 cycles consisting of denaturation at 95 °C for 1 minute, primer- Annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute and finally a final extension at 72 °C for 5 minutes. Both positive and negative controls were processed simultaneously.

HPV Genotyping

Samples that were positive for HPV presence were proceeded further for high-risk HPV 16 & 18 genotyping. HPV genotyping was done with the previously described type specific primers for HPV 16 and 18 (Sharma et al., 2005). PCR amplification was carried out in a 20µl reaction volume containing 50ng/µl of genomic DNA, 10pmol each of forward and reverse primer, and 10µl of hot start PCR master mix (Invitrogen, USA). Amplification was performed in thermal cycler (S100TM, Bio-Rad, USA) under the following PCR conditions: An initial denaturation at 94ºC for 10min, followed by 35 cycles of 94ºC for 1min (cycle denaturation), primer annealing at 52°C for 1min for HPV 16 & 62°C for 1min for HPV 18, extension at 72ºC for 1min and a final extension of 72ºC for 5min. PCR products were confirmed for their respective amplicon size on 2% agarose gel electrophoresis and visualized by UV-transillumination (Gel Doc XR+ , Bio-Rad, USA).

Extraction and analysis of ctHPV DNA

A 3.0 ml of peripheral blood was collected from each patient using an EDTA tube, and the plasma was subsequently separated by two step centrifugations. cfDNA was isolated from 200 µl of plasma using a QIAamp Min Elute Virus Spin kit (Qiagen, USA) following the manufacturer's protocol. Elution was carried out with 25 ml of the provided elution buffer. The eluates obtained from the identical sample were combined and then stored at -20 °C until the HPV ctDNA was analysed.

The QX100 system (Bio-Rad Laboratories) was used for ddPCR reactions according to the manufacturer guidelines. The reaction was carried out in 20µl master mix solution containing 10 ml of 2x ddPCR Super Mix for probes without dUTP (Bio-Rad), relevant primers at a concentration of 450 nmol/L each and corresponding

TaqMan probes at 250 nmol comprised /L each (Applied Biosystems), and 6µl of DNA template and volume was adjusted using nuclease-free water. The PCR reactions were performed on a C1000 thermal cycler (Bio-Rad) following this thermal cycling protocol: 10 minutes at 95 °C, followed by 40 cycles (30 seconds at 94 °C, 1 minute at 60 °C) and finally for 10 Minutes at 98 °C. Quantification of HPV DNA copies were performed using Quanta Soft v1.7.4 software. Three replicates were performed to identify HPV16, HPV18, or HPV integration sites using specific primers and TaqMan probes for HPV16 E7, HPV18 E7, or viral-cellular compounds, multiplexed with a commercial human ddPCR assay targeting RPP30 gene (dHSaCP2500350, Bio-Rad Laboratories), which served as a reference for human DNA. For each patient, genomic DNA extracted from frozen tumor samples was used as a positive control, and a triplicate without DNA was provided for each test plate. Serum samples were classified as HPV positive if at least three droplets containing HPV amplicons were present. Conversely, a sample was considered HPV negative if fewer than three or no droplets containing HPV amplicons were observed, provided that more than 500 copies/mL of the human RPP30 reference gene was detected. Finally, the concentration of HPV ctDNA was reported in copies/ml serum.

Statistical analysis

Statistical analysis was performed using the statistical analysis software SPSS (Statistical Package for Social Science) version 21.0. Categorical variables were presented as numbers and percentages (%) and continuous variables as mean SE. The Shapiro-Wilk test was used to test the normal distribution of the variables. Overall survival was measured from the start of treatment to the date of surgery or last follow-up. The chi-square test was used to calculate the significance of differences between categorical variables. Two-sided p values were reported for all analyses and p values less than 0.05 were considered statistically significant.

Results

Patient characteristics

A total of 67 cases of preneoplastic and neoplastic lesions of the cervix were analysed, including 14 (20.9%) preneoplastic cases and 53 (79.1%) neoplastic cases, all of which were treated in the Department of Obstetrics and Gynaecology. Age of patients enrolled in the study ranged from 29 to 82 years, the mean age of patients was 53.48±11.74 years. Only 16.4% of women were ≤40 years of age and only 4.5% were >70 years. The majority of patients were 41-60 years (58.2%). Out of 53 neoplastic cases enrolled, 49/53 (92.5%) were presented with moderately differentiated squamous cell carcinoma, 03/53(5.7%) were of well differentiated squamous cell carcinoma and 01/53(1.9%) was of poorly differentiated squamous cell carcinoma.

Forty-two patients with locally advanced cervical cancer (LACC) were included. Human papillomavirus (HPV) types 16, 18, and 45 were detected in 23 of 42 (52.27%) biopsy samples. Plasma samples were collected from all 42 LACC patients. Of these, 15 patients received chemoradiotherapy (CRT), three received only external beam radiation therapy (EBRT) due to existing comorbidities, while four patients were treated with primary chemotherapy (cisplatin and paclitaxel) and subsequent CRT or EBRT. In addition, one patient underwent primary surgery followed by CRT. During the follow-up period, 11 patients with locally advanced cervical cancer experienced disease progression. In addition, 11 patients with early-stage cervical cancer were included in the study. Of these, nine out of 11 tested positive for HPV types 16, 18 or 45, and 15 of 22 were previously identified as positive for these HPV types using the PCR method. Pretreatment plasma samples were collected and 6 of 11 (54.54%) patients received adjuvant treatment. Of note, no patient with early-stage cervical cancer experienced disease recurrence during the follow-up period. In addition, 14 premalignant cases were included and plasma samples were also collected from these patients. The ctHPV DNA Copy number in each case is depicted in Table 1.

Association of ctHPV DNA with clinical stages of tumor: ctHPV DNA was not detected in early tumor stage (Stage IB1 and IB2). However, in locally advanced cervical cancer stages, ctHPV was detected in 11.11% of cases with Stage IIB, 40.0% in Stage IIA, 66.7% in Stage IVA, and 100% in Stage IIIB and Stage IVB (n=1 each). There was a statistically significant difference in ctHPV detection across various clinical tumor stages (p=0.006).

Change in ctHPV levels before and after treatment

Post-treatment ctHPV level was 24108.44±25.308.51 copies/µl. A decline of 32387.44±29948.95 copies/µl in the pre-treatment ctHPV level (56495.89±53248.63 copies/microliter) was observed. A statistically significant decline in pre-treatment ctHPV levels was observed after treatment (p=0.012) (Table 2).

Outcome of the Study population

Of the 67 cases included in the study, 2 were lost to follow-up, leaving the results of 65 cases known. Of these 65 cases, 5 (7.7%) had expired, 9 (13.8%) were undergoing chemoradiotherapy, and the remaining 51 (78.5%) were in follow-up. In addition, 3 (4.6%) had completed the 6-month follow-up period after treatment.

Comparison of Survival duration of ctHPV not detected and detected Data

While the duration of survival was longer for cases undetected on ctHPV compared to those detected on ctHPV (11.474±0.298 vs. 8.091±0.695 months), this difference was not found to be statistically significant (p = 0.023) (Table 3 and Figure 1).

Discussion

Cervical cancer remains a major health problem worldwide and is the fourth most common cancer in women after breast, colon and lung cancer. This is particularly alarming in low- and middle-income

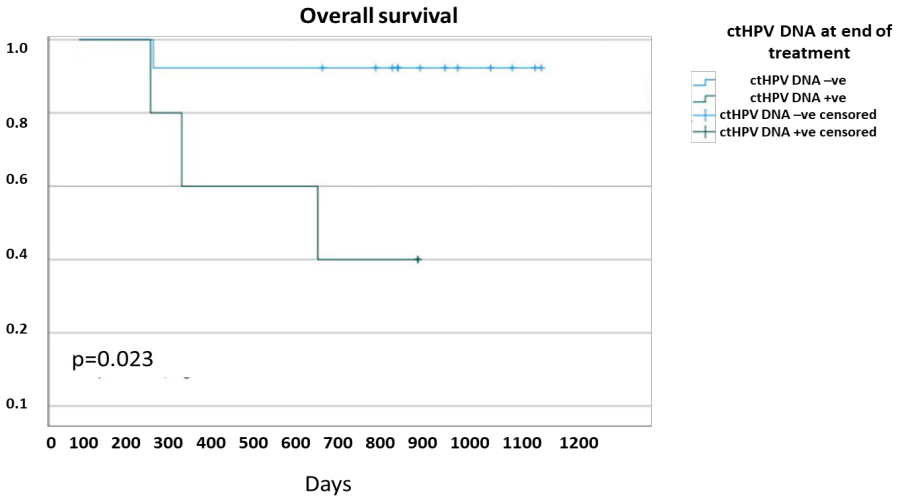

Figure 1. Kaplan-Meier Graph Depicting Overall Survival for Locally Advanced Cervical Cancer Patients in association to Plasma ctHPV-DNA Status at End of Treatment

Table 1. Distribution of ctHPV DNA Copy Number in each Case

	Pretreatment ctHPV DNA levels from plasma			Post treatment ctHPV DNA levels from plasma	
S. No.	ctHPV 16 (copies/microlitre)	ctHPV 18 (copies/microlitre)	ctHPV DNA from tissue (copies/microlitre)	ctHPV 16 (copies/microlitre)	ctHPV 18 (copies/microlitre)
Case 1	47,658	-	1,508	18,400	
Case 2	61,535	-	45,,678	25,760	
Case 3	121,380	-	3,765	Patient expired	
Case 4	120,360	-	48,543	No treatment received	
Case 5	19,860	-	12,765	6,630	
Case 6	121,370	-	313,068	70,685	
Case 7	61,636	-	39,654	24,800	
Case 8	161,020	-	38,678	60,410	
Case 9	-	8127	1,224	-	4,061
Case 10	-	8038	1,034	-	630
Case 11	19219		Outside treated case, FFPE tissue not available	5,600	

countries, as cervical cancer is the third most common cancer in women, accounting for approximately 85% of new cases and 90% of disease-related deaths [20]. The global prevalence of human papillomavirus (HPV) infection in women is estimated at 31%. Several factors contribute to the increased risk of cervical cancer, including HPV infection, lower socioeconomic status, tobacco use, early marriage (before age 18), early initiation of sexual activity, multiple sexual partners, and experiencing multiple pregnancies. HPV, particularly strains 16, 18, 31, 33 and 45, is identified as the primary etiological agent in the development of cervical cancer and significantly influences its progression [21].

In recent years, the field of liquid biopsy has attracted considerable attention, particularly with regard to the use of cell-free tumor DNA (ctDNA) as a non-invasive approach to identify cancer-specific genetic abnormalities in plasma. Numerous studies have demonstrated the effectiveness of ctDNA in detecting genetic alterations associated with advanced stages of cancer. Assays that analyse ctDNA provide comprehensive insight into tumor heterogeneity, facilitate real-time assessment of treatment effectiveness, and are critical for early identification of

Table 2. Change in Pre-Treatment ctHPV Level

	Mean	S.D.
Before treatment	56495.89	53248.63
After treatment	24108.44	25308.51
Change after treatment	32387.44	29948.95
Paired 't' test	't'=3.244; p=0.012	

Table 3. Comparison of Survival duration of ctHPV not Detected and Detected Data

ctHPV	Mean	SE	95% CI
Not detected	11.474	0.298	10.890-12.057
Detected	8.091	0.695	6.728-9.454

Log-Rank (Mantel-Cox) χ^2=3.584; p=0.023

resistance mutations. Furthermore, recent discussions have suggested ctDNA analysis as a promising tool for early detection and detection of cancer [22, 23].

The association between circulating cell-free tumor HPV DNA levels and cervical cancer and their correlation with disease stage and treatment has been documented in numerous studies [2, 8, 24, 25]. In the current investigation, we examined the levels of circulating cell-free tumor HPV DNA in both preneoplastic and neoplastic lesions of the cervix, focusing on their association with treatment modalities and different stages of cervical cancer. To achieve this, a prospective observational study was designed, which included a total of 67 cases of preneoplastic and neoplastic lesions of the cervix. Prospective studies are characterized by their forward-looking approach, allowing the collection of data specifically tailored to capture relevant exposure information, thereby increasing the potential for comprehensive analysis [26]. In present study, the age of patients ranged from 29 to 82 years, mean age of patients was 53.48±11.74 years. Majority of patients were 41-60 years (58.2%), 16.4% women were ≤40 years of age and only 4.5% were >70 years.

Sathish et al. conducted a study involving 68 cases, of which 58 were diagnosed with invasive cervical cancer [8]. Among these, three cases were classified as Stage IV, with a mean age of 47 ± 7.55 years. Eighteen cases were categorized as Stage IIIB, with a mean age of 47.6 ± 7.66 years, while 21 cases were identified as Stage IIB, with a mean age of 49 ± 9.17 years. Additionally, one case was classified as Stage IIA, with a mean age of 47.5 ± 6.36 years, and 15 cases were identified as Stage IB, with a mean age of 42.3 ± 8.94 years. The study also included 10 cases of preneoplastic lesions, with a mean age of 40.8 ± 11.1 years, comprising one case each of CIN I and CIN II, and eight cases of CIN III [9].

Sanjose et al. conducted a study involving 14,249 women, of whom 10,575 cases of invasive cervical cancer were identified. Among these cases, 8,977 (85%) tested positive for HPV DNA. The analysis revealed that women with invasive cervical cancers associated with HPV types 16, 18, or 45 were diagnosed at a younger average age compared to those with other HPV types, with mean ages of 50.0 years (49.6-50.4), 48.2 years (47.3-49.2), 46.8 years (46.6-48.1), and 55.5 years (54.9-56.1), respectively [2]. Therefore, all of these studies, which were similar to the present study, reported a mean age of cases between 40 and 55 years, but there were minor differences in the proportion of malignant and premalignant cases.

The results of the current investigation show that of the 53 neoplastic cases examined, 49 (92.5%) were classified as moderately differentiated squamous cell carcinoma, while 3 (5.7%) were identified as well-differentiated squamous cell carcinoma and 1 (1.9%) than poorly differentiated squamous cell carcinoma. A significant proportion of cases, both with and without detectable ctHPV, were moderately differentiated at 92.9% and 90.9%, respectively. In contrast, well-differentiated and poorly differentiated cases were rare, with only three cases of well-differentiated carcinoma and a single case of poorly differentiated carcinoma. Notably, all three well-differentiated cases were negative for ctHPV, whereas the one poorly differentiated case tested positive. Nevertheless, no statistical association was found between histological grade and ctHPV status. Sivars et al. reported 17 cases of squamous cell carcinoma, 13 cases of adenocarcinoma, 1 case of clear cell carcinoma, 1 case of adenosquamous carcinoma, and 1 case of Sarcomatoid carcinoma [27]. Furthermore, Young et al. identified squamous cell carcinoma as the most common type of cervical cancer; However, their research did not examine the association between histological grade, type and ctHPV [28].

Of the 67 patients included in this study, only 6 (8.9%) underwent liquid-based cytology examination. Among them, 4 (5.9%) were diagnosed with NILM (negative for intraepithelial lesion or malignancy) and the remaining 2 (2.9%) were diagnosed with LSIL (low-grade squamous intraepithelial lesion). After further histological confirmation, one of the NILM cases and one LSIL case were diagnosed with CIN 1 (cervical intraepithelial neoplasia grade 1), while the other LSIL case was diagnosed with early-stage cervical cancer. None of them had ctHPV detection. Caliskan et al. studied a group of 172 patients, representing 42.1% of all patients who tested positive for HPV DNA; Within this group, 107 individuals (26.2%) had hrHPV. The most common HPV genotypes were 59, 16, 33, 52, and 51, accounting for 16.6%, 15.9%, 13.4%, 13.4%, and 8.9%, respectively. Epithelial cell abnormalities were detected in 11.5% of LBC test results. Of note, HPV genotypes 33, 56, 66, and 68 were observed more frequently in patients with epithelial cell abnormalities than in patients without such findings [29].

The present study demonstrates the feasibility of identifying circulating tumor HPV (ctHPV) DNA in plasma samples from individuals with cervical preneoplastic and neoplastic diseases. Real-time polymerase chain reaction (PCR) was used to determine circulating cell-free tumor HPV DNA levels. To check the integrity of the extracted DNA, all samples were tested for the human housekeeping gene human actin. The overall detection rate of ctHPV DNA was 16.4% (n = 11/67). Specifically, the detection rate for ctHPV DNA type 16 was 13.4% (n = 9/67), while for type 18 it was 2.9% (n = 2/67). Of note, the study did not detect detectable ctHPV levels in preneoplastic lesions. Of the 14 cases of preneoplastic lesions associated with cervical cancer, 5 were classified as CIN I and CIN I/II, with one case each categorized as CIN II and CIN III, and 2 cases identified as CIN II/III. In none of these cases was the presence of ctHPV DNA detected, consistent with the results of most previous studies.

The present study included a total of 53 neoplastic cases from which samples were collected before treatment. Posttreatment samples were collected from 49 cases because 2 cases were lost to follow-up and 2 cases were within 2 months of diagnosis. Of these cases, 11 were classified as early-stage cervical cancer (ESCC) and 42 as locally advanced cervical cancer (LACC). Among neoplastic cases, the detection rate was 20.7% (n = 11/53) for locally advanced cervical cancer (stages

IIB, IIIA, IIIB, IVA, IVB) and none for early-stage cervical cancer (IB1 and IB2). All locally advanced neoplastic cases of cervical cancer underwent chemoradiotherapy. A decrease in ctHPV levels was noted after treatment. Of the 11 patients with detectable ctHPV, posttreatment follow-up was performed for 9 patients, with 1 patient dying before follow-up and 1 still in follow-up. After treatment, a statistically significant reduction in pre-treatment ctHPV levels was observed ($p = 0.012$). These results suggest that ctHPV DNA may not be effective as an early detection marker for cervical cancer. However, in patients with advanced stage cervical cancer, ctHPV DNA levels serve as a promising tool to assess tumor burden and help assess treatment response and disease monitoring. Cable et al. [12] analysed blood and tumor samples from 55 patients with HPV-positive locally advanced cervical cancer (LACC) who underwent chemoradiotherapy (CRT). These included a retrospective cohort of 41 patients and a prospective cohort of 14 patients. HPV circulating tumor DNA (ctDNA) was detected using genotype-specific droplet digital PCR (ddPCR). Prior to chemoradiotherapy for locally advanced cervical cancer (LACC), HPV circulating tumor DNA (ctDNA) was detected in 69% of patients ($n = 38/55$), including nine patients with an unusual genotype. HPV ctDNA positivity for HPV18 (20%, $n = 2/10$) was significantly lower than for HPV16 (77%, $n = 27/35$) or other types (90%, $n = 9/10$, $P = 0.002$). Considering all samples collected at the end of chemoradiotherapy (CRT) and during follow-up in the prospective cohort, detection of positive HPV circulating tumor DNA (ctDNA) was associated with decreased disease-free survival (DFS) ($P = 0.048$). associated overall survival (OS) ($P = 0.0013$). It was found that HPV ctDNA was cleared in the majority of patients by the end of treatment. The presence of residual HPV ctDNA at the end of CRT or during follow-up could serve as a valuable indicator for identifying patients who may be at higher risk of later relapse [12].

The present study also shows that pretreatment ctHPV DNA copy number increases with increasing FIGO stage ($p = 0.006$), which is consistent with previous studies. Jeannot et al. performed an analysis of serum samples from 94 cases of cervical cancer (CC) associated with HPV16 or HPV18 within the prospective cohort of BioRAIDs. These samples were collected both before and after treatment and during an 18-month follow-up period. Pretreatment circulating HPV DNA (HPV ctDNA) was found in 63% (59/94) of patients. Detection of HPV ctDNA in serum samples correlated with advanced FIGO stage ($P = 0.02$). Complete clearance of HPV ctDNA by the end of treatment was significantly associated with longer PFS ($P < 0.0001$). Patients with persistent HPV ctDNA in serum experienced a relapse a median of 10 months (range: 215) after HPV ctDNA was detected [13].

To validate the results, we correlated our results of ctHPV DNA levels from plasma samples from patients with formalin-fixed, paraffin-embedded tissue and showed similar results. The amount of ctHPV DNA extracted from FFPE samples was lower compared to plasma samples due to the degradation of DNA quality and quantity in formalin. This was further confirmed using p16 immunohistochemistry. Various studies reported the similar results [8, 25, 30]. The ctHPV DNA detection rate in locally advanced cervical cancer is 26.2%, a lower rate attributed to the use of real-time PCR in the present study Comparison to the use of ddPCR in the other study. Jeannot et al. conducted a retrospective analysis of a series of 70 serum samples collected between 2002 and 2013. These samples were obtained from patients diagnosed with human papillomavirus 16 or human papillomavirus 18-related carcinomas using ddPCR. Sixty-one of 70 serum samples from patients with carcinomas showed detectable circulating human papillomavirus DNA, accounting for 87% of samples tested. Digital droplet PCR is an attractive technique. It allows the DNA to be largely divided into numerous nanoliter-sized droplets, with each droplet containing either no or only a few copies of the DNA. These droplets then undergo individual endpoint enhancement processes [21].

In conclusion, pretreatment ctHPV DNA copy number increases with increasing FIGO stage ($p = 0.006$). In the present study, ctHPV DNA was not found in any of the preneoplastic lesions of cervical cancer analysed. The current study showed that ctHPV can be detected in pretreatment plasma in the majority of patients with locally advanced cervical cancer. Furthermore, it suggests an association between pretreatment ctHPV DNA copy number and stage. Increasing evidence suggests that post-treatment ctHPV DNA status is a promising biomarker for cervical cancer.

Author Contribution Statement

All authors contributed equally in this study.

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