RESEARCH ARTICLE

Monitoring of Cell-free Human Papillomavirus DNA in Metastatic or Recurrent Cervical Cancer: Clinical Significance and Treatment Implications

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Funding: This work was supported by the Key R&D Program of Zhejiang (2022C04001), the Zhejiang Province Medicine and Health Science and Technology Program (2020KY454), the Zhejiang Science and Technology Department Public Welfare Project (LGF22H160075).

Potential competing interests: No potential competing interests to declare.

Abstract

Purpose: Monitoring of circulating human papillomavirus (HPV) cell-free DNA (cfDNA) is a minimally invasive approach for surveillance in HPV-associated cancers, particularly cervical cancer. The aim of this study was to monitor circulating HPV cfDNA levels in patients with recurrent or metastatic cervical cancer during treatment and follow-up to assess the utility of HPV cfDNA as a tumor marker for disease surveillance and in guiding clinical treatment decisions.

Experimental Design: In this prospective pilot observational study, levels of HPV cfDNA in serum samples from 28 patients with recurrent or metastatic HPV+ cervical cancer were measured via digital droplet polymerase chain reaction. Results for HPV cfDNA levels were matched to clinical outcomes and to serum levels of squamous cell carcinoma antigen (SCC-Ag) to assess the clinical potential of HPV cfDNA as a tumor marker.

Results: HPV cfDNA was detected in all 28 patients (100%). Notably, median baseline HPV cfDNA levels varied according to the metastatic pattern in individual patients (P=0.019). Specifically, patients with a combined multiple-metastasis pattern had higher median baseline HPV cfDNA levels than patients with a single metastasis (P=0.003). All participants exhibited changes in HPV cfDNA levels over a median monitoring period of 2 months (range 0.3-16.9) before evaluations for treatment response or disease progression. Among 26 patients initially diagnosed with squamous cell cervical cancer, the positivity rate was 100% for HPV cfDNA and 69.2% for SCC-Ag (P=0.004, 95% confidence interval, 0-0.391). Among 20 patients longitudinally monitored for squamous cell cervical cancer, the concordance with changes in disease status was 90% for HPV cfDNA and 50% for SCC-Ag (P=0.014, 95% confidence interval, 0.022-0.621).

Conclusions: HPV cfDNA is a promising tumor marker for HPV+ cervical cancer that offers advantages over SCC-Ag. In the context of precision medicine, HPV cfDNA is poised to play an increasingly pivotal role in monitoring treatment efficacy, providing valuable insights into disease progression, and guiding clinical decisions.

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Introduction

Cervical cancer (CC) is the fourth most prevalent malignancy in terms of both incidence and mortality among females globally, and is the primary human papillomavirus (HPV)-associated cancer[1][2]. Despite advances in CC treatment, challenges persist regarding recurrence and metastasis[3]. The treatment landscape for recurrent or metastatic CC has changed with the emergence of targeted immunotherapy drugs. However, the lack of effective biomarkers hinders assessment of treatment efficacy and the ability to predict patient outcomes in this setting[4]. There is a pressing need for more effective and minimally invasive biomarkers for serial monitoring of treatment responses and prognostication of patient outcomes.

As a liquid biopsy modality, measurement of circulating cell-free DNA (cfDNA) released from tumor cells into the bloodstream has extensive utility in optimizing various facets of cancer management, including early diagnosis[5], noninvasive genotyping, pretreatment assessment, drug target identification, resistance detection[6][7], treatment efficacy monitoring, post-treatment follow-up, and relapse prediction[8][9][10]. HPV is the cause of most CC cases[11]. Viral DNA from high-risk HPV subtypes integrates into host cell genomes, resulting in widespread expression of virus-specific E6/7 protein[12]. As HPV-associated cancers release HPV cfDNA into the host's bloodstream, circulating HPV cfDNA in free or integrated form is an attractive potential biomarker that can be detected in blood[13][14]. Typically, circulating cfDNA is detected using digital droplet polymerase chain reaction (ddPCR) or next-generation sequencing (NGS) technology[15]. ddPCR allows direct, independent, and absolute quantification of cfDNA in samples, with a DNA detection threshold as low as 1 copy/mL. Results can be obtained within 1 day, so ddPCR is relatively cost-effective[16][17].

We conducted a prospective clinical study (NCT03175848) in primary stage IVB CC to investigate the role of radiotherapy (RT) in combination therapy. Recent advances in targeted therapies and immunotherapies have significantly enhanced the treatment efficacy for recurrent or metastatic CC and have increased overall survival (OS)[4]. Some patients may require maintenance therapy for up to 2 years, and others may survive with their tumor for an extended period. Consequently, it is imperative to closely monitor each patient's condition and adjust their treatment plan accordingly. However, conventional imaging methods occasionally fail to reflect disease changes in a timely manner[18], while blood biomarkers such as squamous cell carcinoma antigen (SCC-Ag) have limited clinical effectiveness for monitoring[19]. Considering these clinical challenges, we hypothesized that circulating HPV cfDNA levels correlate with metastatic patterns and treatment response in CC. To validate this hypothesis, we prospectively recruited an observational pilot cohort comprising patients with primary stage IVB or recurrent HPV-positive CC and measured HPV cfDNA levels for analysis of HPV cfDNA copy numbers in relation to disease parameters and treatment responses.

Methods

Study design

From August 2017 to February 2023, a total of 33 patients with pathologically confirmed primary stage IVB or recurrent HPV+ CC were enrolled at Zhejiang Cancer Hospital. Five patients were excluded from the analysis, as outlined in Figure 1. The final analysis cohort comprised 28 cases: 21 with primary stage IVB CC and seven with recurrent CC. Notably, 19 cases in the primary CC group participated in the prospective clinical study (NCT03175848). The main eligibility criteria were: pathologically confirmed diagnosis of CC; pathological evidence of at least one metastatic lesion; and PCR positivity for a high-risk HPV subtype in pretreatment exfoliated cervical cells or a serum sample. All patients consented to the study protocol, including collection of blood samples throughout the study. Patients received chemotherapy with or without immunotherapy, targeted therapy, or RT. For patients with primary stage IVB CC who had a single sample collected, this occurred at treatment initiation. For the group undergoing longitudinal sampling, patients with primary stage IVB CC had three to five blood samples collected at treatment initiation, mid-treatment, and during follow-up, while patients with recurrent CC had three 3 blood samples collected during treatment, starting from enrollment. For all serum specimens, HPV cfDNA was quantified using ddPCR. Concurrent SCC-Ag testing was conducted for patients with squamous cell CC in the sequential sampling group (at a time point matching or closely following HPV cfDNA sampling). The 2018 International Federation of Gynecology and Obstetrics staging criteria were applied. Each patient underwent routine imaging assessments, and treatment efficacy was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST version 1.1). We defined lymph node metastasis as metastasis in para-aortic or distant lymph nodes (e.g., supraclavicular, inguinal, mediastinal nodes). We classified patterns of CC recurrence or metastasis into five groups: local recurrence (LR); lymph node metastasis (LNM); hematogenous metastasis (HM); lymph node+hematogenous metastasis (LN+HM); and lymph node+ hematogenous+diffuse serosal metastasis (LN+H+DSM). DSM encompasses metastases to the peritoneal, pleural, or pericardial regions. The study was approved by the Ethics Committee of Zhejiang Cancer Hospital.

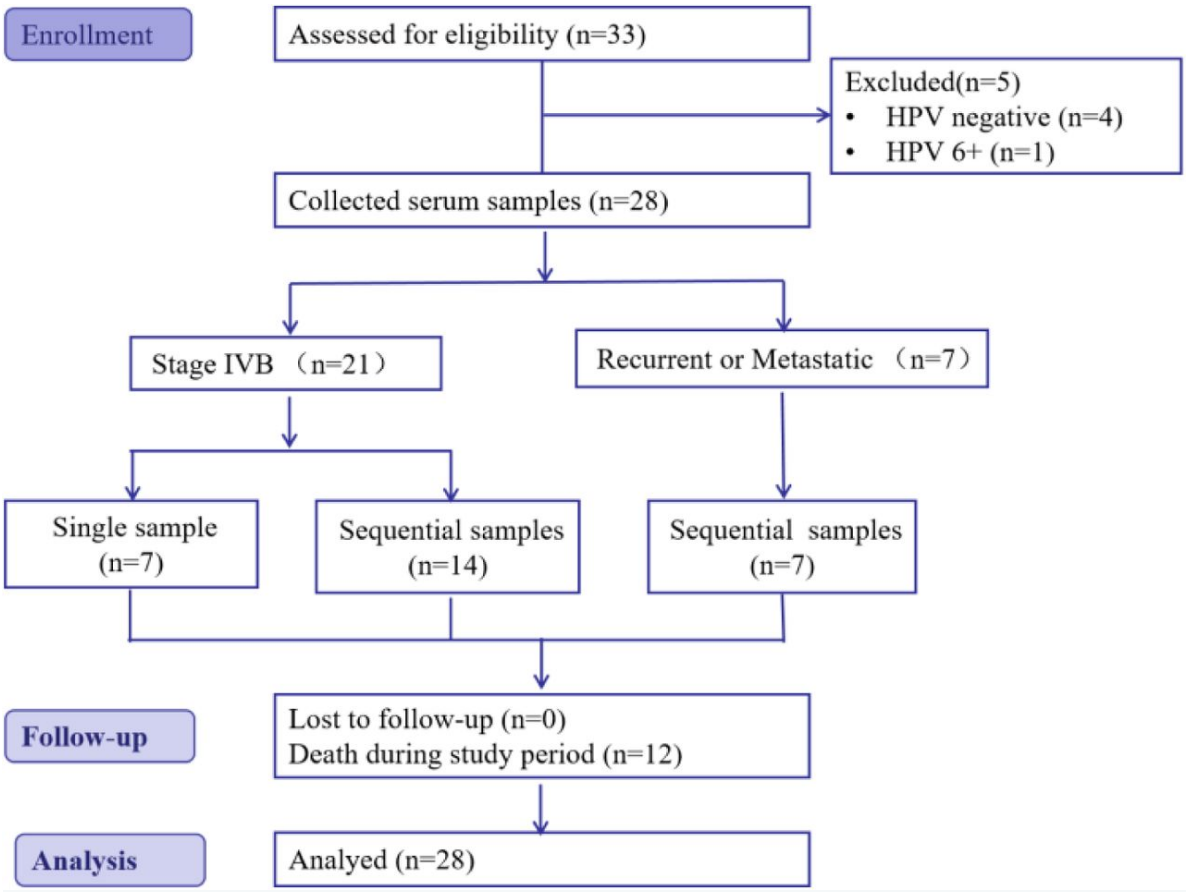

Figure 1. CONSORT flow diagram showing the enrollment of an observational cohort of patients diagnosed with recurrent or metastatic HPV-cervical cancer undergoing serum human papillomavirus (HPV) cell-free DNA surveillance.

HPV diagnostics and typing

HPV subtypes were determined via routine PCR for exfoliated cervical cells or serum samples collected before treatment. Patients were excluded from the study if both samples tested negative. A commercial PCR testing kit designed primarily for qualitative genotyping of HPV DNA extracted from exfoliated cervical cells was routinely used. This kit can detect 21 HPV virus subtypes (8 low-risk and 13 high-risk subtypes): HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, and HPV68.

Samples

Serum was extracted from peripheral whole blood samples at the Radiobiology Laboratory of Zhejiang Cancer Hospital. A 5-mL aliquot of whole blood was collected into a yellow-top blood collection tube and allowed to clot at room temperature for 30 min. After centrifugation at 2000×*g* for 10 min in a refrigerated centrifuge, the serum was carefully transferred into polypropylene tubes in 1-mL aliquots and stored at –80°C. Frozen serum samples were subsequently transported to the Oncology Research Institute of Zhejiang Cancer Hospital for DNA extraction.

Procedure for ddPCR analysis

Before analysis, serum samples were thawed and centrifuged at 2000×*g* at 4°C for 10 min for DNA extraction. In accordance with the manufacturer's protocol, cfDNA was isolated from 2 mL of serum using a QIAamp Circulating Nucleic Acid Extraction Kit (Qiagen, Hilden, Germany). The DNA was eluted twice through a column for purification, resulting in 60 µL of eluate that was stored at –80°C until analysis. Primers and probes for ddPCR detection were designed on the basis of E7 gene sequences of the target HPV subtypes to generate amplicons of varying length (Supplementary Table 1). Each ddPCR reaction used 30 µL of DNA template. According to the manufacturer's instructions for the QIAcuity QX-200 ddPCR platform (Qiagen), ddPCR reactions consisted of 40 µL of reaction mixture per well that included the primers, probes, and template. The reactions were amplified in QIAcuity 26,000 24-well Nanoplates (Qiagen) under the following conditions: initial enzyme activation at 95°C for 2 min, followed by 50 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 30 s. The exposure time for imaging of partitions was 400 ms for fluorescein amidite (FAM) and 300 ms for 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC). Data analysis was performed using QIAcuity software version 2.1.7 (Qiagen) to quantify HPV copy numbers.

HPV cfDNA monitoring protocol

Participants were enrolled on a rolling basis, and the first serum sample collected was regarded as the baseline sample. A baseline sample was categorized as a treatment initiation sample if it was collected between Day –14 and Day +30 preceding initial treatment in patients with primary stage IVB CC, or before treatment for relapse or disease progression in patients with recurrent CC.

For patients with sequential samples, the time at which the initial blood sample was collected was designated as time 0. Up to four additional samples were collected at intervals ranging from 8 to 962 days (median 73 days, mean 128 days), and blood collection continued for up to 1513 days. Blood sample collection was coordinated with patient treatment and follow-up times to the greatest extent possible for measurement of HPV cfDNA levels (copies/mL). Serum SCC-Ag levels are routinely assessed multiple times before, during, and after treatment for patients with squamous cell CC at our hospital using an Abbott Architect instrument (Abbott Laboratories, Abbott Park, IL, USA). To match the analysis, we selected SCC-Ag values measured at the same time as the HPV serum sample or at the most recent time point. To ensure consistency in the analysis, we chose SCC-Ag values measured concurrently with the HPV serum sample or at the latest time point available. SCC-Ag levels <1.5 ng/mL were classified as normal, while levels ≥1.5 ng/mL; the upper limit of detection limit was 70 ng/mL. For statistical purposes, clinical test results exceeding 70 ng/mL were treated as 70 ng/mL.

Following the literature[20][21], serum samples were deemed HPV+ if at least three droplets containing HPV amplicons were identified. Samples with fewer than three droplets containing HPV amplicons or no amplicons detected were categorized as HPV-negative. Serum HPV cfDNA levels were quantified as copies/mL.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 9. The Mann-Whitney U test was used to assess the

difference in the number of viral DNA copies between two groups or two metastasis patterns. Kendall's τ correlation test was used to determine the coefficient of correlation between two factors. Comparisons of the rates were conducted using Fisher's exact test. Kaplan-Meier survival analysis was conducted to calculate the hazard ratio with a 95% confidence interval (CI) using the Cox model. Overall survival (OS) was defined as time from diagnosis until death from any cause or the last follow-up date (December 31, 2023). All *P*-values reported are two-tailed, with statistical significance defined as $P<0.05$.

Results

Patient characteristics

Our study included 28 patients diagnosed with HPV+ advanced CC treated at our hospital, comprising 21 cases (75%) with primary stage IVB CC and seven cases (25%) with recurrence and metastasis after treatment. The clinical characteristics of the patients are listed in **Table 1**. The median age at diagnosis was 52 years (range 34-67). In total, 76 serum samples were obtained from the cohort, consisting of 69 longitudinal samples from 21 patients (3-5 samples per patient) and single-time samples from seven patients at treatment initiation. Twenty-five (89%) of the baseline samples were obtained at treatment initiation. HPV cfDNA was quantified in all serum samples via ddPCR.

Table 1. Characteristics of patients with metastatic or recurrent cervical cancer

N	All patients (n=28)	Primary IVB stage (n=21)	Recurrence or metastasis (n=7)
Age (years)			
Median age (range)	52 (34-67)	51 (34-66)	53 (37-67)
Pathological types(n)			
Squamous cell carcinoma	26	20	6
Adenocarcinoma	1	0	1
Large cell neuroendocrine carcinoma	1	1	0
HPV subtype			
16	20	14	6
58	3	3	0
18	2	2	0
31	1	0	1
66	1	1	0
16,33	1	1	0
Baseline serum sampling time			
Treatment initiation	25	20	5
During treatment	3	1	2
Pattern of metastasis			
Local recurrence	1	0	1
Lymphatic node metastasis	11	10	1
Hematogenous metastasis	4	2	2
Lymph node + hematogenous metastasis	9	8	1
Lymph node + hematogenous + diffuse serosal metastasis	3	1	2
Treatment modality			
Neoadjuvant chemotherapy	18	18	0
Surgeries	2	0	2
Adjuvant chemotherapy	25	18	7
Radiotherapy/concurrent chemoradiotherapy	25	18	7
Targeted therapy	7	3	4
Immunotherapy	9	3	6

Validity of the test for HPV cfDNA detection and genotyping

To validate the consistency of HPV genotyping between serum and exfoliated cervical cells from the same patient, we conducted conventional PCR genotyping of HPV cfDNA in baseline serum. The analysis revealed that only 12/28 patients (42.9%) tested positive; however, the genotyping outcomes were entirely consistent with those obtained from HPV genotyping in matched cervical exfoliated cells. Subsequently, the 28 baseline serum samples underwent qualitative and quantitative assessment using ddPCR. The results revealed that all 28 patients (100%) tested positive for HPV cfDNA, with HPV typing showing complete concordance (100%) with HPV PCR outcomes from matched cervical exfoliated cells.

HPV genotyping revealed that HPV16, HPV58, HPV18, and other subtypes (31, 66, 33) accounted for 72.4%, 10.3%, 6.9%, and 10.3% of cases, respectively. (Figure 2A).

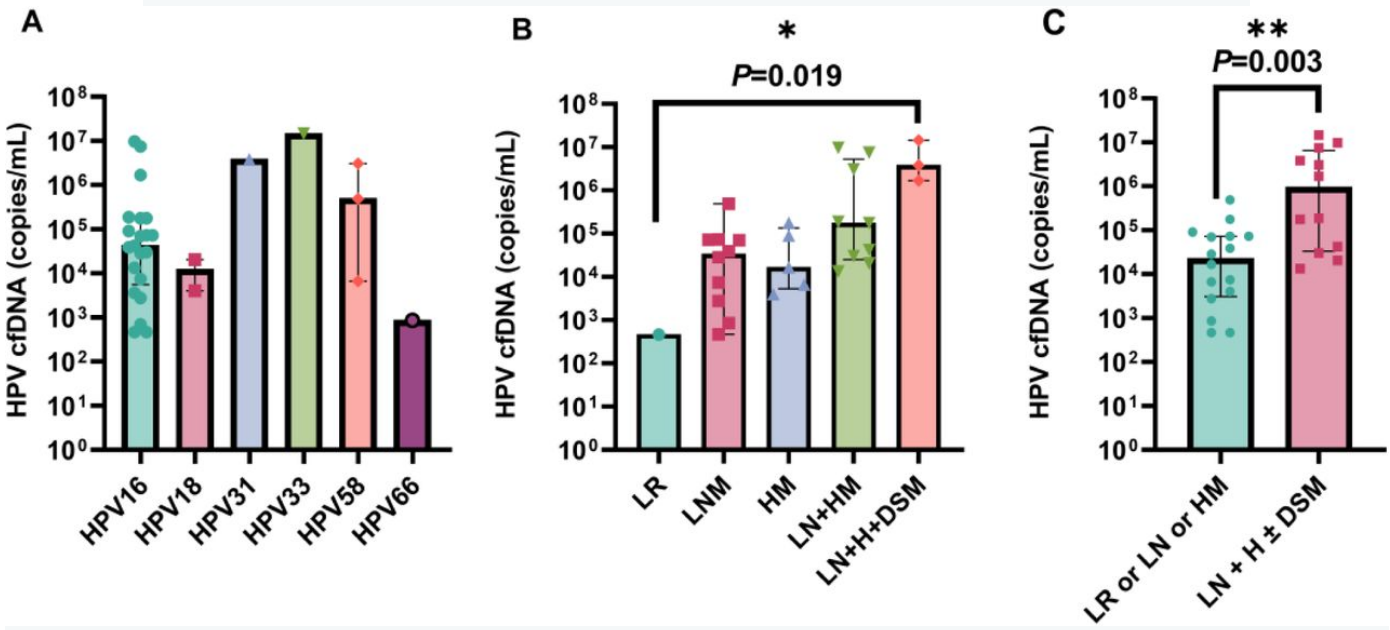

Figure 2. (A) Serum HPV cfDNA copy number for six HPV subtypes at baseline. (B) Baseline HPV cfDNA copy numbers for five recurrence/metastasis pattern subgroups. Statistical significance was determined using a two-sided Kruskal-Wallis test. (C) Relationship between HPV cfDNA viral copy number and metastatic pattern. Statistical significance was determined using a two-sided Mann-Whitney U test. All plots show the median and interquartile range on a $\log_{10}$ scale.

Correlation between tumor metastasis pattern and baseline HPV cfDNA

Analysis for the study cohort revealed an association between tumor metastatic pattern and baseline HPV cfDNA levels. According to their metastatic status at baseline, patients were categorized into two groups: the single-metastasis pattern (SMP) group (LR, LNM, or HM); and the multiple-metastasis pattern (MMP) group (LN+H±DSM). The baseline copy number significantly differed among the five recurrence/metastatic pattern groups (P=0.019) and tended to gradually increase with the degree of recurrence/metastasis (Figure 2B). The median baseline HPV cfDNA copy number was significantly higher in the MMP group than in the SMP group (P=0.003; Figure 2C).

HPV cfDNA as a predictor of treatment response or failure

We followed the enrolled patients for an average of 24.1 months (range 2.1-77.8). Figure 3 shows temporal changes in serum HPV cfDNA levels. In all patients with longitudinal testing, changes in HPV cfDNA levels occurred at a median of 2 months (range 0.3-16.9) before confirmation of a treatment response or disease progression via imaging assessment. Six patients with clinically progressive disease (as per RECIST) exhibited elevation of HPV cfDNA copy number before imaging-confirmed disease progression. The median time from detection of elevated plasma HPV cfDNA to imaging confirmation of disease progression was 4.2 months (range 1.9-16.9; Figure 3A1). Likewise, we observed a consistent decrease in HPV cfDNA copy numbers in 16 patients before imaging confirmed a treatment response. The median time

from detection of a decrease in HPV cfDNA to imaging confirmation of disease regression was 1.2 months (range 0.3-2.8; Figure 3B1-D1).

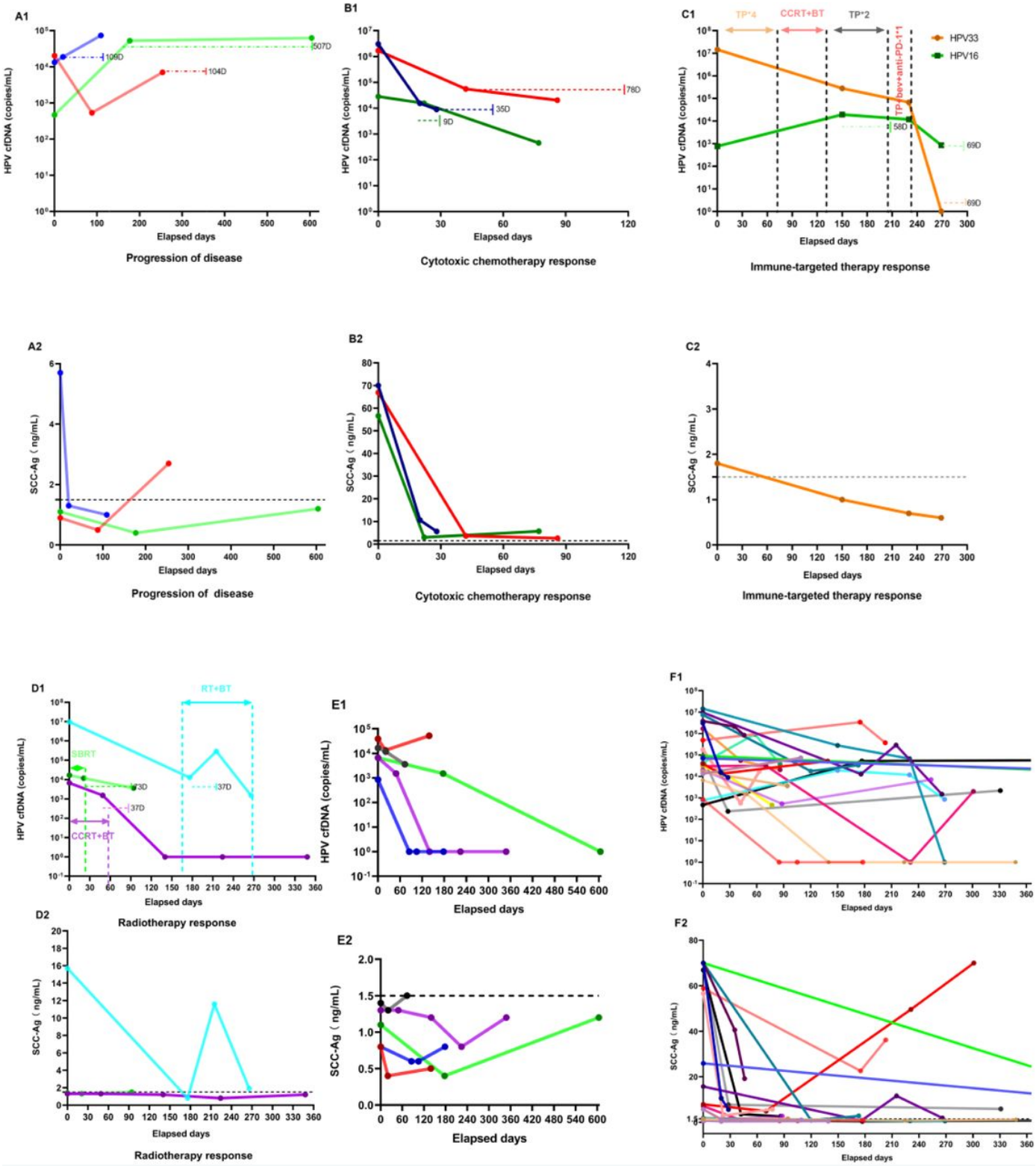

Figure 3. Paired plots for serum HPV cfDNA (copies/mL) and squamous cell carcinoma antigen (SCC-Ag) (ng/mL) levels were measured longitudinally in patients with metastatic or recurrent HPV+ cervical cancer (lines of the same color for each patient). HPV cfDNA levels in serum were scaled using log10. (A1, A2) Patients whose disease progressed during treatment. (B1, B2) Selected patients who showed a response to cytotoxic chemotherapy. (C1, C2) Response to immune therapy in a patient with stage IVB cervical cancer positive for HPV subtypes 33 and 16,

treated with paclitaxel + cisplatin, concurrent chemoradiotherapy, brachytherapy, and bevacizumab. (D1, D2) Selected patients with a response to radiotherapy. (E1, E2) HPV cfDNA levels were significantly higher than normal at some of the longitudinal time points in five patients, but SCC-Ag levels were within the normal range (<1.5 ng/mL) at all time points. (F1, F2) HPV cfDNA levels for all patients with longitudinal samples (*n*=21) and matched SCC-Ag levels for the patients with squamous cell cancer (*n*=20). Each colored line corresponds to one patient, except in C. Horizontal dashed lines indicate the days between plasma HPV cfDNA levels suggestive of a response or progression (rise or fall in levels) and imaging-confirmed changes in disease status. The associated number of days (D) is listed adjacent to the horizontal dashed line for comparison. It should be noted that not all HPV cfDNA data points are plotted for each patient.

For patients in whom systemic cytotoxic chemotherapy was effective, higher HPV cfDNA copy number at baseline was correlated with a more substantial decrease in copy number after chemotherapy. For two patients with high HPV cfDNA levels of 3.1×10^6 copies/mL and 1.7×10^6 copies/mL at baseline, the HPV cfDNA copy number decreased significantly following one or two cycles of paclitaxel+cisplatin (TP) chemotherapy (median 98.2%, range 96.7-99.5). The rate of decline in viral load slowed after the subsequent cycle of TP chemotherapy, with a median decline of 51.3% (range 39-63.5%). In addition, a patient with baseline HPV cfDNA of 2.8×10^4 copies/mL experienced a 45% reduction in copy number following one cycle of TP chemotherapy (Figure 3B1).

We observed that changes in HPV cfDNA levels may indicate a response to combined immunotherapy and targeted therapy. One patient diagnosed with primary stage IVB CC and multiple metastases (LN+H+DSM) tested positive for HPV33 and HPV16 in exfoliated cervical cells and serum. The patient received four cycles of TP and concurrent chemoradiotherapy plus brachytherapy for the pelvic-abdominal primary focus, followed by two additional TP cycles. The HPV33 viral load decreased from a pretreatment level of 1.5×10^7 copies/mL to 6.7×10^4 copies/mL after treatment; however, the HPV16 viral load increased from 7.7×10^2 to 1.2×10^4 copies/mL. Subsequent imaging evaluation after 58 days indicated the emergence of new foci in the lungs, prompting adjustments to the patient's treatment plan. Guided by the immunohistochemical presence of PD-L1-positive cells (combined positive score of 10) in the primary cervical lesion, the patient received one cycle of TP + bevacizumab + anti-PD-1 treatment. This regimen led to a swift decrease in viral levels of both HPV subtypes after 36 days, with HPV 33 reaching undetectable levels and HPV 16 decreasing to 7.8×10^2 copies/mL (Figure 3C1).

Serum HPV cfDNA levels also changed in response to RT. A transient rise (20.8-fold) in HPV cfDNA copy number was observed in a patient with stage IVB CC who experienced a 2-week interruption of RT because of grade IV thrombocytopenia. Following stereotactic body RT to lung metastases, a patient with pulmonary oligometastases experienced a 29% decrease in HPV cfDNA copy number. Another patient diagnosed with stage IVB CC and pelvic bone metastases who underwent radical concurrent chemoradiotherapy targeting both primary and metastatic lesions exhibited a notable reduction in HPV cfDNA levels (Fig. 3D1).

Correlation between HPV cfDNA and SCC-Ag

There were 26 patients with squamous cell CC in the study cohort. All 26 (100%) had elevated serum HPV cfDNA at baseline, but only 18/26 patients (69.2%) had elevated SCC-Ag at baseline (*P*=0.004, 95% CI, 0-0.391). Among 72 serum

samples from patients with squamous cell CC, the median HPV cfDNA level was 1.7×10^4 copies/mL (range 0-1.4×10^7) and the median SCC-Ag level was 2.6 ng/mL (range 0.4-70). There was no significant correlation between SCC-Ag and HPV cfDNA levels (R^2=0.034, *P*=0.120). For patients with squamous cell CC who had longitudinal monitoring (*n*=20), the concordance with disease change was 90% for HPV cfDNA and 50% for SCC-Ag (*P*=0.014, 95% CI, 0.022-0.621). Comparison of matched serum HPV cfDNA and SCC-Ag levels for patients with squamous cell CC (Figure 3A–D) revealed that HPV cfDNA exhibited dynamic fluctuations, while serum SCC-Ag levels in the majority of patients rapidly decreased to near or below the normal range (<1.5 ng/mL) following initiation of treatment. During the course of treatment, SCC-Ag levels remained consistently within the normal range (<1.5 ng/mL) at all time points in five patients, but matched serum HPV cfDNA showed fluctuating changes above normal values at some time points. (Figure 3E1, E2).

Correlation between HPV cfDNA and survival

The 5-year OS rate for the entire cohort was 42.3%, with median OS of 52.1 months at median follow-up of 42.3 months (range: 10.2-88.5 months). As of December 31, 2023, there were 12 patient deaths and 20 disease progression events. Analysis of survival by HPV subtypes revealed that the difference in OS between the HPV16+ group and the non-HPV16+ group was not statistically significant (*P*=0.052; Figure 4A). Patients with primary stage IVB CC (*n*=21) were stratified by baseline median HPV cfDNA level using 3.9×10^4 copies/mL as the dichotomization threshold. The difference in OS between the groups with ≥3.9×10^4 copies/mL and <3.9×10^4 copies/mL at baseline was not statistically significant (*P*=0.111; Figure 4B).

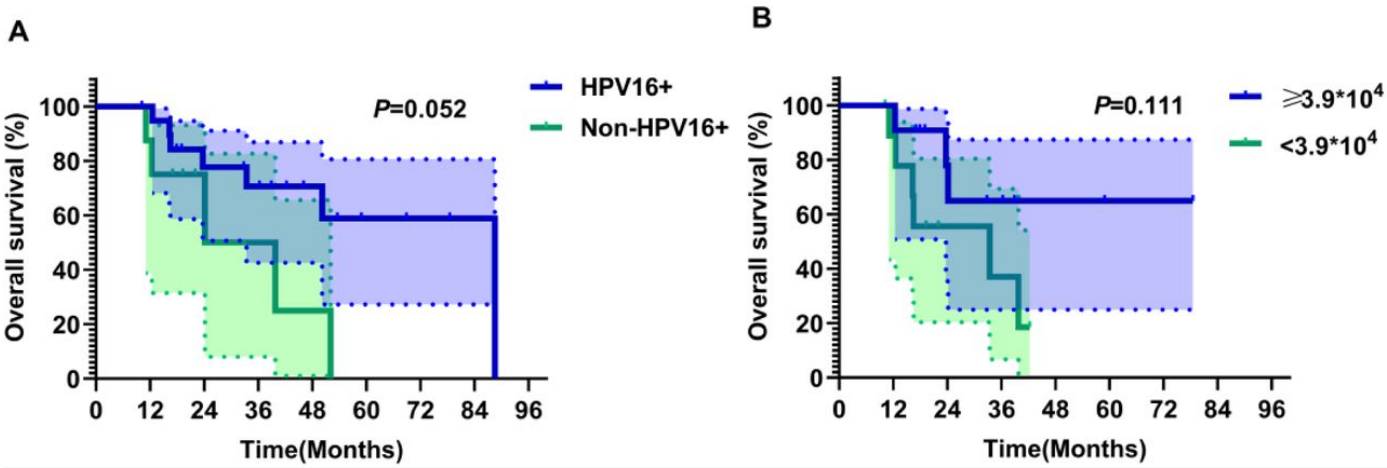

Figure 4. (A) Overall survival for patients stratified by HPV subtype (HPV 16+ versus non-HPV16+). (B) Overall survival for patients stratified by baseline serum HPV cfDNA copy number using a cutoff of 3.9×10^4 copies/mL. *P*-values were calculated using a two-sided log-rank test.

Discussion

We conducted a prospective pilot observational study in patients with metastatic or recurrent CC to analyze ddPCR HPV cfDNA results in relation to SCC-Ag levels, clinical treatment responses, and prognosis. The study findings confirm the significant clinical potential of dynamic HPV cfDNA surveillance for CC. First, we found a correlation between baseline

HPV cfDNA copy number and recurrence/metastasis patterns. Second, HPV cfDNA predicted treatment response or disease progression at an earlier time point than imaging assessments. Finally, we demonstrated the superiority of serum HPV cfDNA over SCC-Ag in monitoring metastatic or recurrent CC.

Our positivity rate for HPV cfDNA was 42.9% (12/28) when using PCR and 100% (28/28) when using ddPCR. We found 100% agreement of HPV typing results between exfoliated cervical cells and serum samples. These findings demonstrate the high sensitivity and specificity of HPV cfDNA detection via ddPCR. A meta-analysis comparing the accuracy of different methods for HPV cfDNA detection in HPV+ tumors revealed that NGS outperformed ddPCR and quantitative PCR in terms of sensitivity, while specificity remained consistent across all three methods[15]. We found that HPV cfDNA positivity in HPV+ CC correlated positively with tumor stage, tumor load, and lymph node status. Consistent with our findings, a recent study demonstrated a serum HPV cfDNA positivity rate of 100% in HPV+ metastatic CC[22]. Consequently, HPV cfDNA appears to be an ideal serum tumor marker for HPV+ metastatic or recurrent CC given the highly sensitive and specific detection methods available.

Studies have shown that HPV cfDNA levels correlate with disease stage, tumor size, tumor load, and lymph node status[23][24][25]. Accurate quantification of tumor load can be challenging, particularly in settings involving diffuse serosal metastases. Therefore, we classified recurrence/metastatic patterns into five categories and observed a significant difference in median baseline HPV cfDNA copy number among these (P=0.019). Patients were categorized into SMP and MMP groups according to their recurrence/metastasis status at baseline. The MMP group (LN+H±DSM) had higher median HPV cfDNA copy number at baseline than the SMP group (LR or LNM or HM; P=0.003). The recurrence/metastasis pattern appears to reflect tumor load and spread. A study by Mittelstadt et al. involving 35 patients with CC also revealed correlation between HPV cfDNA levels and tumor load and spread[24]. Patients with multiple metastases are likely to have a higher tumor load with greater shedding of HPV-containing DNA fragments, which can enter the blood circulation via several pathways, resulting in higher HPV cfDNA levels.

In our study, changes in HPV cfDNA levels frequently preceded confirmation of disease changes on imaging scans. Several studies demonstrated that changes in HPV cfDNA copy number are associated with response to therapy for HPV+ tumors[22][24][25]. We found that the median time from the onset of a change in HPV cfDNA copy number to imaging confirmation of a treatment response or disease progression was 2 months (range 0.3-16.9). Another study reported analogous findings in HPV-associated oropharyngeal cancer: changes in HPV cfDNA copy number were observed at a median of 16 days (range 12-38) before imaging confirmation of treatment response or disease progression in all 22 patients enrolled[26]. These findings suggest that HPV cfDNA can serve as a sensitive marker and a valuable clinical indicator. In addition, our results indicate that HPV cfDNA can serve as a tool for monitoring the effectiveness of RT. Among patients receiving effective RT, HPV cfDNA levels gradually declined throughout the treatment; however, interruptions in RT could lead to transient increases in HPV cfDNA levels. Moreover, our results demonstrate that the combination of immunotherapy and targeted therapy, along with cytotoxic chemotherapy, enhances tumor cell death and clearance of HPV cfDNA in vivo, surpassing the effects of cytotoxic chemotherapy alone. Our findings suggest that HPV cfDNA holds promise as a tool for evaluating the effectiveness of novel therapies such as immunotherapy and targeted therapy and informing subsequent maintenance treatment strategies for patients.

While SCC-Ag is acknowledged as a serum tumor marker for squamous cell CC[27][28][29], its clinical sensitivity and specificity for monitoring responses to therapy are low[30][31]. We compared HPV cfDNA and corresponding SCC-Ag levels in 72 serum samples and found no correlation between the two data sets (R^2=0.03, *P*=0.11). HPV cfDNA exhibited several advantages as a serum tumor marker in our study cohort. First, the positivity rate was 100% for HPV cfDNA versus 69.2% for SCC-Ag at baseline (*P*=0.004, 95% CI, 0-0.391). Second, changes in HPV cfDNA copy number showed greater concordance with disease progression in the group of 20 patients with squamous cell CC with longitudinal monitoring, with a concordance rate of 90% versus 50% for SCC-Ag (*P*=0.014, 95% CI, 0.022-0.621). Finally, HPV cfDNA provides more comprehensive information for dynamic monitoring in comparison to SCC-Ag, as SCC-Ag levels consistently remained within the normal range in some patients. Thus, HPV cfDNA is a more useful serum marker than SCC-Ag in patients with metastatic or recurrent CC.

The 5-year OS rate for the study cohort was 42.3% and the median OS time was 52.1 months. This good outcome was due to active systemic treatment (chemotherapy+immunotherapy) and local treatment (RT to primary and metastatic foci). Therefore, noninvasive dynamic monitoring using serum tumor markers is important for this population. Our data showed that correlation between OS and HPV subtypes (HPV16+ vs non-HPV16+) did not reach statistical significance (*P*=0.052). However, we observed a trend towards better prognosis for patients with HPV16+ than for patients with positivity for other HPV subtypes, and further expansion of the sample size may yield positive results. In the analysis of baseline copy number in relation to prognosis, the difference in OS between the groups with baseline

HPV cfDNA levels ≥3.9×10^4 and <3.9×10^4 copies/mL was not significant (*P*=0.111). Previous studies reported a positive correlation between high baseline HPV cfDNA levels and poor prognosis in oropharyngeal cancer[32][33][34], so further in-depth studies with larger sample sizes are needed to assess this correlation in CC.

Our study has some obvious limitations, including the small sample size and the heterogeneous sequential sampling protocol, for which both the number of blood draws and time interval during treatment varied. We are currently conducting a prospective study of stage IVB CC. Owing to the absence of literature support for HPV cfDNA sampling protocols, we designed this initial exploratory study with a small sample size to clarify the value of HPV cfDNA monitoring and explore various sampling times and intervals. Our study results suggest that monitoring of HPV cfDNA is valuable before, during, and after treatment. Assessment of HPV cfDNA levels in every chemotherapy cycle (monthly) during treatment and every 3-6 months during follow-up may be a reasonable approach.

In conclusion, our prospective study demonstrated that HPV cfDNA detection has high sensitivity and specificity for prediction of recurrence or metastasis in HPV-related CC, making it a suitable tumor marker for treatment monitoring and long-term follow-up. Studies have shown that the baseline HPV cfDNA copy number correlates with the patient's metastatic pattern, which reflects in part the extent of tumor load and spread. As a serum tumor marker, HPV cfDNA outperforms SCC-Ag in reflecting changes in disease dynamics and provides a timely measure of response to various treatments, including RT and emerging immune and targeted therapies. These preliminary findings show the potential of HPV cfDNA in monitoring the efficacy of treatments for HPV-associated cancers and predicting disease progression and

recurrence, but large-scale prospective validation in clinical trials is required.

Supplementary Material

HPV subtype	Forward primer	Reverse primer	Probe	Amplicon size	Annealing temperature
HPV16	TCCAGCTGGACAAGCAGAAC	CACAACCGAAGCGTAGAGTC	ACAGAGCCCATTACAAT	88 pb	60°C
HPV18	AACATTTACCAGCCCGACGA	TCGTCTGCTGAGCTTTCTAC	AACCACAACGTCACACAA	106 pb	60°C
HPV31	CGTTACCTTTTGTTGTCAGTGT	GAACAGTTGGGGCACACGA	ACAGAGCACACAAGTAG	123 pb	56°C
HPV33	CAGATGAGGATGAAGGCTTGGA	ACTGTTGACACATAAACGAACTG	CTTGTCCATCTGGCC	119 pb	56°C
HPV58	CAGACGAGGATGAAATAGGCTTG	ATGTAGTAATTAGCTGTGGCCGG	CTTGTCCATCTGGCC	70 pb	56°C
HPV66	CCGTTAACACCGGAGGAAAA	ATGACCCGGTCCATGCATAT	TGAACATAAAAGACGATTTC	82 pb	56°C

Supplementary Table 1. Primer and probe sequences for ddPCR, fragment sizes, and annealin temperature.

Statement and Declarations

Conflict of Interests

The authors make the statement that there is no conflict of interest to disclose.

Funding

This work was supported by the Key R&D Program of Zhejiang (2022C04001), the Zhejiang Province Medicine and Health Science and Technology Program (2020KY454), the Zhejiang Science and Technology Department Public Welfare Project (LGF22H160075).

Ethical conduct of research

This study was approved by the Medical Ethics Committee of Zhejiang Cancer Hospital and informed consent was obtained from all subjects.

Acknowledgements

We thank the follow-up in the follow-up room of Zhejiang Provincial Cancer Hospital.

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