Received: 4 June 2024 | Revised: 10 October 2024 | Accepted: 24 October 2024 | Published online: 8 November 2024

DOI: 10.1002/ijgo.16011

CLINICAL ARTICLE

Gynecology

Circulating tumor HPV DNA as a specific biomarker for cervical cancer

Tao Chen[1,2] | Tao Wu[3] | Rumei Sun[1] | Jianan Sun[4] | Xuanran Tao[2,3] | Juming Su[3] | Shujun Kong[4]

[1]Guangzhou KingMed Transformative Medicine Institute Co. Ltd. and Guangzhou KingMed Diagnostics Group Co. Ltd., Guangzhou, China

[2]KingMed School of Laboratory Medicine, Guangzhou Medical University, Guangzhou, China

[3]Department of Pathology, Guangzhou KingMed Center for Clinical Laboratory, Guangzhou, China

[4]Department of Obstetrics and Gynecology, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, China

Correspondence
Shujun Kong, Department of Obstetrics and Gynecology, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, China.
Email: ksj198778@126.com

Funding information
Science and Technology Program of Guangzhou, China, Grant/Award Number: 2023A03J0542

Abstract

Objective: The aim of this study was to determine whether circulating tumor human papillomavirus (HPV) DNA is a potential specific biomarker for cervical cancer (CC).

Methods: This retrospective matched study included 87 patients with cervical intraepithelial neoplasia (CIN), 29 CC patients (FIGO IA1-IVA) and 29 HPV-negative controls at Yuhuangding Hospital of Qingdao University (from July 2022 to September 2023). The digital droplet PCR (ddPCR) was used to detect and quantify ctHPV DNA in the plasma of patients with HPV16, 18, 33, 52, or 58-associated CC.

Results: The ctHPV DNA was exclusively detectable in HPV-positive samples, with no detection in patients across various CIN stages ($n = 87$) or HPV-negative controls ($n = 29$). Additionally, ctHPV DNA was identified in nine out of 10 late-stage patients (90%) and six out of 19 early-stage patients (31.6%).

Conclusion: The ctHPV DNA serves as a specific biomarker for the diagnosis CC. Additionally, this discovery addresses the knowledge gap in ctHPV DNA research in the early stages of CC and promotes clinical diagnosis and treatment strategies.

KEYWORDS
cervical cancer, cervical intraepithelial neoplasia (CIN), circulating tumor HPV DNA (ctHPV DNA), digital droplet PCR (ddPCR), HPV

1 | INTRODUCTION

Persistent human papillomavirus (HPV) infection is widely acknowledged as the primary causative factor in cervical cancer (CC).[1,2] Despite significant progress in controlling CC through preventive screening and HPV vaccination, it remains the fourth most common cancer among women globally.[3] The progression of CC involves distinct stages including LSIL (CIN1), HSIL (CIN2 and CIN3) and invasive carcinoma. Timely detection and intervention in these precancerous lesions are essential to prevent the progression of CC to invasive carcinoma, potentially improving the 5-year survival rate for patients.[4] However, there is a deficiency in liquid biopsy methods with high sensitivity for distinguishing between cervical precancerous lesions and invasive CC in clinical applications.

CC typically arises from persistent infection with high-risk human papillomaviruses (HR-HPV), which linearly integrate their DNA into the cervical host genome. Upon necrosis or apoptosis of CC cells, viral DNA is released into patients' peripheral blood, resulting in circulating tumor HPV DNA (ctHPV DNA). This ctHPV DNA serves as a highly convenient, sensitive, and minimally invasive liquid biopsy marker for cancer detection and disease monitoring.[5] In a groundbreaking 2016, digital droplet PCR (ddPCR) technology was first used to detect ctHPV DNA in the plasma of CC patients. Sivars et al. and Jeannot et al. analyzed 47 HPV16/18-positive CC patients revealing that 83% of ctHPV

[Correction added on 14 February 2025, after first online publication: the author names were re-ordered in this version.].

Tao Chen, Tao Wu, and Rumei Sun have contributed equally to this work and share first authorship.

DNA was detected in pretreated plasma using ddPCR, compared to 69% using qPCR technology.[6,7] In recent years, multiple studies have reported results from ddPCR-based detection of ctHPV DNA in the peripheral blood of CC patients. Detection rates have exhibited some variation across different studies, ranging from 31% to 100%.[6,8–14] Moreover, if we consider ctHPV DNA as a diagnostic biomarker, it is essential to address conflicting reports concerning its presence in the peripheral blood of potential cervical precancerous lesions.[15–17]

In this study, we utilized the highly sensitive ddPCR technique to investigate ctHPV DNA at various stages of CIN patients, CC patients, and HPV-negative control group. We found that ctHPV DNA could serve as a specific biomarker for CC and was associated with pathologic staging of CC. These findings are intended to offer guidance for the formulation of clinical diagnosis and treatment strategies.

2 | MATERIALS AND METHODS

2.1 | Patients

All patients diagnosed with CC from July 2022 to September 2023 at Yuhuangding Hospital of Qingdao University were included in the study. The studies involving human participants were reviewed and approved by the Medical Research Ethics Committee of Yuhuangding Hospital of Qingdao University (no. 2021–382). The inclusion criteria were as follows: (1) age over 18 years and (2) willingness to supply blood samples for research purposes and provide informed consent. Cases included newly diagnosed or suspected untreated patients with LSIL (CIN1), HSIL (CIN2 and CIN3) and invasive carcinoma.

The HPV negative controls comprised patients who tested negative for HPV. The HPV-positive control comprised purified DNA extracted from cervical biopsies of relevant CC cases. Blood samples were taken from all patients and they subsequently received a research ID. All subsequent analyses were conducted in a single-blind fashion.

2.2 | Molecular diagnosis of HPV infection

The HPV status of all patients was determined using the 23 HPV Genotyping Real-time PCR Kit (Hybribio, China), according to the manufacturer's instructions.

2.3 | Blood sample collection

To detect ctHPV DNA, each participant provided a total of 8–10 mL of whole blood collected in Cell-Free DNA BCT collection tubes (Streck, La Vista, NE, USA). Following the manufacturer's instructions, samples were stored at room temperature for up to 14 days before DNA extraction. Plasma was obtained by low-speed centrifugation, followed by an additional high-speed centrifugation, yielding approximately 3–5 mL of plasma per sample. Plasma samples were stored at −80°C until ctDNA extraction.

2.4 | cfDNA extraction and ddPCR analysis for ctHPV DNA detection

Total cfDNA was extracted from 3 to 4 mL of plasma using a QIAamp circulating nucleic acid kit (Qiagen) following the manufacturer's instructions. Bio-Rad QX200 Droplet Digital PCR system was used according to the manufacturer's instructions (Bio-Rad Laboratories, USA). Briefly, 10 ng of DNA used as a positive control, or 5.4 μL cfDNA isolated from plasma were mixed with ddPCR supermix for probes (no dUTPs, Bio-Rad laboratories) and primer/probe set (Bio-Rad Laboratories). Droplets were generated using 20 μL of the reaction mixture and 70 μL of droplet generation oil. The following cycling conditions were used for PCR amplification: 95°C for 5 min, 40 cycles of 94°C for 30 s and 55°C for 1 min, 98°C for 10 min, and 4°C for infinite hold. The droplets were examined using the QX200 Droplet Reader (Bio-Rad) to measure fluorescence emitted by FAM and HEX probes.

All primers were designed to target conserved regions of HPV E6 for HPV 16, 18, 33, 52, and 58 (amplicons spanning 57–70 base pairs, Table S2). The probes were verified to exhibit no cross-genotype reactivity (Table S3) and to demonstrate a linear relationship with $R^2 > 0.97$ for all genotypes (Figure S1). Additionally, we incorporated the human reference *FGFR1* as a DNA quality control.[18]

The HPV read count results were quantified in copies/mL using the following formula: (copies per well)/(mL input of sample)×(mL DNA extracted/mL processed plasma). The quantification of the target molecule was reported as the number of copies (HPV and *FGFR1*) per sample in each reaction.

As previously described,[19] we determined the cutoff value by analyzing ctHPV DNA from negative controls. ctHPV DNA extracted from 29 HPV negative samples was utilized to establish the cutoff value of the ddPCR assays. In this cohort, the maximum measurable quantity of ctHPV DNA was 4.17 copies per mL plasma (Table S4). Hence, we set a conservative cutoff value for ctHPV DNA positivity of >5 copies/mL.

2.5 | Statistical analysis

All ddPCR data were presented as log10-transformed. Student's *t*-test was used for analyzing the data in this study. All tests were performed using R version 4.1.0, with statistical significance defined as *P* values less than 0.05, and 95% confidence interval.

3 | RESULTS

3.1 | Patient characteristics

The case cohort comprised 87 patients with CIN, encompassing CIN1, CIN2, CIN2-3, or CIN3, who tested positive for HPV16, 18, 33, 52, or 58, while the control cohort consisted of 29 HPV negative patients. Moreover, we included 29 patients with CC, containing 19 early stage

and 10 late stage (Table 1). Histologically, the CC cases included 25 (86.2%) squamous cell carcinomas (SCC), two (6.9%) adenocarcinomas (AC), and two (6.9%) adenosquamous carcinomas (ASC). Among the 29 CC patients, 21 (72.4%) had tumors that tested positive for HPV16, four (13.8%) had tumors positive for HPV33, three (10.3%) had tumors positive for HPV52, and one (3.4%) had tumors positive for HPV18. Table 1 shows the patient and tumor characteristics, and all relevant clinical and biological data are available in Table S1.

3.2 | ctHPV DNA as a specific diagnostic marker for CC

The presence of ctHPV DNA in the blood of patients with precancerous CIN3 lesions, which may progress to CC, has been a subject of conflicting reports.[15–17] To address this, we conducted a study involving 87 cases of CIN patients at various stages to investigate the detectability of ctHPV DNA in this group. Through the analysis of ctHPV DNA in HPV-positive controls, CIN patients, and HPV-negative samples, we observed that ctHPV DNA was only detectable in HPV-positive controls (Figure 1a). In contrast, neither CIN patients nor HPV-negative samples exhibited detectable ctHPV DNA (Figure 1a). Moreover, to address potential issues related to sample DNA quality that could lead to the failure of ctHPV DNA detection, we conducted parallel examinations of the internal reference gene *FGFR1* in each sample. Our findings confirmed the detectability of *FGFR1* in all 87 CIN samples, signifying the adequacy of sample DNA quality (Figure 1b). In conclusion, our findings substantiated the absence of ctHPV DNA in the blood of CIN patients.

Next, we encompassed patients with cervical CIN ($n=87$) and CC ($n=29$). We observed that ctHPV DNA was only detectable in 15 out of 29 (51.7%) CC patients (Figure 2). All in all, our findings

TABLE 1 Patient characteristics for the CIN and cervical cancer.

Patients	CIN $N=87$	Early-stage cervical cancer $N=19$	Late-stage cervical cancer $N=10$
Age (mean ± SD)	46 ± 11	43 ± 10	52 ± 13
Histology	***n*%**	***n*%**	***n*%**
CIN1	6 (6.9)		
CIN2	27 (31.0)		
CIN2 ~ 3	23 (26.5)		
CIN3	31 (35.6)		
SCC		17 (89.5)	8 (80.0)
ASC			2 (20.0)
AC		2 (10.5)	
FIGO 2018[a]	***n*%**	***n*%**	***n*%**
IA1		9 (47.4)	
IB1		5 (26.3)	
IB2		5 (26.3)	
IB3			2 (20.0)
IIA			1 (10.0)
IIB			2 (20.0)
IIIB			2 (20.0)
IIIC			3 (30.0)
Tumor size (mm) (mean ± SD)[b]		12.4 ± 16.4	41.4 ± 11.6
HPV genotype tissue[c]	***n*%**	***n*%**	***n*%**
16	47 (54.0)	13 (68.4)	8 (80.0)
18	1 (1.1)	1 (5.3)	
33	13 (14.9)	2 (10.5)	2 (20.0)
52	12 (13.8)	3 (15.8)	
58	14 (16.1)		

Abbreviations: AC, adenocarcinoma; ASC, adenosquamous carcinoma; CIN, cervical intraepithelial neoplasia; HV, human papillomavirus; SCC, squamous cell carcinoma; SD, standard deviation.

[a]Disease stage according to FIGO 2018. For details, see Table S1.

[b]Largest diameter of tumor. For the early-stage subgroup, tumor size was evaluated pathologically after surgery based on the removed tissue. For the late-stage subgroup, tumor size was evaluated based on clinical examination or magnetic resonance imaging (MRI) prior to treatment.

[c]Cervical tissue biopsy tested with 23 HPV genotyping real-time PCR kit (Hybribio).

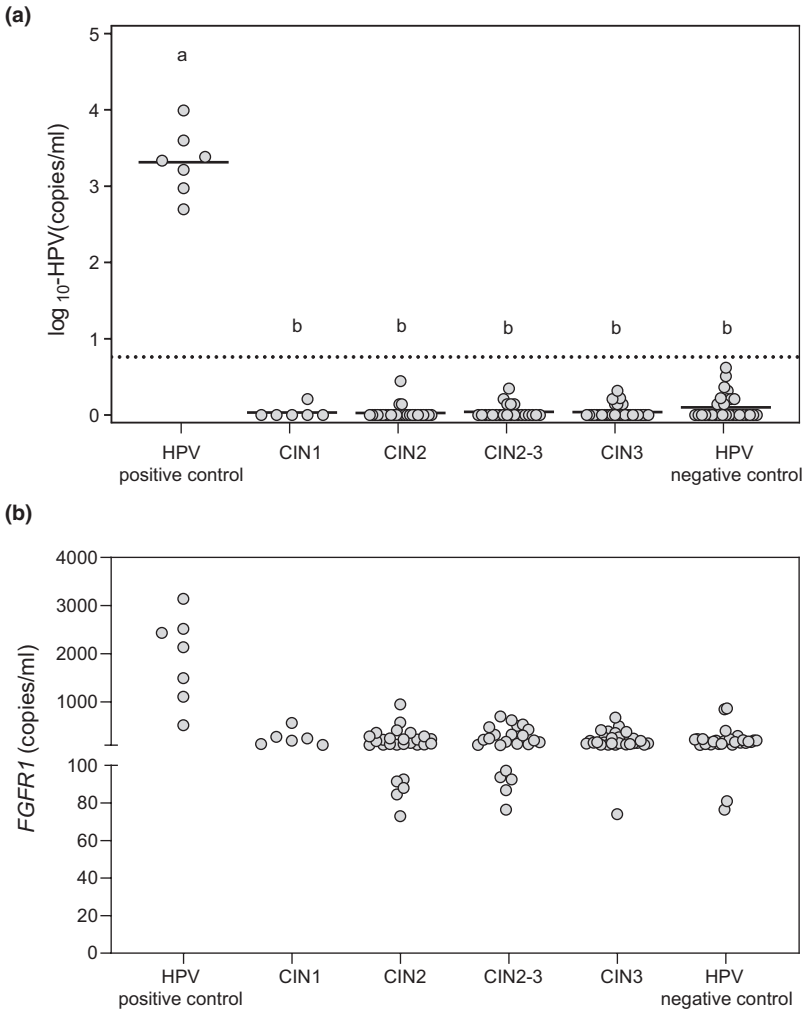

FIGURE 1 Detection of ctHPV DNA in cervical intraepithelial neoplasia (CIN) patients. (a) Seven cervical biopsies of cervical cancer samples (human papillovirus [HPV] positive control), 87 plasma samples from CIN patients containing CIN1 ($n=6$), CIN2 ($n=27$), CIN2-3 ($n=23$), and CIN3 ($n=31$), and 29 plasma samples from HPV negative control. The black dashed line represents cutoff value for HPV positivity (>5 copies/mL). Different letters (a, b) denote statistically different groups ($P<0.001$). Black lines represent median values. (b) Detection of *FGFR1* by digital droplet PCR (ddPCR) in plasma samples.

suggested that ctHPV DNA could effectively serve as a specific biomarker for CC diagnostic.

3.3 | Distinct ctHPV DNA profiles in early and late-stage CC

Moreover, our study aimed to investigate the effectiveness of ctHPV DNA in distinguishing between early- and late-stage CC. Notably, our analysis revealed a significant difference among CC patients (Figure 3a,b). In the early-stage group ($n=19$), ctHPV DNA was detectable in six out of 19 patients (31.6%), with viral loads ranging from 0 to 27.78 copies/mL plasma (Figure 3a). In contrast, among late-stage CC patients ($n=10$), ctHPV DNA was nearly universally detectable in nine out of 10 patients (90%), with viral loads ranging from 0 to 601.85 copies/mL plasma (Figure 3a). These findings underscored the potential of plasma ctHPV DNA levels as a discriminatory marker between early and late-stage CC.

3.4 | Correlation of ctHPV DNA levels with clinical and biological parameters

To evaluate the relationship between ctHPV DNA levels and clinical or biological parameters, we compared the ctHPV DNA results with the patient's age, and tumor size. Our analysis revealed that ctHPV DNA levels displayed a weak but significant positive correlation with tumor size ($R^2=0.34$, $P=0.002$) (Figure 4a) and a slight but significant positive correlation with patient age ($R^2=0.12$, $P=0.070$) (Figure 4b).

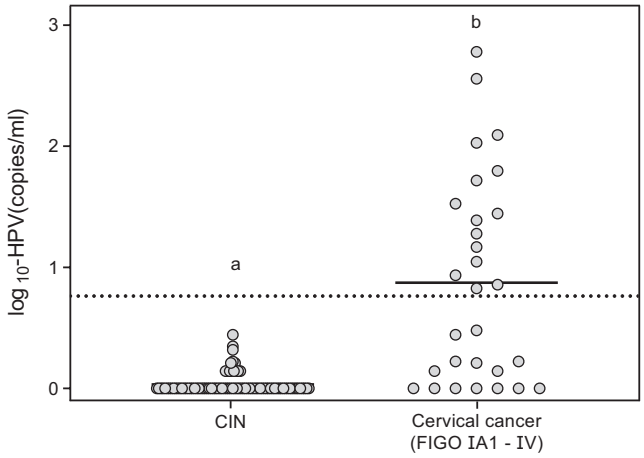

FIGURE 2 Distinct level of ctHPV DNA between cervical intraepithelial neoplasia (CIN) and cervical cancer patients. A total of 87 plasma samples from CIN patients and 29 plasma samples from cervical cancer patients. Black dashed line represents cutoff value for human papillovirus (HPV) positivity (>5 copies/mL). Different letters (a, b) denote statistically different groups ($P < 0.001$). Black lines represent median values.

4 | DISCUSSION

In this study, we examined the presence of ctHPV DNA in the blood of CIN patients and assessed its potential as a biomarker for the detection of CC, with the goal of improving clinical diagnosis. Through the utilization of ddPCR technology, we first established that ctHPV DNA was absent in CIN patients. Subsequent analysis demonstrated that as many as 90% of samples from late-stage CC patients yielded positive results for ctHPV DNA. However, among early-stage CC patients, only 31.6% of samples showed detectable ctHPV DNA levels.

Presently, research on liquid biopsies utilizing ddPCR technology for CC diagnosis has been conducted. However, due to variations in threshold levels set by different studies, the detection sensitivity ranges widely from 31% to 100%.[6,8–14] As a result, the common threshold set by most researchers defined ctHPV DNA positivity as having more than three copies/mL plasma. In our study, we identified that applying the common threshold for HPV-negative control cases led to false positives. To address this issue, we established a more conservative threshold, requiring more than five copies/mL for ctHPV DNA positivity. Each ddPCR run included a non-template

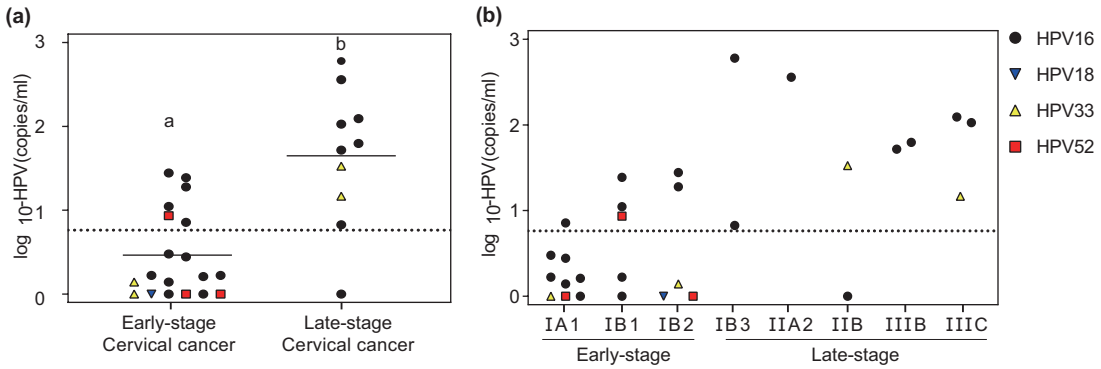

FIGURE 3 ctHPV DNA detection by digital droplet PCR (ddPCR) in early or late-stage cervical cancer patients. (a) Detection of ctHPV DNA by ddPCR in plasma samples from the early or the late-stage cervical cancer patients, respectively. (b) ctHPV levels according to FIGO stages. Black dashed line represents cutoff value for human papillovirus (HPV) positivity (>5 copies/mL). Different letters in (a) (a, b) denote statistically different groups ($P < 0.001$). Black lines represent median values.

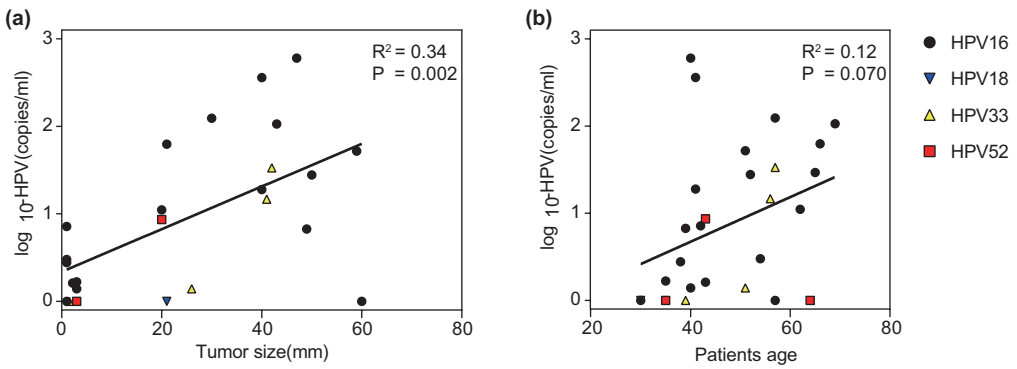

FIGURE 4 Relationship between ctHPV DNA and tumor size or patient age. Statistical significance was tested using the Kruskall–Wallis test followed by linear regression for (a and b).

control, a healthy control, and an HPV-positive control. The HPV-positive control consisted of purified DNA from cervical biopsies of relevant CC cases. Furthermore, we included an internal reference gene, *FGFR1*, to serve as a human reference control, ensuring the presence of human DNA in all plasma samples.[18]

In our study, we revealed a significant correlation between ctHPV DNA levels and tumor size ($P=0.002$). As is known, larger tumors are often more prone to metastasize to adjacent tissues or lymph nodes. Reder et al. reported higher ctHPV DNA levels in lymph node-positive and distant metastases patients,[20] and tumor size was associated with prognosis, with larger tumors typically correlated with poorer survival rates and prognosis. Han et al. demonstrated an association between ctHPV DNA levels at the end of chemoradiation (CRT) and inferior progression-free survival (PFS).[21] These findings underscore the importance of ctHPV DNA levels in clinical decision making, facilitating the determination of optimal treatment strategies and providing more accurate prognostic assessments for patients.

Indeed, our study had certain limitations. First, the relatively small cohort size may have influenced the detection rate of ctHPV DNA, which was higher in late-stage CC patients. Furthermore, future endeavors should prioritize expanding the study cohort and employing advanced analytical methods, such as artificial intelligence, to examine the correlation between ctHPV DNA level and the progression of CC. This approach will significantly contribute to the precise diagnosis of CC.

5 | CONCLUSION

In conclusion, our study clearly established that ctHPV DNA is a highly specific biomarker for CC diagnosis. Additionally, our research uncovered a positive correlation between ctHPV DNA levels and patient age, especially tumor size. In summary our findings significantly advance the prospects of precise diagnostic methods for CC.

AUTHOR CONTRIBUTIONS

T.C and S.J.K: Conceptualized the study. J.N.S, T.W and R.M.S.: Collected the samples and performed biological experiments. T.C, S.J.K, R.M.S., T.W, X.R.T, J.M.S. and J.N.S: Performed the data analysis. T.C, T.W and R.M.S.: Wrote the manuscript with input from all authors. T.C and S.J.K: Revised the manuscript. All authors read and approved the final version of the manuscript.

FUNDING INFORMATION

This study was supported by the Science and Technology Program of Guangzhou, China (grant no. 2023A03J0542).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

All data are contained within the study.

REFERENCES

1. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol*. 1999;189(1):12-19.
2. Andersson S, Rylander E, Larsson B, Strand A, Silfversvard C, Wilander E. The role of human papillomavirus in cervical adenocarcinoma carcinogenesis. *Eur J Cancer*. 2001;37(2):246-250.
3. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209-249.
4. Ghaleh HEG, Shahriary A, Izadi M, Farzanehpour M. Advances in early diagnosis of cervical cancer based on biosensors. *Biotechnol Bioeng*. 2022;119(9):2305-2312.
5. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer*. 2011;11(6):426-437.
6. Jeannot E, Latouche A, Bonneau C, et al. Circulating HPV DNA as a marker for early detection of relapse in patients with cervical cancer. *Clin Cancer Res*. 2021;27(21):5869-5877.
7. Sivars L, Palsdottir K, Crona Guterstam Y, Falconer H, Hellman K, Tham E. The current status of cell-free human papillomavirus DNA as a biomarker in cervical cancer and other HPV-associated tumors: a review. *Int J Cancer*. 2023;152(11):2232-2242.
8. Bonlokke S, Stougaard M, Sorensen BS, et al. The diagnostic value of circulating cell-free HPV DNA in plasma from cervical cancer patients. *Cells*. 2022;11(14):2170.
9. Cheung TH, Yim SF, Yu MY, et al. Liquid biopsy of HPV DNA in cervical cancer. *J Clin Virol*. 2019;114:32-36.
10. Galati L, Combes JD, Le Calvez-Kelm F, et al. Detection of circulating HPV16 DNA as a biomarker for cervical cancer by a bead-based HPV genotyping assay. *Microbiol Spectr*. 2022;10(2):e0148021.
11. Kang Z, Stevanovic S, Hinrichs CS, Cao L. Circulating cell-free DNA for metastatic cervical cancer detection, genotyping, and monitoring. *Clin Cancer Res*. 2017;23(22):6856-6862.
12. Rungkamoltip P, Temisak S, Piboonprai K, et al. Rapid and ultrasensitive detection of circulating human papillomavirus E7 cell-free DNA as a cervical cancer biomarker. *Exp Biol Med (Maywood)*. 2021;246(6):654-666.
13. Sivars L, Hellman K, Crona Guterstam Y, et al. Circulating cell-free tumor human papillomavirus DNA is a promising biomarker in cervical cancer. *Gynecol Oncol*. 2022;167(1):107-114.
14. Thangarajah F, Busshoff J, Salamon J, et al. Digital droplet PCR-based quantification of ccfHPV-DNA as liquid biopsy in HPV-driven cervical and vulvar cancer. *J Cancer Res Clin Oncol*. 2023;149(14):12597-12604.
15. Hsu KF, Huang SC, Hsiao JR, Cheng YM, Wang SP, Chou CY. Clinical significance of serum human papillomavirus DNA in cervical carcinoma. *Obstet Gynecol*. 2003;102(6):1344-1351.
16. Jeannot E, Becette V, Campitelli M, et al. Circulating human papillomavirus DNA detected using droplet digital PCR in the serum of patients diagnosed with early stage human papillomavirus-associated invasive carcinoma. *J Pathol Clin Res*. 2016;2(4):201-209.
17. Cocuzza CE, Martinelli M, Sina F, et al. Human papillomavirus DNA detection in plasma and cervical samples of women with a recent history of low grade or precancerous cervical dysplasia. *PLoS One*. 2017;12(11):e0188592.
18. Siravegna G, O'Boyle CJ, Varmeh S, et al. Cell-free HPV DNA provides an accurate and rapid diagnosis of HPV-associated head and neck cancer. *Clin Cancer Res*. 2022;28(4):719-727.
19. Milbury CA, Zhong Q, Lin J, et al. Determining lower limits of detection of digital PCR assays for cancer-related gene mutations. *Biomol Detect Quantif*. 2014;1(1):8-22.
20. Reder H, Taferner VF, Wittekindt C, et al. Plasma cell-free human papillomavirus oncogene E6 and E7 DNA predicts outcome in oropharyngeal squamous cell carcinoma. *J Mol Diagn*. 2020;22(11):1333-1343.

21. Han K, Zou J, Zhao Z, et al. Clinical validation of human papilloma virus circulating tumor DNA for early detection of residual disease after chemoradiation in cervical cancer. *J Clin Oncol*. 2024;42(4):431-440.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Chen T, Wu T, Sun R, et al. Circulating tumor HPV DNA as a specific biomarker for cervical cancer. *Int J Gynecol Obstet*. 2025;169:148-154. doi:10.1002/ijgo.16011