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Liquid biopsy of HPV DNA in cervical cancer

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ABSTRACT

Background: A blood test to serve as a tumor marker for cervical cancer would be useful to clinicians to guide treatment and provide an early signal for recurrence. The development of droplet digital PCR has enabled the detection of HPV DNA in patient serum, providing a potential marker for cervical cancer.
Objectives: To report on a blood-based test for HPV-specific E7 and L1 genes, which may serve as a tumor marker to guide treatment and detect early recurrence in cervical cancer.
Study design: Pre-treatment plasma samples were investigated from 138 Hong Kong Chinese women with primary invasive squamous cell carcinoma and adenocarcinoma of the cervix with tumor samples expressing HPV16 or HPV18. Two genes specific to the human papillomavirus, E7 and L1, were measured in cell free DNA (cfDNA) extracted from plasma using droplet digital PCR. Analysis of detectable E7 and L1 levels was performed to investigate the potential of liquid biopsy of E7 and L1 as a clinically useful molecular biomarker.
Results: The majority of patients had HPV16 (71.7%), squamous cell carcinoma (78.3%) and stage IB-II disease (82.6%). HPV E7 and L1 sequences were detected in plasma cfDNA from 61.6% (85/138) of patients. Patients with high viral load (defined as ≥ 20 E7 or L1 copies per 20 μL reaction volume) had increased risk of recurrence and death at 5 years on univariate analysis but not multivariate analysis.
Conclusions: HPV DNA can be quantitatively detected with the use of cfDNA. This has the potential to provide a clinically useful tumor marker for patients with cervical cancer that can aid in post-treatment surveillance and estimating the risk of disease relapse.

1. Background

Cervical cancer remains the fourth most common malignancy worldwide in women, despite Pap smear screening [1]. This problem continues despite the introduction of the human papillomavirus (HPV) vaccine in 2006, as most women in the world remain unvaccinated [2]. A simple and sensitive blood test to inform prognosis, guide treatment, monitor treatment response and provide an early signal for treatment failure would be desirable to maximize the cure rate.

Current pre-treatment assessment and post-treatment surveillance for cervical cancer include physical examination and imaging studies. Various imaging studies are useful prior to treatment, but frequent and repeated imaging studies for postoperative surveillance are impractical. Overall survival from cervical cancer is highly correlated with stage,

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with > 50% mortality at 5 years for stage III-IV disease [3]. A blood tumor marker would represent a preferred first-line test for post-treatment surveillance. Squamous Cell Carcinoma Antigen or TA-4 has been studied as a tumor marker for cervical carcinoma. However, TA-4 is of limited value due to poor sensitivity [3].

HPV is a critical step in the development of cervical cancer. It has been shown that HPV genetic material can be found in almost all cervical cancer tissue [4]. Two particular HPV subtypes, HPV 16 and 18, are responsible for over 70% of cervical cancers including both squamous cell and adenocarcinomas of the cervix [5]. Recent research has shown that most patients with cervical cancer beyond stage I have detectable amounts of circulating free DNA (cfDNA), and the concentration of cfDNA reflects tumor burden [6]. Since the integration of HPV into the human genome is a crucial part in tumorigenesis [7] and the sequence of viral genes is distinct from human genes, detection of HPV DNA in the blood may reflect tumor burden before treatment and signal recurrence during post-treatment surveillance.

2. Objectives

The aims of this study were to determine whether circulating HPV DNA is a sensitive tumor marker and to evaluate whether the amount of circulating HPV DNA was correlated with prognosis.

3. Study design

Patients with pathologically confirmed primary invasive cervical cancer diagnosed at the Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, Prince of Wales Hospital from 1997 to 2007 were included. Informed consent was obtained from participants to collect blood and tumor tissue before treatment for molecular genetic studies. HPV typing was performed on cervical cancer tissue, and only patients whose cancer tissue was positive for HPV16 or HPV18 DNA were included in the study. HPV DNA was detected by the INNO-LiPa HPV Genotyping Extra kit (Innogenetics, Belgium). This was a retrospective study approved by the joint The Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee (Reference #2013.349).

Radical surgery was offered to patients with stage I-IIA disease unless there was a contraindication. Chemo-irradiation or primary radiotherapy were offered to patients with advanced-stage disease, or those with a contraindication to radical surgery. Pelvic lymphadenectomy was performed as part of the surgical treatment procedure. Enlarged pelvic or para-aortic nodes identified on pre-treatment imaging studies were removed before chemo-irradiation or radiotherapy to improve disease control and modify the radiation field if necessary [8].

Persistent disease was defined as refusal or failure of treatment. Follow-up included evaluation every 4 months in the first 2 years and every 6 months thereafter. Failure of treatment and recurrences were diagnosed clinically with or without histological confirmation.

Plasma samples were collected at the time of study entry and stored at −70 °C prior to analysis.

Plasma samples were later thawed and centrifuged at 2000 *g* at 4 °C for 10 min before DNA extraction. DNA was extracted from plasma samples using the QIAamp Circulating Nucleic Acid Kit Extraction (Qiagen, Hilden, Germany) per the manufacturer's instructions. Three mL of plasma was treated with Proteinase K and Buffer ACL. The lysate was passed over the QIAamp Mini columns on a vacuum manifold, followed by two washing steps and eluted in 150 µL. The extracted DNA was stored at −70 °C until use.

ddPCR was carried out using the ddPCR Supermix for Probes (no dUTP), the Droplet Generation Oil for Probes, the QX200 Droplet Generator, the QX200 Droplet Reader, the C1000 Touch Thermal Cycler, and the PX1 PCR Plate Sealer (all from Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The ddPCR reactions contained 10 µL of 2x ddPCR Supermix in a final reaction volume of 20 µL, consisting of 7.2 µL diluted DNA. Four sets of primers and probes were designed to target E7 and L1 genes (Table S1) of HPV16 and HPV18. E7 was chosen because it is highly conserved and allows for more options for primer and probe design. L1 was chosen as it is a commonly used target for HPV detection. The primer and probe concentrations used were 0.45 µM and 0.25 µM, respectively for the four sets of ddPCR. Twenty microliters of reaction mix were transferred to a DG8 cartridge for a QX200 Droplet Generator followed by 70 µL of Droplet Generation Oil into oil wells. After droplet generation, 40 µL of the reaction were then transferred to a 96-well plate (Eppendorf, Hamburg, Germany) and heat-sealed with pierceable sealing foil sheets using a PX1 PCR Plate Sealer. The cycling conditions were 95 °C for 10 min, 50 cycles of 94 °C for 30 s and 55 °C for 1 min, followed by 98 °C for 10 min and a hold at 12 °C using thermal cycler C1000. After PCR amplification, the plate was read by the QX200 Droplet Reader for analysis. Droplets from each well were aspirated and went through a two-color fluorescent detector for identifying the fluorescent amplitude of each well. The DNA targets were quantified using QuantaSoft Software (Bio-Rad). HPV positivity was determined using automated cutoff values relative to controls. A cut off value of ≥ 20 copies in a 20 µL reaction volume was arbitrarily chosen to represent a high viral load, while 0–20 copies in a 20 µL reaction volume was chosen to represent a low viral load. The 20 copy cut off was chosen to minimize the chance of false positivity.

4. Results

One hundred thirty-eight cervical cancer patients were included (Table 1). Most patients had FIGO stage IB-II squamous cell carcinoma. Ninety-nine patients (71.7%) had tumors that were HPV16 positive and 39 (28.3%) were HPV18 positive by genotyping. Thirty-nine patients

Table 1
Patient demographic, histologic and clinical characteristics according to plasma HPV DNA status.

Characteristic (N = 138)	Plasma HPV DNA High Viral Load (≥ 20 copies) (N = 34)	Plasma HPV DNA Low Viral Load (0–20 copies) (N = 104)	P-value
Age at diagnosis	56.4 (31–80)	52.3 (25–85)	0.110
Histologic type			
Squamous cell carcinoma	31	77	0.0532
Adenocarcinoma	3	27	
FIGO stage			
1 (52)	5	47	0.0027
2 (62)	19	43	
3 (21)	8	13	
4 (3)	2	1	
Pelvic lymph node			
Positive (34)	9	25	0.9550
Negative (104)	25	79	
Abdominal lymph node			
Positive (6)	3	3	0.1593
Negative (132)	31	101	
Persistence of primary disease			
Yes (12)	2	10	0.7300
No (126)	32	94	
Recurrence within 3 years			
Yes (38)	16	22	0.0071
No (88)	16	72	
Recurrence within 5 years			
Yes (45)	16	29	0.0820
No (81)	16	65	
Recurrence pattern (number of patients)			
Locoregional	4	22	
Distant	14	25	
Median overall survival in months (range)	33 (2–202)	114 (3–200)	

underwent primary surgical management and 99 patients were treated by primary chemo-irradiation or radiotherapy alone. Recurrence rates at 3- and 5-years after treatment were 50% and 57%, respectively. All 138 patients had DNA samples available for E7 testing, however for 20 patients DNA samples were exhausted, so L1 testing was performed for 118 patients with sufficient DNA available.

Through testing for E7, 61.6% (61/99) and 41% (16/39) were found to have positive HPV16 and HPV18 DNA in a 20 μL reaction volume. HPV DNA was positive in 55.8% (77/138) of the entire study population. Using a cut off value of ≥10 copies and ≥20 copies in a 20 μL reaction volume, the positive test rate of the entire study population dropped to 29.0% (40/138) and 24.6% (34/138), respectively. The 34 patients with ≥20 copies of HPV DNA in a 20 μL reaction volume were classified as having a high viral load (Table 1).

Through testing for L1, 61.0% of HPV16+ and 51.6% of HPV18+ patients were found to have HPV DNA. HPV DNA was positive in 58.5% (69/118) of the entire study group. Eight patients who had no identifiable HPV E7 gene in the first set of ddPCR were found to be positive for the HPV L1 gene in the second set of ddPCR. By combining ddPCR test results for E7 and L1, we could detect HPV DNA in 61.6% (85/138) of cervical cancer patients (Table 2).

On univariate analysis, there was a trend toward a significant relationship between HPV DNA copy number and FIGO stage at diagnosis ($p = 0.076$). There was no correlation between HPV DNA copy number and lymph node status ($p = 0.717$). Kaplan–Meier survival analysis revealed that patients with high viral load had significantly worse overall survival (Fig. 1). In univariate analysis, high viral load level was associated with relative risk of progression (RR = 1.69, $p = 0.030$) and risk of death (RR = 1.70, $p = 0.007$). FIGO stage ≥ IB2 and positive pelvic lymph nodes were also associated with risk of recurrence and death, while patient age and HPV type were not. In multivariate analysis, only FIGO stage ≥ IB2 and positive pelvic lymph nodes remained as an independent factor related to the risk of recurrence and overall survival, but high viral load was not.

5. Discussion

HPV DNA can be found in 99.7% of cervical cancer tissue and HPV infection is considered as a pre-requisite for cervical squamous cell carcinoma and adenocarcinoma [9]. Sixteen HPV serotypes related to carcinogenesis are classified as high-risk serotypes. Among these, HPV16 and HPV18 are the two most important serotypes and together account for 70% of cervical carcinomas worldwide [10].

There does not appear to be a role for a serum marker in the screening process for cervical cancer, as the cervix is readily accessible by clinical examination for screening and diagnostic purposes. The recent addition of HPV DNA testing in cervical fluid in addition to cervical cytology increased the sensitivity of screening [11]. However, once a diagnosis of cervical cancer is established, a sensitive blood marker could guide treatment and improve surveillance after treatment.

Measurement of tumor-associated antigen (TA-4) in the blood of cervical cancer patients was first described by Kato in 1977 [12]. Higher levels of TA-4 are related to advanced stage of disease and nodal metastasis [13]. Among 70 patients who developed recurrence, 80% were found to have raised levels of TA-4, which preceded clinical diagnosis of recurrence by an average of 6.9 months [14]. However, only 15.8% of patients with stage I squamous cell carcinoma and none of the patients with adenocarcinoma had an elevated TA-4 [2]. A more sensitive blood test is therefore needed.

Despite the high sensitivity and specificity of HPV16 E6 antibodies in oropharyngeal cancer [15], an HPV antibody has limited use as an effective tumor marker for cervical carcinoma, as patients with current or previous HPV infection could be HPV antibody-positive regardless of whether they harbor a cervical malignancy [16]. Furthermore, the antibody level remains elevated after successful treatment which precludes its use as a tumor marker for surveillance [15].

Circulating nucleic acid was first reported in 1940s and includes DNA and RNA that are genomic, mitochondrial and viral in origin [17]. In healthy individuals, most circulating free DNA (cfDNA) originates from apoptotic cells and some living cells may also actively release DNA fragments into the circulation. The amount of cfDNA contributed by tumor cells depends on the tumor volume as well as other tumor characteristics, such as turnover rate.

The specific changes of tumor DNA in terms of oncogene and tumor suppressor mutations, microsatellite alterations, and hypermethylation can be similarly found in cfDNA [18,19]. Detection of these specific changes in cfDNA may provide a tumor marker with high specificity, though high test sensitivity is needed as mutant DNA fragments may represent a small proportion of total cfDNA [20].

Cervical cancer differs from other cancer types because HPV infection is a crucial step in tumorigenesis and HPV DNA material is nearly universal within tumor cells. As HPV's DNA sequence is profoundly distinct from the human genome, HPV DNA can be more readily detected in cfDNA, and a test to identify HPV DNA material in the serum could be considered a "liquid biopsy." Unfortunately, HPV DNA can only be found in the blood of 12–45% of cervical cancer patients using conventional PCR [20,21].

The recent development of Digital PCR offers higher sensitivity than conventional PCR and therefore was used in this study to examine the potential role of circulating HPV DNA as a tumor marker for cervical carcinoma. This proof of concept was recent demonstrated using ddPCR to show that total tumor burden strongly correlated with HPV cfDNA levels in oropharyngeal cancer [22].

We explored the use of a set of ddPCR to detect a fragment (183 bp of HPV16, 93 bp of HPV18) of HPV E7 in the blood samples of patients with cervical cancer. The HPV E7 gene was targeted because it is one of the key viral genes that inhibits the tumor suppressor genes to promote carcinogenesis and is constitutively found in tumor cells.

We found that 55.8% (77/138) of patients had detectable HPV E7 DNA, however fewer patients had a "high viral load" which was defined as ≥20 copies per μL. Since the size of cell-free DNA fragments from apoptotic tumor cells is generally around 180 bp [23], PCR primer pairs that target a shorter DNA fragment may identify more patients with detectable HPV DNA. Thus, we also explored the use of additional primer pairs to detect a fragment (107 bp of HPV16 and 98 bp of HPV18) of the HPV L1 gene, to see if the HPV DNA detection rate could be increased. We found 8 HPV E7 DNA-negative patients who were positive for HPV L1 DNA and therefore increased the sensitivity from 55.8% to 61.6% if two primer pairs were used. With more primer pairs, a further increase in the detection rate may be possible.

The number of copies of HPV DNA in the tumor cells and thus the number of circulating HPV DNA fragments varied significantly. There is no clear standard on how many copies of HPV DNA should be used as a cut-off to define a positive test. We found that the test positive rate of using E7 primer decreases significantly from 55.8% to 29% if the threshold was increased from any detectable copy to ≥10 copies. We

Table 2
Risk of recurrence within 5 years and death within 5 years among patients with low and high viral load by ddPCR.

Characteristic (N = 138)	Plasma HPV DNA High Viral Load (≥20 copies) (N = 34)	Plasma HPV DNA Low Viral Load (0–20 copies) (N = 104)	Relative Risk (95% CI)	P-value
Recurrence within 5 years (%)	16 (47.1%)	29 (27.9%)	1.69 (1.05-2.71)	0.030
Death within 5 years (%)	20 (58.5%)	36 (34.6%)	1.70 (1.16-2.50)	0.007

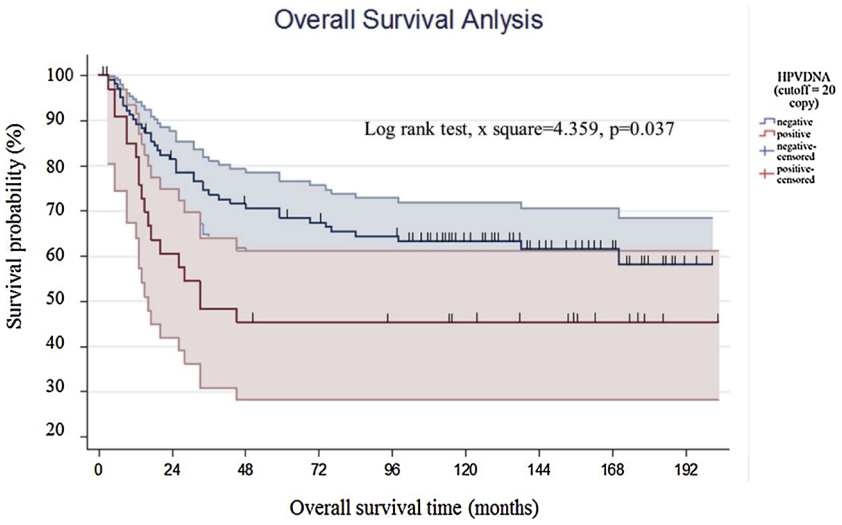

Fig. 1. Kaplan–Meier survival analysis of HPV positivity by ddPCR on overall survival time in cervical cancer.

believe that future prospective work should determine the proper cut-off to determine treatment failure or recurrence.

It has been hypothesized that the concentration of cfDNA is related to advanced clinical disease and pathological characteristics of the tumor [23]. We studied the use of HPV DNA copy number as a prognostic marker. We found that high viral load was a risk factor for recurrence at 5-year follow-up in univariate analysis, but it was not an independent risk factor for recurrence after controlling for other risk factors in multivariate analysis.

Our study is limited by the small sample size and the use of stored blood which can degrade DNA and thus impair sensitivity for HPV detection. For those with a test-positive case, with low levels of HPV DNA, we did not have enough plasma to verify the findings by repeating the test. For those with negative test results, we could not conduct further testing using additional primer sets. The 61.6% positive detection rate is far from satisfactory and further improvement in the cfDNA extraction method and inclusion of additional primer sets to cover a larger part of the HPV genome are needed before HPV DNA in cfDNA can be used clinically as a tumor marker. Nonetheless we believe that further study of ddPCR in cervical cancer could ultimately yield a clinically meaningful test for monitoring patients during and after treatment for this common and lethal malignancy.

Conflict of interest statement

Author Rossa WK Chiu holds equity in GRAIL Inc. All other authors have no conflicts of interest to declare.

CRediT authorship contribution statement

Tak Hong Cheung: Conceptualization. **So Fan Yim:** Investigation. **Mei Yun Yu:** Investigation. **Michael J. Worley:** Formal analysis. **Stephen J. Fiascone:** Formal analysis. **Rossa W.K. Chiu:** Methodology. **Keith W.K. Lo:** Investigation. **Nelson S.S. Siu:** Investigation. **Martin C.S. Wong:** Formal analysis. **Apple C.M. Yeung:** Methodology. **Raymond R.Y. Wong:** Investigation. **Zi Gui Chen:** Methodology. **Kevin M. Elias:** Formal analysis. **Tony K.H. Chung:** Investigation. **Ross S. Berkowitz:** Conceptualization, Writing. **Yick Fu Wong:** Conceptualization, Writing. **Paul K.S. Chan:** Conceptualization, Writing.

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