Available online at www.sciencedirect.com

SCIENCE DIRECT

Gynecologic Oncology 99 (2005) 615 – 621

Gynecologic Oncology

www.elsevier.com/locate/ygyno

Detection and quantitation of human papillomavirus type 16, 18 and 52 DNA in the peripheral blood of cervical cancer patients

Chih-Ming Ho[a,b,c,d], Sien-Sing Yang[e], Tsai-Yen Chien[a], Shih-Hung Huang[f], Cherng-Jye Jeng[a], Shwu-Fen Chang[d,*]

[a]*Gynecologic Cancer Center, Department of Obstetrics and Gynecology, Cathay General Hospital, Taipei, Taiwan, ROC*
[b]*School of Medicine, Taipei Medical University, Taipei, Taiwan, ROC*
[c]*School of Medicine, Fu Jen Catholic University, Hsinchuang, Taipei Hsien, Taiwan, ROC*
[d]*Graduate Institute of Cell and Molecular Biology, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan, ROC*
[e]*Liver Unit, Cathay General Hospital, Taipei, Taiwan, ROC*
[f]*Department of Pathology, Cathay General Hospital, Taipei, Taiwan, ROC*

Received 3 March 2005
Available online 11 August 2005

Abstract

Objective. To prospectively evaluate the feasibility of detecting human papillomavirus (HPV) type 16, 18 and 52 DNA in the peripheral blood of patients with cervical cancer using real-time polymerase chain reaction (PCR) and to determine its prognostic importance.

Methods. Blood and cervical swab specimens from 135 consecutive patients with 60 invasive cervical cancers, 10 microinvasions, 20 cervical intraepithelial neoplasias (CIN) III, 10 CIN II, 10 CIN I and 25 controls were collected and examined for HPV type 16, 18 and 52 DNA using real-time PCR to investigate the prevalence and viral load of HPV DNA at the time of diagnosis and during follow-up in patients with positive blood samples.

Results. Of the 60 patients with invasive cervical cancer, 27% had positive test results for HPV DNA in blood samples in contrast to 0% of patients with microinvasions, CIN III, CIN II, CIN I and normal controls. The DNA detection rates of viral subtypes in blood samples of cervical cancer patients were 5% for HPV-16, 16.7% for HPV-18, 8.3% for HPV-52, 1.7% for both HPV-16 and HPV-18 and 1.7% for both HPV-18 and HPV-52, while the detection rates in cervical swab specimens were 36.2% for HPV-16, 15.5% for HPV-18 and 17.2% for HPV-52. During follow-up, 8 of 10 cervical cancer patients with viral DNA detected in blood within 3 months after treatment had recurrence, and a high percentage (87.5%, 7/8) of this recurrence involved distant metastases.

Conclusions. In this study, real-time PCR detected HPV-16, -18 or -52 DNA in the peripheral blood of more than one-fourth of invasive cervical cancer patients. The association between risk of cancer recurrence and the amount of viral DNA detected in blood among cervical cancer patients after treatment is intriguing and deserves further investigation.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Polymerase chain reaction; Cervical neoplasia; Cervical carcinoma; Metastasis; Microinvasion

Introduction

Multiple studies have established infection with high-risk types of human papillomavirus (HPV) as a cause of cervical cancer [1,2]. More than 35 different types of HPV infect the genital tract, and at least 20 of these are associated with cervical cancer [3]. The etiological role of most common high-risk HPV types in pre-invasive and invasive cervical cancers, such as HPV types 16 and 18, has been demonstrated by epidemiological evidence and molecular studies [1,2]. In cervical cancer, up to 99.7% of cases are associated with HPV infection [2].

Accumulated evidence shows that tumor DNA can be found in the circulation of patients with cervical cancer [4,5]. The presence of tumor DNA in the blood may be of diagnostic and prognostic value. Genetically or even

* Corresponding author. Fax: +886 2 23778620.
E-mail address: cmbsfc21@tmu.edu.tw (S.-F. Chang).

0090-8258/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygyno.2005.07.004

epigenetically altered tumor DNA present in the primary tumor can also be detected in the serum or plasma [6,7]. HPV DNA has been found in serum or plasma samples from cervical cancer patients with detection rates ranging from 7% to 45% [8–12]. The discrepancy in these rates may be due to differences in target materials (serum or plasma), methods used to extract DNA, tools used to analyze DNA (either conventional PCR, real-time PCR or PCR-enzyme immunoassay) and in primers selected (L1, E6, E7). Few studies have compared detection rates of HPV DNA in circulating blood. In addition, several studies have suggested that HPV viral load might play an important role in the progression from HPV infection to cervical cancer development [13,14]. Testing of this hypothesis via prospective study with HPV DNA detection at diagnosis and during follow-up is mandatory.

Cervical cancer is the leading cancer among women in Taiwan. Liaw et al. studied the association between HPV infection and cervical cancer in Taiwanese women and found that 10% of low-grade and 33% of high-grade cervical neoplasias could be explained by infection with HPV subtypes 52 and/or 58 [15]. Huang et al. reported that HPV-52 and -58 were as prevalent as the high-risk HPV types 16 and 18 in Chinese women [16]. The present hospital-based study sought to determine the prevalence of type 16, 18 and 52 HPV infections not only in cervical swabs but also in blood among women with pre-invasive and cervical cancer residing in Taipei, Taiwan. This study provides new information regarding the occurrence of cervical cancer in patients with detection of HPV subtype 52 in circulating blood, a relatively uncommon finding among cervical cancers in the Americas, Europe, Africa and Southeast Asia.

This prospective study examined blood and cervical swabs from women with benign tumors who served as controls, women with cervical intraepithelial neoplasia (CIN) or women with cervical cancer for the presence of HPV types 16, 18 and 52 using real-time PCR. The association of HPV DNA detection with CIN lesions and the severity of cervical cancer were analyzed. In addition, we investigated whether HPV DNA viral load in blood specimens of cervical cancer patients before and/or after treatment was predictive of recurrence.

Materials and methods

The study protocol was reviewed and approved by the Institutional Review Board and Research Grant Committee of Cathay General Hospital. A total of 135 patients treated in the gynecologic cancer center of Cathay General Hospital (CGH) from January 2003 to December 2003 were recruited. Patients with CIN lesions were recruited consecutively from those with diagnosis during examination from January to June 2003. Among the total 135 patients, 25 patients with benign tumors who received simple hysterectomy performed by the same surgeon were recruited from January to March 2003 to serve as controls. Controls had no pathological findings of CIN or cancer of the cervix. Sample size justification was described as below. This study was powered to detect a clinically meaningful difference in the prevalence rate of positive test results for HPV DNA in blood samples between patients with invasive cervical cancer and patients with CIN or controls. Assuming that the incidence rate of positive test results for HPV DNA in blood samples for patients with CIN or controls is less than 2% [9], the selected sample size of 130 (65 per group) will give a 97% power for correctly detecting a 25% difference in the prevalence rate of positive test results for HPV DNA in blood samples at the 5% level of significance. Both blood specimens and cervical swabs were collected from all patients for the viral load detection of HPV types 16, 18 and 52 using real-time PCR. However, 2 of the 60 invasive cervical cancer specimens from cervical swabs could not be obtained due to lack of permission from patients.

All patients underwent complete physical and gynecologic examinations, and the cervical cancer was staged according to the guidelines of the International Federation of Gynecology and Obstetrics (FIGO). By the end of December 31, 2004, all patients had follow-up after primary treatment at 3-month intervals for the first 2 years or until death. All surviving patients were followed up for at least 1 year. The follow-up investigations included physical examination, cervical cytology, chemistry profiling and analysis of tumor markers such as serum squamous cell carcinoma-antigen (SCC-Ag) or CA-125. Further examinations, such as computed tomography (CT) of the pelvis, bone scan, chest radiography or biopsy of any suspected lesions, were performed when clinically indicated. Distant metastasis was defined as disease that occurred outside the pelvis. The association was evaluated between the HPV DNA level in the blood and clinical parameters such as tumor size, clinical staging, tumor marker, lymph node metastasis, lymphovascular space involvement (LVSI), histological type and adjuvant therapy. Among patients with an HPV-positive blood sample, the correlation between the amount of HPV DNA in blood and clinical parameters was evaluated before treatment and at 3 months after treatment.

DNA from cervical swab or blood samples was extracted using the QIAamp Blood Kit following the manufacturer's instruction (Qiagen, Hilden, Germany). Quantitative real-time PCR fluorescent assays for each of the HPV genotypes and for the HLA-DQα gene were performed with the Qgene HPV16, HPV18, HPV52 and housekeeping gene HLA-DQα detector kits (Qgene Biotecnology, Kaohsiung, Taiwan) along with SYBR Green dye using the ABI 5700 apparatus (Applied Biosystems, Foster City, CA). The real-time PCR assay had a dynamic range from 10^0 to more than 10^7 copies, allowing documentation of a wide range of HPV DNA copies found in clinical specimens. The HPV16, HPV18, HPV52 and HLA-DQα primer sequences from each kit are shown in Table 1.

Table 1
HPV detector primer set

	Primer set	Amplicon size
Type 16	MY74 5′ CAT TTG TTG GGG TAA CCA AC3′ MY75 5′ TAG GTC TGC AGA AAA CTT TTC 3′	412 bp
Type 18	MY76 5′ TGT TTG CTG GCA TAA TCA AT 3′ MY77 5′ TAA GTC TAA AGA AAA CTT TTC 3′	415 bp
Type 52	MY81F 5′ CAC TTC TAC TGC TAT AAC TTG T 3′ MY82R 5′ CCT TTC CTT TAG GTG TGT 3′	50 bp
HLA-DQα	GH26 5′ GTG CTG CAG GTG TAA ACT TGT ACC AG 3′ GH27 5′ CAC GGA TCC GGT AGC GGT AGA GTT G3′	242 bp

An aliquot of 5 μl of DNA sample was added to 10 μl of PCR reagent mixture consisting of HPV optimal buffer (15 mM KCl, 20 mM Tris–HCl (pH 8.3), 0.2% Triton X-100, nuclease-free bovine serum albumin, $MgCl_2$), 0.3 pmol/μl of each primer, 1 mM each dATP, dCTP, dTTP, dGTP, 3.75 μl 1X SYBR Green and 0.07 U/μl Ampli*Taq* Gold (Roche Molecular Systems, Foster City, CA). The reaction was started with a 10-min incubation at 95°C to activate the Ampli*Taq* Gold followed by 50 cycles of 95°C for 30 s, then 60°C for 30 s and 72°C for 45 s, finally with an additional dissociation stage. The PCR product was verified by electrophoresis on 2% agarose gel. A linear plot of the log of copy numbers vs. numbers of threshold cycles (C_T) was consistently obtained for HPV type 16, 18 and 52 genes, and the correlation coefficient for each target gene was between 0.995 and 1.00 in each run.

Quantitation was done using an external standard curve. The HPV type 16 viral DNA fragment (412 bp) was amplified from genome of the CaSki cell line which derived from HPV-16 integrated human cervical carcinoma [17], and a 415-bp long HPV-18 viral DNA fragment was amplified from the genome of a HeLa cell line derived from HPV-18 integrated human cervical adenocarcinoma [18]. Both CaSki and HeLa cell lines were obtained from the ATCC. A 50-bp-long HPV type 52 viral DNA was amplified from positive clinical samples and confirmed by direct sequencing. The accurate molecular weight and copy numbers of each viral DNA fragment were determined based on UV absorption at a wavelength of 260 nm and then used as the template DNA for establishment of the standard curve. A standard curve with each template at 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 copy numbers was generated in parallel with the clinical samples. We also used CaSki DNA and HeLa DNA to further validate the accuracy of our real-time PCR. In our hand, there were about 10,000 copies of HPV-16 genome estimated in 1.1 ng of CaSki DNA that was consistent with previous report [17]. Both the HPV and HLA-DQα PCR reactions were performed in duplicate. Multiple negative water blanks were included in every analysis.

Primers and probes to a housekeeping gene (HLA-DQα) were run in parallel to standardize the input DNA. Concentrations of blood HPV DNA were expressed as copies of HPV genome/ml of blood and were calculated using a previously described equation [19]. The real-time PCR products were purified and sequenced with the HPV type 16, 18 and 52 specific primers (Table 1) and a DNA sequencing kit and finally analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster, CA). Sequence alignments were obtained using returned results from the GeneBank on-line Blast server (available from URL: http://www.ncbi.nlm.nih.gov/BLAST/).

Differences in proportions were evaluated using Fisher's Exact Test. A P value <0.05 was considered significant. Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS, Chicago, IL).

Results

Both blood and cervical swab specimens from 135 patients with 70 cervical cancers and 65 CIN lesions or controls were obtained for HPV type 16, 18 and 52 DNA. Among the 70 patients, 60 had invasive cervical cancers including 23 IB, 2 IIA, 17 IIB, 5 IIIB, 1 IVB and 12 recurrences, and 10 had microinvasions (IA1). Among the 65 patients, 40 had CIN lesions including 20 CIN III, 10 CIN II and 10 CIN I, and 25 had controls. The mean age of patients with cervical cancer was 53 years old, ranging from 33 to 82 years old. Histological diagnoses included squamous cell carcinoma (n = 56), adenocarcinoma (n = 8) and adenosquamous carcinoma (n = 6). We obtained swab samples from 58 of the total 60 patients with invasive cervical cancer, and 33 out of these 58 patients had a positive test result for HPV DNA type 16, 18 or 52 from cervical swabs. Sixteen out of 33 (48%) patients with cervical cancer were found to have HPV DNA in blood. As shown in Fig. 1A, HPV type 16 DNA was found in cervical swabs of 4% (1/25) of the control group, 0% (0/10) of patients with CIN I, 30% (3/10) with CIN II, 25% (5/20) with CIN III, 50% (5/10) with microinvasion and 36.2% (21/58) with invasive cervical cancer. By contrast, HPV type 18 DNA was found in cervical swabs of 0% (0/25) of the control group, 10% (1/10) of patients with CIN I, 20% (2/10) with CIN II, 0% (0/20) with CIN III, 0% (0/10) with microinvasion and 15.5% (9/58) with invasive cervical cancer. Type 52 DNA was (0/25) detected in none of the cervical swabs of patients in the control group, in 30% (3/10) of swabs from patients with CIN I, 20% (2/10) with CIN II, 30% (6/20) with CIN III, 20% (2/10) with microinvasion and 17.2% (10/58) with invasive cervical cancer.

Of the 60 patients with invasive cervical cancer, 27% (16/60) had positive test results for HPV DNA in blood.

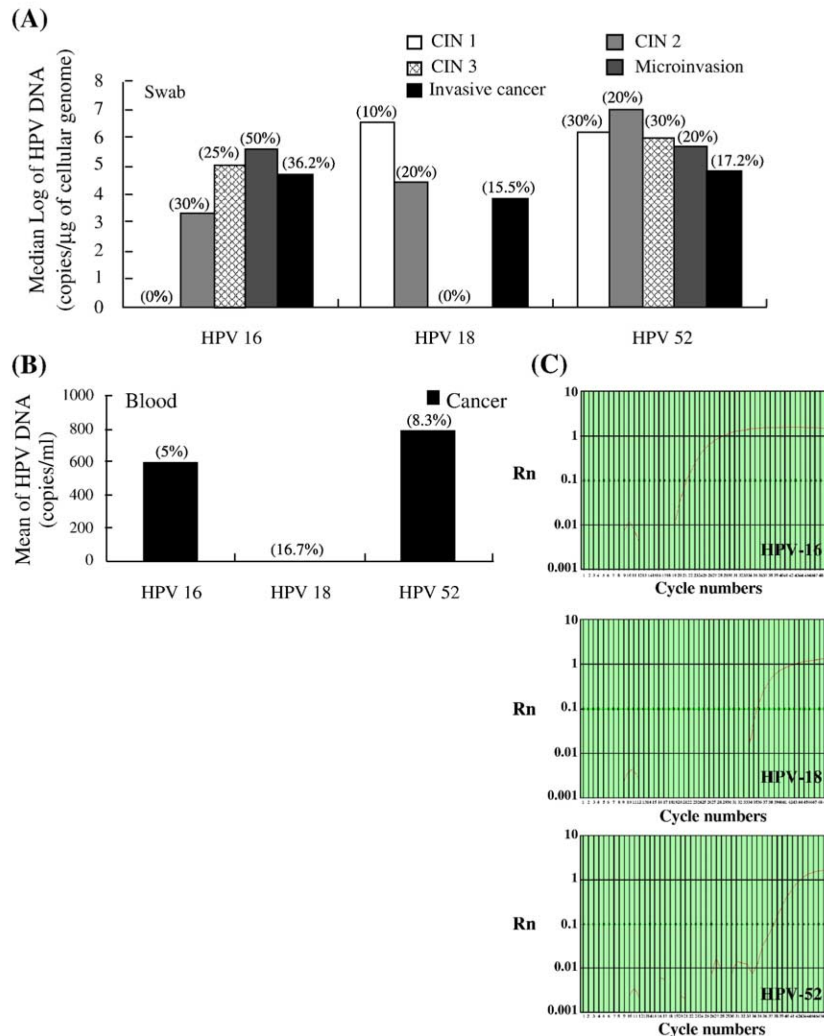

Fig. 1. Dynamic change in concentration (copies/μg of cellular genome) of HPV type 16, 18 and 52 DNA detected in cervical swabs (A, indicated as copies/genome) and blood (B, indicated as copies/ml) specimens. The amount of viral DNA was detected by real-time PCR using a sub-type specific primer. The viral load in blood and the amount of viral DNA in cervical swab samples were plotted for each type of HPV and for different stages of cervical cancer (CIN1, CIN2, CIN3, microinvasion and invasive cancer). The prevalence of each diagnosis is indicated at the top of each column (A). (C) A representative amplification curve for each type of HPV DNA from a patient sample is also shown.

In contrast, no patient with microinvasion (0/10), CIN III (0/20), CIN II (0/10), CIN I (0/10) or controls (0/25) had HPV DNA detected in blood (96% power for CIN, 86% power for controls). Furthermore, five percent (3/60) of patients with invasive cervical cancer had HPV-16, 16.7% (10/60) had HPV-18 and 8.3% (5/60) had HPV-52 DNA detected in blood (Fig. 1). Three percent (2/60) of patients with invasive cervical cancer had multiple types of HPV DNA detected in blood (one had both types 16 and 18, the other types 18 and 52).

The dynamic change in the median log of HPV DNA viral concentration (copies/μg of cellular genome) detected in cervical swab specimens varied for each subtype of HPV, as shown in Fig. 1A. The median values for HPV types 16, 18 and 52 DNA detected in invasive cervical cancer were 1.0×10^5 (range $0.39–5.73 \times 10^6$), 5.9×10^3 (range $295.22–1.20 \times 10^7$) and 8.9×10^5 (range $442.14–3.0 \times 10^9$) copies/μg of cellular genome, respectively. In general, the viral loads of HPV type 16 and 52 DNA increased from CIN to invasive cancer in contrast to that of HPV type 18 DNA, which revealed a fluctuating pattern. The viral load was higher for both HPV type 52 DNA (1.34–3700 copies/ml in blood with mean value of 771.26) and type 16 DNA (1.035–1756 copies/ml with mean value of 586) than for HPV type 18 DNA (0.003–7.94 copies/ml with mean value of 1.8) (Fig. 1B). The viral loads of the different HPV DNA types found in blood and the disease stage and sites are shown in Table 2. The prevalence of HPV DNA detection in blood of cervical cancer patients was not significantly different for each cancer stage ($P > 0.05$; stage IB: 26% vs. IIB: 18% vs. IIIB: 20% vs. IV: 100% vs. recurrence: 45%). All three women with HPV type 16 detected in blood also had HPV type 16 in cervical swabs. Of the ten women who had HPV type 18 detected in blood, six had positive HPV type 18 DNA detected in a cervical swab and one had HPV type 16 detected in a cervical swab, while three had no

Table 2
Detection of HPV-16, -18 or -52 DNA by real-time quantitative PCR

Cases with HPV-positive blood	Age	HPV typing		HPV concentration		Disease stage and site	SCC	Tumor size (cm)
		Blood	Swab	Before	After treatment			
1	63	16	16	1.035	ND	IB	0.3	2.0
2	53	16	16	111.5	1756	R, bone metastasis	0.8	
3	69	16	16	1.83	ND	R, abdominal metastasis	0.4	3.0
		18		ND	<1, 7.94			
4	48	18	18	<1	ND	IB	0.2	4.2
5	43	18	ND	<1	<1	IB, R in abdomen	6.7	4.5
6	56	18	18	<1	ND	R, pelvic metastasis, NED after treatment	0.8	8.0
7	51	18	16	<1	5.07, 0.32	IIB	2.2	5.0
8	78	18	18	<1	ND	IB	1.8	3.0
9	53	18	ND	1.17	<1	R, bone metastasis Adeno CA		
10	36	18	18	<1	2.64	IB, R in pelvis	1.9	3.1
11	56	18	18	6.0	ND	IB	5.6	3.0
12	57	52	52	689	3700, 584, 92, ND	IIB, LN metastasis	4.0	6.0
13	56	52	16, 52	1.34	38	IIIB, R in neck LN metastasis	5.8	7.0
14	47	52	52	8.2	ND	IB Adeno CA	3.0	
15	68	52	52	69	ND	IIB, persistent, abdominal metastasis	5.3	5.0
		18	18	ND	<1			
16	84	52	52	17.8	77.75	IVB, persistent	4.5	5.5

ND: not detectable; R: recurrence; NED: no evidence of disease; LN: lymph node.

evidence of HPV in cervical swabs, including one with bone metastasis without local recurrence. Of the five women with HPV-52 detected in blood, four also had type 52 viral DNA detected in a cervical swab, and one had both HPV type 16 and 52 DNA detected in a cervical swab. Seven of the 12 HPV-positive blood samples from primary invasive cervical cancer patients had bulky tumors (>4 cm). Six of the 12 HPV-positive blood samples from invasive cervical cancer patients showed abnormal SCC levels (>2.5 ng/ml).

Among the 16 patients with HPV DNA detectable in blood before treatment, seven had stage IB disease, three had stage IIB, one had stage IIIB, one had stage IVB, three had stage IB disease with distant recurrence (two had bone metastases and the other had abdominal metastases) and one had stage IIB with pelvic recurrence. HPV DNA was detected in blood during follow-up in the three patients of stage IB disease with distant recurrence. One patient with stage IIB disease with pelvic recurrence had undetectable HPV DNA in blood after treatment and was free of disease during follow-up. Among the remaining 12 patients with invasive cervical cancer, three patients (two initially had stage IB, and one initially had stage IIIB disease) developed recurrence at 5 months, 6 months and 8 months after treatment, respectively; in addition, the other two had persistent disease (one initially had stage IIB with para-aortic lymph node metastasis confirmed by fine needle biopsy under CT guidance, and the other initially had stage IVB disease). HPV was detected in blood after treatment of all 5 patients. At 3 months after treatment, eight out of ten patients with cervical cancer who had HPV DNA detected in blood had recurrence (including two with persistent disease). Among these eight patients, seven (87.5%) had distant metastases. The site of distant metastasis was bone in two, abdomen in three, neck lymph nodes in one and vulva in one. Of the remaining two patients, one was negative for HPV DNA in blood 9 months after treatment and was clinically free of disease, while HPV DNA was still detected in blood in the other patient who was clinically free of disease at the last follow-up visit. The other six cervical patients with negative HPV DNA results in blood after treatment showed no recurrence until December 31, 2004, a median follow-up duration of more than 18 months.

Discussion

The results of our study confirm that HPV DNA can be detected and quantified using real-time PCR from blood samples of patients with invasive cervical carcinoma. Circulating HPV DNA was not present in controls with benign tumors or patients with CIN lesions or microinvasive cervical cancers. Moreover, type 52 HPV DNA was prevalent in the blood of 8.3% and in the cervical swab of 17.2% of patients with invasive cervical cancer. We used unfractionated whole blood to test for HPV DNA because it combines all blood components that can harbor it. This might provide a more accurate estimate of the absolute viral loads in the blood circulation of patients. At the beginning of the study, we compared HPV viral loads between plasma and whole blood but found no significant difference between these two measurements (data not shown). Use of whole blood has the additional advantage of being collected simply and in a uniform specimen size.

Only one study using real-time PCR for detection and quantitation of HPV DNA in the plasma of patients with cervical cancer has been previously reported. In that study, Dong et al. found HPV-16 or HPV-18 E7 DNA in only 6.9% of invasive cervical cancers by conventional PCR [9]. The authors noted that the major limitations of their study included the retrospective nature of the analysis in which it subjected to selection bias and lack of detailed follow-up information. Here, we provide data from a prospective study with HPV DNA detection at the time of diagnosis and at follow-up after treatment. Because low viral loads of HPV DNA in blood may be detectable by real-time PCR, use of this method may improve detection rates compared to conventional PCR. More than one-fourth (27%) of patients with invasive cervical cancer had HPV DNA detected in their blood samples. Approximately 50% of patients with confirmed HPV type 16, 18 or 52 DNA positive cervical cancers had HPV DNA detected in their blood using our assay system. These results suggest that our assay is superior to those of other reports [9,11,12]. Moreover, this study also provides serial follow-up data on HPV DNA viral load in cervical cancer patients after treatment suggesting its prognostic significance.

Although the exact pathway by which tumor DNA is released into the bloodstream remains unclear, its presence and quantity are likely to be a reflection of tumor load or metastasis and may have prognostic value for patients with cancer [20–22]. Because the life cycle of HPV occurs entirely within epithelial tissues, the virus is not usually found in the bloodstream. A possible hypothesis to explain the detection of circulating viral DNA in cervical cancer patients may be due to the lysis of circulating cancer cells (necrosis or apoptosis) or micrometastases (active release of tumor DNA) shed by the tumor [23]. Circulating cervical cancer cells have been found by analyzing HPV-specific mRNA in peripheral blood cells of advanced stage cervical cancer patients with metastasis [24]. Detection of HPV DNA in blood may be an early sign of distant cervical cancer metastasis. Thus, even in the absence of active cell shedding, DNA and proteins could be translocated from neoplastic cells to a distant site via the bloodstream.

In our study, six cervical cancer patients negative for HPV DNA after treatment showed no recurrence during follow-up. During follow-up, eight out of ten cervical cancer patients with HPV DNA detected in the blood at 3 months after treatment had either recurrence or failure of treatment. Among them, seven of eight patients had distant metastases. There was no significant difference in HPV DNA detection rates in the early or advanced stage at the time of diagnosis. Besides, three of four patients with distant metastasis did not have increased tumor markers at the time of diagnosis. These findings may suggest that distant metastasis is related to persistent detection of HPV DNA after treatment, supporting the idea that circulating HPV DNA may play a crucial role in metastasis. Therefore, detection of HPV DNA in blood could be a specific, although not very sensitive, genetic marker that has a strong association with cervical cancer metastasis. Detection of circulating HPV DNA after treatment could predict recurrence. We postulate that detection of HPV DNA in blood might be a useful marker to select subsets of patients who need more aggressive treatment.

Cervical cancer is the most common female gynecologic cancer in Taiwan and has a high mortality rate. With HPV vaccination studies currently underway, it is important to map the epidemiology of HPV infection in populations worldwide to facilitate the implementation of future prevention programs. Previous studies have reported that HPV types 52 and 58 seem to play a more prominent role in cervical cancer in Asia than HPV types 31, 33 and 45, which are more common in western countries [15,16,19,25]. The distribution of various HPV types among our patients revealed a more prominent role of HPV-52 in Taiwan than in other parts of the world. Among our patients, HPV-16 (36.2%), 18 (15.5%) and 52 (17.2%) were the most common HPV types in invasive cervical cancers, together accounting for almost 70% of the samples. Moreover, HPV type 52 DNA was detected in the blood of 8.3%, HPV type 16 in 5% and HPV type 18 in 16.7% invasive cervical cancer patients. The high prevalence of HPV-52 in the blood was unexpected. Our assay was also validated with HPV-52 E6 primer (data not shown), confirming that there was no misclassification. In general, the viral load of HPV type 16 and 52 increased as cancer stage increased from CIN to invasive cancer. This was in contrast to findings for HPV type 18 DNA which revealed fluctuating loads with cancer stage. Our clinical data revealed that two patients with invasive cervical cancer and heavy viral loads of HPV type 52 DNA ($>10^7$ copies/genome) died within 8 months. Three patients with HPV type 52 DNA detected in blood before treatment were found to have undetectable blood levels after treatment and were clinically free of disease at the last follow-up. Of the two patients with HPV type 52 DNA detected in blood after treatment, one initially had stage IIIB disease with neck lymph nodes recurrence, and the other had persistent stage IVB disease.

The detection rate for HPV type 18 DNA in our study was higher than that of previous reports [9,12]. In our patients, most of the detectable viral loads for HPV type 18 DNA were very low (<1 copy/ml). We confirmed all positive real-time PCR products including those for HPV type 18 by direct sequencing and testing them with CaSki and HeLa cell lines bearing a known amount of HPV genomes. We found that six of ten patients with HPV type 18 DNA detectable in blood developed in recurrence.

In this study, we developed an assay to detect and quantitate viral loads of HPV DNA using real-time PCR, which is a rapid, sensitive and specific method. The same HPV DNA type was detected in the blood and genital tract of 12 of 16 (75%) women whose blood specimens were

either HPV type 16, 18 or 52 DNA positive. The reason for the discrepancy between findings for cervical swabs and blood samples observed in the remaining 4 women whose blood specimens were HPV type 18 DNA positive could not be determined. However, we confirmed our data using different primers and methods. One possible reason for this discrepancy may be that virus from the genital tract was eliminated after treatment or it may be that only one type of viral load was detected in the genital tract in patients with multiple latent infections of different HPV types. Dong et al. also reported that the same HPV type was detected in the plasma and genital tract of 9 of 14 (64.3%) women whose plasma specimens were HPV-16/18 positive [9]. Contamination was considered unlikely because all HLA-DQα gene negative blood samples were also HPV DNA negative. All PCR negative controls were also negative.

There are a number of limitations to the present analysis. First, the patients were studied for a limited period of time, and the sample size was small. Extended follow-up and study of greater numbers of patients may be required before a definite conclusion regarding the usefulness of blood HPV DNA to detect tumor recurrence and its impact on the survival of patients can be reached.

Acknowledgment

This research was supported by the Research Fund (mr9201) from Cathay General Hospital, Taipei, Taiwan, ROC.

References

[1] IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans: Human Papillomaviruses, vol. 64. Lyon: International Agency for Research on Cancer; 1995.

[2] Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al. Human papillomavirus is a necessary cause of invasive cancer worldwide. J Pathol 1999;189:12–9.

[3] Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International Biological Study on Cervical Cancer (IBSCC) study group. J Natl Cancer Inst 1995;87:796–802.

[4] Stroun M, Maurice P, Vasioukhin V, Lyautey J, Lederrey C, Lefort F, et al. The origin and mechanism of circulating DNA. Ann NY Acad Sci 2000;906:161–8.

[5] Anker P, Mulcahy H, Chen XQ, Stroun M. Detection of circulating tumor DNA in the blood (plasma/serum) of cancer patients. Cancer Metastasis Rev 1999;18:65–73.

[6] Nawroz H, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med 1996;2:1035–7.

[7] Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, et al. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res 2000;60:892–5.

[8] Capone RB, Pai SI, Koch WM, Gillison ML, Danish HN, Westra WH, et al. Detection and quantitation of human papillomavirus (HPV) DNA in the sera of patients with HPV-associated head and neck squamous cell carcinoma. Clin Cancer Res 2000;6:4171–5.

[9] Dong SM, Pai SI, Rha SH, Hildesheim A, Kurman RJ, Schwartz PE, et al. Detection and quantitation of human papillomavirus DNA in the plasma of patients with cervical carcinoma. Cancer Epidemiol Biomark Prev 2002;11:3–6.

[10] Widschwendter A, Blassnig A, Wiedemair A, Muller-Holzner E, Muller HM, Marth C. Human papillomavirus DNA in sera of cervical cancer patients as tumor marker. Cancer Lett 2003;202:231–9.

[11] Pornthanakasem W, Shotelersuk K, Termrungruangleert W, Noravud N, Niruthisard S, Mutirangura A. Human papillomavirus DNA in plasma of patients with cervical cancer. BioMed Central Cancer 2001;1:2.

[12] Liu vW, Tsang P, Yip A, Ng TY, Wong LC, Ngan HY. Low incidence of HPV DNA in sera of pretreatment cervical cancer patients. Gynecol Oncol 2001;82:269–72.

[13] Josefsson AM, Magnusson PK, Ylitalo N, Sorensen P, Qwarforth-Tubbin P, Andersen PK, et al. Viral load of human papillomavirus 16 and risk of cervical carcinoma in situ: a nested case-control study. Lancet 2000;355:2189–93.

[14] Ylitalo N, Sorensen P, Josefsson AM, Magnusson PK, Andersen PK, Ponten J, et al. Consistent high viral load of human papillomavirus 16 and risk of cervical carcinoma in situ: a nested case-control study. Lancet 2000;355:2194–8.

[15] Liaw KL, Hsing AW, Chen CJ, Schiffman MH, Zhang TY, Hsieh CY, et al. Human papillomavirus and cervical neoplasia: a case-control study in Taiwan. Int J Cancer 1995;62:565–71.

[16] Huang S, Afonina I, Miller BA, Bechmann M. Human papillomavirus types 52 and 58 are prevalent in cervical cancers from Chinese woman. Int J Cancer 1997;70:408–11.

[17] Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA, Howley PM. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J Virol 1987; 61:962–71.

[18] Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau A, et al. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature 1985;314:111–4.

[19] Sasagawa T, Basha W, Yamazaki H, Inoue M. High-risk and multiple human papillomavirus infections associated with cervical abnormalities in Japanese women. Cancer Epidemiol Biomark Prev 2001;10:45–52.

[20] Castells A, Puig P, Mora J, Boadas J, Boix L, Urgell E, et al. *K-ras* mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: diagnostic utility and prognostic significance. J Clin Oncol 1999;17:578–84.

[21] Sidransky D. Circulating DNA: what we know and what we need to learn. Ann NY Acad Sci 2000;906:1–4.

[22] Jen J, Wu L, Sidransky D. An overview on the isolation and analysis of circulating tumor DNA in plasma and serum. Ann NY Acad Sci 2000;906:8–12.

[23] Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 2001;61:1659–65.

[24] Tseng CJ, Pao CC, Lin JD, Soong YK, Hong JH, Hsueh S. Detection of human papillomavirus types 16 and 18 mRNA in peripheral blood of advanced cervical cancer patients and its association with prognosis. J Clin Oncol 1999;17:1391–6.

[25] Clifford GM, Smith JS, Plummer M, Munoz N, Franceschi S. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. Br J Cancer 2003;88:63–73.