Detection and Quantitation of Human Papillomavirus DNA in the Plasma of Patients with Cervical Carcinoma[1]

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Abstract

Human papillomaviruses (HPVs) play a central role in the development of cervical carcinoma. Plasma DNA from 232 patients taken at diagnosis or after treatment for invasive cervical cancer ($n = 175$) or carcinoma *in situ* ($n = 57$) and 60 normal controls were examined for HPV-16 or HPV-18 *E7* DNA by conventional and real-time quantitative PCR assays. We found HPV-16 or HPV-18 *E7* DNA in 6.9% (11 of 175) of invasive cervical cancer cases (18.1% of cases positive for HPV-16 or HPV-18 at the genital tract), 1.8% (1 of 57) of carcinoma *in situ*, and 1.7% (1 of 60) of normal controls by conventional PCR. Quantitative PCR identified the highest concentrations of HPV DNA (copy number of HPV/ml of plasma) in patients with invasive cervical cancer (mean, 11,163; median, 183.5), followed by a level of 8 in the single carcinoma *in situ* case and 0 copies in the normal control initially positive by conventional PCR. HPV DNA can be detected in the plasma of some patients with HPV-positive cervical tumors. It remains to be demonstrated whether quantitative PCR analysis of HPV DNA in plasma may have utility in patients at high risk of recurrent disease.

Introduction

Cervical cancer is the third most common cancer in women worldwide (1). Several lines of evidence indicate that high-risk HPV[3] infection is an important event in the progression of cervical cancer. Multiple epidemiological studies identified a consistent association of HPV infection with the development of cervical cancer (2). The discovery and molecular cloning of HPV-16 and HPV-18 from cervical cancers and subsequent demonstration of these viral DNAs in most cases of cervical cancer established a biological link between the virus and this disease (3, 4). Moreover, two HPV gene products, E6 and *E7*, inactivate the *p53* and *Rb* gene products, respectively, and are consistently retained and expressed in cervical cancers (5–7). These gene products of oncogenic HPV-16 and HPV-18 are known to play an important role in the early stages of malignant transformation and immortalization of cervical epithelial cells (7–9). All of these observations point to an important and even essential role for HPV in carcinogenesis of the uterine cervix.

Circulating tumor DNA has been identified in the serum and plasma of cancer patients (10, 11). This tumor-specific DNA can be detected in the serum or plasma via specific genetic and epigenetic alterations found in the primary tumor (12, 13). Although the exact pathway by which tumor DNA is released into the bloodstream remains unclear, the presence of circulating tumor DNA is likely to be a reflection of tumor load or metastasis and may have prognostic value for the cancer patient (14–16). Similarly, recent studies have documented the presence of viral sequences as a surrogate for circulating tumor DNA in the peripheral blood of certain groups of cancer patients. EBV DNA was detected in the serum and plasma of patients with NPC (17, 18). HPV mRNA was detected in the peripheral blood of advanced cervical cancer patients with metastasis who were also positive for HPV DNA in the cervical cancer tissues (19). Additionally, Lo *et al.* (17) quantified EBV DNA in the plasma of NPC patients and correlated these findings with NPC stage. A recent study by Capone *et al.* (20) detected and quantified HPV DNA in the sera of patients with HPV-associated head and neck cancer. Furthermore, several studies have suggested that HPV viral load could be an important factor for progression from HPV infection to cervical cancer (21, 22).

In light of these finding, we examined plasma from a large subgroup of women enrolled in a multicenter, case-control study of cervical cancer for the presence of HPV DNA by PCR. Moreover, we used a real-time PCR assay to investigate whether the viral load of HPV DNA in the plasma correlated with the type of cervical lesion present and/or the stage of cervical cancer. Real-time quantitative PCR can distinguish invasive cancer cases from *in situ* lesions and normal controls.

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[3] The abbreviations used are: HPV, human papillomavirus; NPC, nasopharyngeal cancer.

Materials and Methods

Sample Collection and DNA Isolation. A multicenter, case-control study of cervical adenocarcinoma and other glandular tumors ($n = 195$), cervical squamous carcinomas ($n = 234$), and population controls ($n = 307$) was conducted between July 1994 and March 1996, as described in detail previously (23, 24). From among this population, plasma specimens from 292 participants were selected for the present investigation. These 292 individuals were selected to include all HPV-16- and HPV-18-positive controls ($n = 18$), a random sample of the remaining HPV-negative controls ($n = 42$), all HPV-positive *in situ* cases ($n = 37$), a sample of the remaining *in situ* cases for whom HPV status was uncertain ($n = 20$), and a random sample of 175 invasive cases. HPV typing was performed by testing cervical cells collected into STM (Digene Corp., Gaithersburg, MD) using PCR-based methods, as described previously by Lacey *et al.* (23). Blood samples were collected at the time of the interview. Because many cases were recruited retrospectively, some blood samples were collected before (25.4%) and others after (66.0%) treatment. 8.6% of cases, all of them *in situ*, had unknown timing of blood collection. One ml of the plasma samples was used for DNA extraction, and DNA was prepared as described previously (12). Briefly, plasma samples were digested in SDS and proteinase K at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation of DNA.

PCR Amplification. Purified genomic DNA was amplified by PCR for the *HPV-16* and *HPV-18 E7* genes as well as for an internal reference gene, *β-globin*. Oligonucleotide primers were as follows: *HPV-16 E7*, 5′-ATTAAATGACAGCTCAGAGGA-3′ (sense) and 5′-GCTTTGTACGCACAACCGAAGC-3′ (antisense); and *HPV-18 E7*, 5′-AAGAAAACGATGAAATAGATGGA 3′ (sense) and 5′-GGCTTCACACTTACAACACA-3′ (antisense). Primers for *β-globin* were 5′-GAAGAGCCAAGGACAGGTAC-3′ (sense) and 5′-CAACTTCATCCACGTTCACC-3′ (antisense). DNA made from a confluent culture of the human cervical carcinoma cell lines SiHa (HPV-16) and CII4 (HPV-18; American Type culture Collection, Manassas, VA) was used as a positive control for each HPV subtype.

Each PCR reaction was performed under standard conditions in a 20-μl volume containing 5 μl of template DNA, 2.5 μM each primer, 50 μM deoxynucleotide triphosphate, 3.75 mM $MgCl_2$, 1.25 unit of Ampli Taq Gold (Perkin-Elmer, Foster City, CA), and 2 μl of 10× PCR Gold buffer (Perkin-Elmer). Reactions were denatured for 10 min at 95°C and incubated for 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, followed for 10 min at 72°C). Each PCR reaction included the following controls: (*a*) specimen DNA integrity controls (*β-globin*) amplification; (*b*) positive controls, SiHa cells (HPV-16) and CD4II cells (HPV-18); and (*c*) negative controls, K5622 cells. Reactions were analyzed on Spreadex EL 400 gels in the SEA 2000 Apparatus System (Elchrom Scientific, Cham, Switzerland), stained with SYBR Gold (Molecular Probes, Eugene, OR), and visualized under UV illumination.

Real-Time Quantitative HPV DNA PCR. Plasma HPV DNA concentrations were measured using a real-time quantitative PCR system. Real-time PCR reactions were set up in a reaction volume of 25 μl using the TaqMan Universal PCR Master Mix (Perkin-Elmer). HPV-16 *E7* primers and probe were used as described previously (20). HPV-18 *E7* primers and probe were designed using the primer Express Software (Perkin-Elmer). Fluorogenic probe for HPV-18 *E7* was 5′-(FAM)-CCCCAAAGGACTCAAAGAACCT-(TAMRA)-3′ (Synthetic Genetics, San Diego, CA). PCR primers of HPV-18 *E7* were 5′-TGTATTGCATTTAGAGCCCCAA-3′ (sense) and 5′-CTTCCTCTGCGCTTAATTGC-3′ (antisense; Life Technologies, Inc., Gaithersburg, MD). As an internal positive control, real-time PCR analysis was performed on the *β-globin* gene in parallel. The *β-globin* gene primers/probes used were as described previously (25). DNA amplifications were carried out in a 96-well reaction plate format in a PE Applied Biosystems 7700 Sequence Detector (Perkin-Elmer). Both the HPV and *β-globin* PCR reactions were carried out in triplicate. Multiple negative water blanks were included in every analysis.

Standard curves were run in parallel with each analysis using DNA extracted from HPV-positive cell lines [SiHa (HPV-16, 20 copy/μl) and C4II (HPV-18, 20 copy/μl)]. SiHa and C4II DNA was diluted to provide from 1 to 100 copies of HPV-16 and HPV-18.

Concentrations of circulating cell-free HPV DNA were expressed as copies of HPV genome/ml of plasma and were calculated using the following equation (26):

$$C = Q \times \frac{Vdna}{Vpcr} \times \frac{1}{Vext}$$

in which C represents the target concentration in plasma, expressed as copies/ml; Q represents the copy number as determined by the sequence detector; *Vdna* represents the total volume of DNA obtained after DNA extraction (50 μl); *Vpcr* represents the volume of DNA used for the PCR reaction (5 μl); and *Vext* represents the volume of plasma used to extract the DNA. Samples were run in triplicate, and a SD was calculated.

Results

We performed a case-control study on a subset of participants from a large multi-institutional study aimed at studying the relations between HPV infection, other exogenous factors, and cervical carcinoma. Plasma samples ($n = 292$) from this trial, including 175 cases of invasive cervical cancers, 57 cases of carcinoma *in situ*, and 60 normal controls, were first examined for the presence of HPV-16 or HPV-18 DNA, using conventional PCR with type-specific oligonucleotide PCR primers. HPV genomic material, using *E7*-specific primers of HPV-16 or HPV-18, was detected in 14 of 292 individuals (4.8%). Twelve of 175 (6.9%) cases with invasive cervical cancer (8 HPV-16 and 4 HPV-18), 1.8% (1 of 57) of carcinoma *in situ* (HPV-18), and 1.7% (1 of 60) of normal controls (HPV-16) were positive (Table 1). The distribution of HPV-positive plasma by conventional PCR was not statistically significantly different among women by diagnosis ($P = 0.19$ by Fisher's exact test). Of the 9 women positive for plasma HPV-16, 6 had evidence of HPV-16 in the genital tract, 1 had evidence of HPV-18, 1 had evidence of HPV types other than 16/18, and 1 was negative for HPV in the genital tract. Of the 5 women positive for plasma HPV-18, 1 had evidence of HPV-16, 3 of HPV-18, and 1 of other HPV subtypes in the genital tract.

In addition, quantitative analysis of HPV DNA was performed on all 14 plasma samples positive for HPV by conventional PCR and 28 negative plasma samples by conventional PCR. Using real-time PCR, varied levels of circulating HPV DNA were detected in 12 plasma samples, but two cases initially positive and all 28 cases initially negative by conventional PCR were negative (*i.e.*, 0 copies). This included an initially positive control (Table 1). The concentration of HPV DNA (copies/ml of plasma) was higher in invasive cervical cancer (mean, 11,163; median, 183.5) than in the single positive

Table 1 Detection of HPV-16 or HPV-18 by real-time quantitative PCR assay

Case of HPV-positive serum	Real-time quantitative PCR	Mean value (HPV copies/ml) ± SD	Histological type
1	*		Normal control
2	*		Invasive cervical cancer
3	HPV-18	8 ± 2	Carcinoma *in situ*
4	HPV-18	6,875 ± 2,704	Invasive cervical cancer
5	HPV-18	950 ± 364	Invasive cervical cancer
6	HPV-18	21,250 ± 8,101	Invasive cervical cancer
7	HPV-18	205 ± 76	Invasive cervical cancer
8	HPV-16	150 ± 59	Invasive cervical cancer
9	HPV-16	100,000 ± 3,8168	Invasive cervical cancer
10	HPV-16	4,100 ± 1,729	Invasive cervical cancer
11	HPV-16	26 ± 9	Invasive cervical cancer
12	HPV-16	104 ± 41	Invasive cervical cancer
13	HPV-16	129 ± 49	Invasive cervical cancer
14	HPV-16	162 ± 60	Invasive cervical cancer

* Negative.

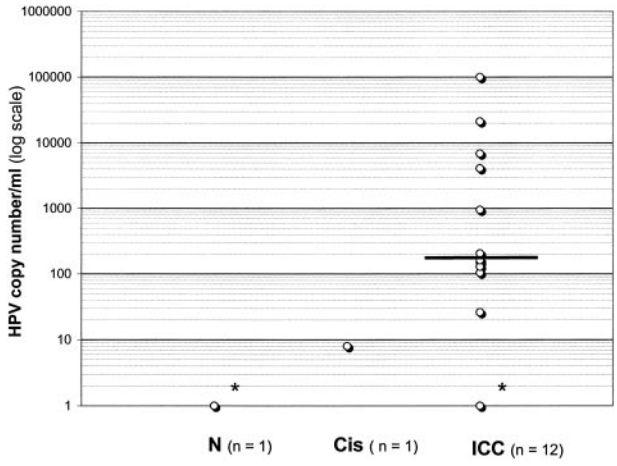

Fig. 1. Plasma HPV DNA concentrations in patients initially positive by standard PCR. Plasma HPV DNA concentrations (copies of HPV/ml of plasma) as determined by real-time quantitative PCR are plotted on the *Y axis* (common logarithmic scale). *, negative (*i.e.*, 0 copy); *Bar*, median value; *N*, normal control; *Cis*, carcinoma *in situ*; *ICC*, invasive cervical cancer.

carcinoma *in situ* case (mean, 8; Fig. 1). When the analysis was restricted to cases and controls known to be infected with HPV-16 or HPV-18 at the cervix/vagina (18 normal controls, 23 carcinomas *in situ*, and 55 cancers), the distribution of plasma positives was as follows: 5.6% of normal controls, 0% of carcinomas *in situ*, and 18.2% among invasive cervical cancers ($P = 0.04$ by Fisher's exact test).

Among the 175 invasive cancer cases, plasma HPV-16/18 *E7* DNA was detected in 9.6% of cases sampled before treatment compared with 5.7% of those sampled after treatment ($P = 0.34$ by Fisher's exact test). No clear pattern was discerned when the proportion of HPV-16/18 *E7* DNA positives was examined by stage at diagnosis among invasive cancer cases (4.7% for stage I, 20.0% for stage II, and 7.7% for stage III/IV; $P = 0.14$ by Mantel-Haenszel test for trend). However, among those positive for plasma HPV-16/18 *E7* DNA, viral load was directly correlated with clinical stage at diagnosis. The average viral load among those diagnosed with stage I was 235.7 (median, 116.5) compared with 7209.3 for stage II (median, 3518.5) and 100,000 for the single positive woman diagnosed with stage III disease.

HPV-16/18 *E7* DNA was detected more frequently in the plasma of those diagnosed with squamous cell carcinoma (10.4% for those diagnosed with squamous tumor compared with 4.1% for those diagnosed with adenocarcinomas and other tumors with a glandular component; $P = 0.13$ by Fisher's exact test). This pattern persisted in analyses restricted to the 55 invasive cancer cases known to be positive at the cervix for HPV-16 and/or HPV-18 ($P = 0.16$ by Fisher's exact test; data not shown).

Discussion

Viral agents are known to be involved in the etiology of several human epithelial tumors. The initiation of cervical cancer by HPV has been well established, as has EBV as a major cause of NPC. Viral DNA has been detected as a component of tumor DNA in the circulation of patients with primary tumors closely linked to viral infection. Elevated levels of EBV DNA were documented in the plasma and serum of patients with NPC (17, 18). The presence of HPV genomic material was detected recently in the serum DNA of HPV-positive head and neck squamous cell carcinoma patients (20). In this study, HPV DNA was detected in the plasma of a subset of patients with cervical cancer. The same HPV type was detected in the plasma and genital tract for 9 of 14 (64.3%) women whose plasma specimens were HPV-16/18 positive. The discrepancy observed between HPV types detected in plasma and the genital tract for the remaining 5 women is unexpected, and we do not have a good explanation for it. Among the other things, it could reflect misclassification of viral type or viral load reductions/elimination of virus at the genital tract that resulted from treatment. Approximately 1 of 5 patients with confirmed HPV-16- or HPV-18-positive cervical cancer was positive by our assay. This percentage might be an underestimate because HPV DNA from other HPV types involved in cervical cancer pathogenesis were not examined, and some participants had blood samples taken after definitive therapy. Thus, this report represents a feasibility study, confirming the ability to detect plasma HPV DNA in some patients with cervical cancer. As such, there are no clinical implications with regard to patient diagnosis or prognosis at present.

The data obtained using the real-time PCR assay were in excellent concordance with conventional PCR results (86%). The real-time assay combines the sensitivity of a PCR reaction with the specificity of a Southern blot analysis. The quantitative nature of the real-time PCR assay also permits evaluation of viral load in the bloodstream. Recent quantitative studies have shown that EBV DNA viral load may be an invaluable tool in monitoring tumor recurrence among patients treated for NPC (26). Likewise, HPV DNA in plasma may be useful to monitor HPV-positive cervical and head and neck cancer patients. Among the 12 positive cases by real-time PCR in our study, we found a consistently increased concentration of HPV (copies/ml of plasma) only in invasive cancer cases. The single positive *in situ* case had a very low concentration of HPV detected (8 copies/ml of plasma), whereas the single normal control initially positive was found to be negative in our quantitative assay (0 copies/ml of plasma). Moreover, nonstatistical differences in HPV DNA detection were observed in invasive cancer cases when pretreatment and posttreatment rates were compared (9.6% *versus* 5.7%). Although no definitive conclusions can yet be drawn, HPV DNA can now be added to an increasing array of circulating tumor DNA markers for various neoplasms, in-

cluding oncogene mutations, microsatellites, and methylation (12, 27, 28).

Among invasive cancer cases, although clinical stage at diagnosis did not correlate with the proportion of cases positive for plasma HPV-16/18 *E7* DNA, a tendency was observed among those positive for HPV-16/18 *E7* DNA for viral load to increase with increasing clinical stage at diagnosis. Also of note is our observation that HPV-16/18 *E7* DNA was detected 2.5 times more likely in the plasma of cases diagnosed with squamous cell carcinomas compared with cases diagnosed with adenocarcinoma and other tumors with a glandular component. These results are not easily explained and should be interpreted with caution, given our modest sample size. If true, however, this finding might point to important differences between these two histologically distinct forms of cervical cancer.

Major limitations of our study include the retrospective nature of the analysis subject to selection bias and lack of follow-up data on our cases. Validation of these data in a prospective study with HPV DNA detection at a single time point (*e.g.*, at diagnosis) is mandatory. Further studies are also needed to determine whether HPV DNA viral load detectable among cervical cancer cases after treatment is predictive of recurrence.

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