

Human Papillomavirus and Disease Status Following Therapy for Cervical Cancer

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We enrolled 85 patients with invasive cervical cancer and collected cervicovaginal lavage samples at each clinical visit for diagnosis, staging, treatment, and follow-up. Lavage samples were tested by L1 consensus polymerase chain reaction for human papillomavirus (HPV). Results were compared with HPV demonstrated in tumor tissue and the clinical status at time of sample collection. Sensitivity and specificity of the lavage for detection of tumor HPV, determined on the basis of results of tests on lavage samples collected prior to therapy, were found to be 56% and 76.9%, respectively. The proportion of lavage samples detecting tumor HPV decreased significantly with treatment, from 0.54 at diagnosis to 0.03 at complete response ($P < .001$). Local treatment failure was associated with increased detection of tumor HPV; however, no samples were positive prior to clinically detected treatment failure. These results suggest that cervicovaginal lavage is not an effective sampling method for epidemiological analysis of HPV in cervical tumors.

Cervical cancer remains a significant public health concern in the United States. While the incidence of invasive cervical cancer has decreased, largely because of secondary prevention by cytological screening, stage-specific cervical cancer death rates have not substantially changed over the last 2 decades. Most patients who receive curative therapy achieve at least an initial complete clinical response; however, there is no effective treatment for cancer unresponsive to initial therapy or for recurrent cervical cancer. In most instances these patients will die of their cervical tumors. If treatment failures could be detected earlier than current clinical methods permit, perhaps salvage therapy would be more successful.

This study was designed to determine whether detection of human papillomavirus (HPV) in cervicovaginal lavage samples was associated with the clinical status of cervical disease for patients with invasive cervical cancer at diagnosis, as well as during therapy and follow-up. The hypothesis was that tumor cells collected in lavage samples would contain HPV DNA detectable with a sensitive PCR assay. HPV DNA in lavage

specimens could then be a useful marker for monitoring patients during therapy to detect minimal residual disease and early recurrence.

Materials and Methods

Patient Population

All patients with cervical cancer of FIGO (International Federation of Gynecology and Obstetrics) stage Ia or greater that had been diagnosed since June 1986 and who attended the Grady Memorial Hospital and Clinics (GMHC) between April 1992 and February 1995 were asked to participate in this study. Once a patient was enrolled, clinical data and specimens were collected at each subsequent routine visit for treatment or follow-up. The patient's disease status at each visit was determined by physical examination, supplemented by radiological and histologic studies as appropriate, and was classified as follows: (1) diagnosis (DX), tumor prior to treatment; (2) partial response (PR), decrease in size of tumor during radiotherapy; (3) complete response (CR), no evidence of tumor during or following treatment; or (4) treatment failure (TF), tumor (confirmed by biopsy) following curative radiotherapy or recurrence after complete response.

Tissue Specimens

Records of the GMHC Anatomic Pathology Department were used to identify all tissue samples from each patient. One of us (E.R.U.) reviewed all histologic material related to cervical cancer before, during, and after treatment. Analysis for HPV was performed on one formalin-fixed paraffin-embedded block of tumor obtained prior to therapy. Serial sections, each 5 μ , were cut for PCR and in situ hybridization, with hematoxy-

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All study participants were volunteers who gave informed consent. Complete study protocols were approved by the Emory University and Centers for Disease Control and Prevention Human Subjects Committees. Human experimentation guidelines of the U.S. Department of Health and Human Services were followed in the conduct of this study.

None of the authors have commercial or other associations that might pose a conflict of interest.

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lin and eosin staining of initial, middle, and final sections to verify that recut sections were representative of tumor histology. A new disposable microtome blade was used for each block to minimize the potential for HPV DNA cross-contamination.

Lavage Specimens

We used cervicovaginal lavage during pelvic examination as a noninvasive means of sampling the lower genital tract at each visit [1]. This provided exfoliated cells of all areas of the lower genital tract (normal as well as tumor). After visualization of the cervix or vaginal apex with a disposable plastic speculum, the cervix and vagina were irrigated with 10 mL of normal saline. The irrigating fluid was collected with gentle aspiration, and the resulting lavage sample was stored within 2 hours of collection at -70°C . Prior to HPV testing, the samples were thawed and cells were concentrated by low-speed centrifugation. Cells were resuspended at 1:10 dilution (volume:volume) in PBS. In the rare instances that the estimated pellet volume was $<50\ \mu\text{L}$, we added $450\ \mu\text{L}$ of saline. A $50\text{-}\mu\text{L}$ aliquot of the resulting suspension was processed for PCR. The remainder of the sample was refrozen for long-term storage. PCR testing was performed as samples accumulated, in batches of 12.

HPV DNA Detection

In situ hybridization. Detection of HPV DNA in tissue sections was achieved with colorimetric *in situ* hybridization adapted for automation as previously described [2]. In brief, the assay used biotinylated probes for HPV types 16, 18, 31, 33, and 35, detected with an avidin alkaline phosphatase conjugate, BCIP/NBT color development, and nuclear fast red counterstain. Optimal pretreatment conditions were determined for each block, with use of results of human placental DNA hybridization (an endogenous positive control) as a guide. Sensitivity of each assay was monitored with detection of HPV 16 in sections of formalin-fixed, paraffin-embedded cell blocks of SiHa cells (1–2 copies of HPV/cell).

L1 consensus primer PCR. L1 consensus PCR was used as previously described [2] to detect HPV DNA in formalin-fixed, paraffin-embedded tissue samples and, with minor modifications, for lavage samples. Proteinase K predigestion was used for both types of samples. In brief, sample DNA integrity and the absence of PCR inhibitors were monitored by amplification of β -globin DNA in replicate tubes. For lavage samples the usual input volume per PCR tube was $5\ \mu\text{L}$ of the 1:10 cell suspension, but occasionally ($\sim 10\%$ of samples) the volume of template had to be increased or decreased to amplify β -globin. If this adjustment was not successful, DNA was extracted from an additional $50\ \mu\text{L}$ of lavage with phenol:chloroform extraction and ethanol precipitation. Successful amplification of β -globin was achieved for all lavages samples. PCR products

were analyzed by type-specific hybridization to probes for HPV 16, 18, 31, 33, 35, and 56. Products not hybridizing were sequenced as described previously [2].

Data Analysis

The type of HPV detected in the primary tumor by *in situ* hybridization or PCR was defined as the *tumor type* HPV. HPV detected in each lavage sample was compared with HPV in tumor. The association between tumor type HPV in the lavage specimen and disease status was tested by means of Fisher's exact test. Lavage samples collected at diagnosis were used to calculate the sensitivity and specificity of this method for detection of tumor type HPV. If multiple samples from an individual patient had been obtained prior to therapy, only the first sample was used for the sensitivity/specificity calculations.

The ability to detect tumor type HPV in lavage as a function of disease status was evaluated for all patients with HPV-positive primary tumors by two approaches. In the first, all samples were grouped according to disease status at lavage specimen collection, and the proportion positive for tumor HPV was calculated. This simple proportion could be inaccurate because some individuals had more than one clinical visit and therefore more than one lavage during a particular disease status (e.g., 6 visits during following up, all in complete response). In the second approach, to account for multiple visits, we initially calculated the proportion of tumor-positive lavage specimens for each patient that were collected during a particular disease status. Then, we estimated the overall proportion of lavage samples positive for tumor type HPV at each disease status by averaging the proportions calculated for each patient. For statistical comparison, proportions across disease status were compared with use of the Fisher's exact test.

One problem with pooling lavage samples from all patients is that those enrolled at diagnosis might not be comparable to those enrolled months to years after therapy, even when grouped by disease status at time of sample collection. To minimize this problem, patients were stratified by cohorts based on their disease status at the time of enrollment. The association of tumor type HPV detection in lavage samples and disease status was determined for each cohort as described above.

Results

Study Population

Characteristics of the 85 study patients are shown in table 1, grouped by disease status at enrollment (cohorts) and summarized for the total population. The DX and CR cohorts accounted for the majority of the patients (45% and 46%, respectively). The patient age at diagnosis of invasive cervical cancer ranged from 28 to 85 years (mean, 51; median, 50). Most patients (82%) were black and most (86%) had early-stage (I or II) disease.

Table 1. Characteristics of study patients, grouped by disease status at enrollment (cohort).

Characteristic*	Data per patient cohort [†]				Total population (n = 85)
	DX (n = 38)	PR (n = 2)	CR (n = 39)	TF (n = 6)	
Age (y)					
Range	28–85	40–41	32–85	41–72	28–85
Mean	47.1	40.7	53.3	58.0	51.4
Disease stage (no. [%] of patients)					
Ia	6 [‡]	0	16	1 [‡]	23 (27)
Ib	16	1	13	1	31 (36)
II	9	0	8	2	19 (22)
III/IV	7	1	2	2	12 (14)
Treatment (no. [%] of patients)					
Not started	5 [‡]	0	0	1 [‡]	6 (7)
Surgery	11	0	18	1	30 (35)
Radiotherapy	19	2	15	2	38 (45)
Surgery + radiotherapy	1	0	6	1	8 (9)
Palliation	2	0	0	1	3 (4)
Follow-up (mo)					
Range	0.2–39	20.4–36.4	2–108.6	5.6–94.2	0.2–108.6
Median	17.3	28.4	40.1	27.6	26
Lavages, per patient					
Range	1–12	5–10	1–12	1–5	1–12
Median	4	7.5	2	2.5	3
Total	186	15	131	16	348
HPV-pos tumor	25 (66)	2 (100)	31 (79)	5 (83)	63 (74)

* Follow-up = time since staging; HPV-pos tumor = no. (%) of patients with human papillomavirus detected in primary tumor by in situ hybridization or PCR; palliation = radiotherapy without goal of cure.

[†] DX = patients enrolled at time of diagnosis; PR = patients enrolled during radiotherapy, tumor responding; CR = patients enrolled during or following curative therapy, with no evidence of tumor; TF = patients enrolled with recurrent or unresponsive tumor.

[‡] Includes two patients (stage Ia) who refused further treatment following diagnostic cone biopsy.

Of the 85 patients, 76 (89%) received curative therapy: surgery (35%), radiation (45%), or combined surgery and radiation (9%). Three patients (4%) received palliative therapy and six (7%) were untreated. Time of follow-up was calculated from date of staging and ranged from 0.2 to 108.6 months. As expected, median follow-up time was shortest for the DX cohort and longest for the CR cohort. We collected 1–12 lavage samples from each patient, for a total of 348 specimens. Of these, 268 samples were collected from the 63 patients with HPV-positive tumors.

We found HPV DNA in 63 (74%) of 85 primary tumors: HPV-16 in 36; HPV-18 in 9; HPV-31, -33, or -35 in 9; mixed types (HPV-18 and -30s or HPV-16 and -18 or HPV-16 and -33) in 4; and HPV-6, -26, -51, -56, and -58 in 1 each. The proportion of HPV-positive primary tumors did not show significant variation between disease cohorts. For the 75 patients receiving curative therapy with known clinical follow-up, detection of HPV in the primary tumor was not associated with response to therapy. HPV-positive primary tumors were found in 11 (79%) of 14 patients whose therapy eventually failed compared with 45 (74%) of 61 patients with disease-free survival.

Sensitivity and Specificity of Lavage

Cervicovaginal lavage samples collected at the time of diagnosis should contain tumor cells. Results for these samples will reflect the sensitivity and specificity of this method of sample collection and analysis for detection of tumor-associated HPV. We collected 49 cervicovaginal lavage specimens from 38 patients with newly diagnosed cervical cancer. In the cases in which samples were obtained more than once prior to therapy, only the first sample was included in the sensitivity/specificity calculations. Results correlating HPV detection in lavage with HPV in the tumor are shown in table 2. The sensitivity of the lavage for detection of tumor HPV was 56% (95% CI, 34.9%–75.6%), with specificity of 76.9% (95% CI, 46.2%–94.9%). HPV was detected in lavage samples at diagnosis for three patients with HPV-negative primary tumors. For two of these patients, lavages repeated prior to therapy were negative for HPV. Only one sample from a patient with an HPV-positive tumor had HPV of a discordant type detected.

Association Between HPV in Lavage Sample and Disease Status

Detection of tumor type HPV in lavage samples could be expected to vary as patients change clinical status due to tumor

Table 2. Association of human papillomavirus (HPV) in lavage specimen at diagnosis with tumor HPV.

Lavage specimen	No. of tumors	
	HPV-negative	HPV-positive
HPV-negative	10	10
HPV-positive: concordant*	0	14
HPV-positive: discordant†	3	1

* Same HPV type detected in both tumor and lavage specimen.

† Different HPV types detected in tumor and lavage.

elimination, persistence, or recurrence. Only the DX cohort had significant numbers of patients who had samples collected at each disease status to permit a longitudinal analysis (table 1).

The proportion of lavages collected at each disease status with detectable HPV are shown for those patients enrolled at diagnosis (table 3). Results in table 3 were calculated as simple proportions; overall proportions, calculated to account for the effect of multiple sampling, were nearly identical (data not shown). Tumor HPV was significantly less likely to be detected in lavage samples collected during CR than during PR, during TF, or prior to therapy ($P < .001$, Fisher's exact test). Detection of tumor HPV in lavage samples was not significantly different for samples collected during DX vs. PR or TF status. By contrast, detection of non-tumor HPV showed no association with disease status. The type of therapy or stage of cervical cancer did not significantly influence these observations (data not shown). In patients from whom samples were obtained multiple times during CR, detection of tumor HPV occurred sporadically and was not persistently observed.

The same influence of disease status on HPV detection was noted if the analysis was restricted to the enrollment lavage of all patients with HPV-positive primary tumors (table 4). In this cross-sectional look at the population, tumor HPV was not

Table 3. Proportion of lavage specimens with detectable human papillomavirus (HPV) for patients enrolled at diagnosis, grouped by disease status at collection.

Disease status at lavage specimen collection†	Proportion of lavage specimens containing	
	Tumor HPV	Nontumor HPV
DX ($n = 35$)	.54‡	.03
PR ($n = 39$)	.36‡	.05
CR ($n = 59$)	.03	.15
TF ($n = 9$)	.44‡	.11

NOTE. Analysis includes only patients with HPV-positive primary tumors.

† CR = complete response, during or following curative therapy; DX = at time of diagnosis, prior to therapy; PR = partial response, during radiotherapy; TF = treatment failure (recurrent or unresponsive tumor following therapy).

‡ Compared with lavage specimens collected at CR: $P < .001$, Fisher's exact test.

Table 4. Proportion of lavages at enrollment revealing human papillomavirus (HPV) for all patients with HPV-positive primary tumors.

Disease status at enrollment*	Proportion of lavage specimens containing	
	Tumor HPV	Nontumor HPV
DX ($n = 25$)	.56	.04
PR ($n = 2$)	.5	.5
CR ($n = 31$)	0	.29
TF ($n = 5$)	.2	.4

* CR = complete response, during or following curative therapy; DX = at time of diagnosis, prior to therapy; PR = partial response, during radiotherapy; TF = treatment failure (recurrent or unresponsive tumor following therapy).

seen in any sample collected during complete clinical response. Again, nontumor HPV did not vary with disease status.

For the patients with HPV-negative primary tumors, the proportion of lavage samples containing any HPV did not vary significantly with disease status. For all samples collected at each disease status, the HPV-positive proportions were as follows: 0.26 at DX ($n = 14$), 0 at PR ($n = 8$), 0.26 at CR ($n = 54$), and 0.25 at TF ($n = 4$). Overall, 11 of the 18 HPV-positive lavage samples contained high-risk types. No patient was repeatedly positive for the same HPV type.

Association Between HPV in Lavage and Treatment Failure

Twelve patients had lavage samples collected during treatment failure (6/6 in TF cohort, 4/38 in DX cohort, 1/2 in PR cohort, and 1/39 in CR cohort); 10 of them had HPV-positive primary tumors. In the 25 lavage samples collected during TF from the patients with HPV-positive tumors, 10 (0.40) were positive for tumor HPV. The positive samples were all collected from patients with local recurrence, and no samples from patients with only distant failure contained tumor HPV (10/18 vs. 0/7; $P = .02$, Fisher's exact test). Review of lavage results for those patients with eventual treatment failure did not demonstrate detection of tumor HPV in any lavage specimen collected prior to clinical evidence of recurrence (data not shown).

Discussion

To date, HPV DNA is the best candidate for a molecular marker of cervical cancer, because HPV is involved in the pathogenesis of the disease [3–5] and HPV DNA is found in most cervical cancers [6, 7]. However, several factors limit the usefulness of HPV as a molecular marker for cervical cancer. Not all cervical cancers have detectable HPV, and HPV DNA can be detected in cervical samples from normal patients without clinical or histologic evidence of lesions [8]. Furthermore, several studies have detected HPV within clinically and morphologically normal epithelium adjacent to cervical cancer or genital warts [9, 10]. These observations raise the possibility

that the genital tract of cervical cancer patients is involved in a "field effect" of latent or occult HPV infection that may not be eliminated by eradication of the neoplastic tissue. In addition, the patient's cervical tumor may be associated with one HPV type and the patient coinfecting with another type.

In our patients, HPV DNA was detected in 74% of primary tumors by in situ hybridization or L1 consensus PCR. The proportion of HPV-negative tumors is higher than that reported in several other studies [6, 11]. This may reflect differences in the patient populations or methodologies for HPV detection. In our study, each assay for detection of HPV in tumor involved histologically confirmed tumor tissue. In most cases HPV was localized to the tumor by in situ hybridization, and all PCR products were verified by type-specific hybridization or sequencing.

In addition to use of a good molecular marker for disease, development of a molecular assay to monitor disease requires samples that are representative of the patient's disease state. Cervicovaginal lavage is a noninvasive method for collection of cells representative of the cervical/vaginal area and is the currently accepted method for sampling cervical disease [1]. This study utilized PCR detection of HPV DNA in cervicovaginal lavage specimens as a molecular assay for monitoring cervical cancer in patients with HPV-positive primary tumors.

We assumed cervicovaginal lavage samples collected from cervical cancer patients prior to therapy would contain tumor cells. However, only about half of these lavage samples from patients with HPV-positive tumors had detectable HPV. Compared with HPV detection within histologically confirmed tumor, the sensitivity and specificity of cervicovaginal lavage for detection of tumor HPV type were only 56% and 76.9%, respectively. This low sensitivity contrasts markedly with the original descriptions of cervicovaginal lavage [1, 12, 13]. These studies, in women referred for colposcopy, all found lavage to be superior to other methods such as scrape or cytobrush cervical sampling. Because the authors assumed that detection of more HPV reflected better sampling, their results could simply indicate a higher yield of cells or the contribution of HPV from sites other than the cervix. Even with cervicovaginal lavage, the authors reported that 10% of their samples yielded inadequate DNA for Southern blot analysis [13].

In the first two studies described [1, 12], no attempt was made to establish an association between results and cytology or biopsy findings. In the third study [13] the authors compared lavage with cervical biopsy ($n = 112$) and found that lavage had 98% sensitivity and 61% specificity for detection of biopsy HPV. Because more HPV was detected in the lavage samples, the authors again concluded that it was a more sensitive method. This assumes lavage results rather than biopsy results to be the reference. The biopsy specimen tested for HPV was divided, with routine histology completed on the portion not extracted. The histology of these particular samples was not specified, but of all 191 biopsy specimens from the total study population, 75.4% were CIN (cervical intraepithelial neoplasia) I–III, 3.1% were carcinoma, and 21.5% had no detectable lesion.

A smaller, more recent study compared hybrid capture detection of HPV in paired lavage and cervical biopsy specimens [14]. For 34 paired samples, the sensitivity and specificity of lavage for detection of biopsy HPV were 77% and 38%, respectively. The low sensitivity resulted from the relatively large numbers of paired lavage-positive/biopsy-negative samples, and the authors attribute this to low-copy HPV infection missed in the biopsy. While the histology of the biopsy specimen tested for HPV was not determined, the findings from additional biopsy specimens obtained at the same colposcopy ranged from normal to high-grade dysplasia. These findings are closer to our results, although our sensitivity was lower because of lavage-negative/tumor-positive samples.

While we did not test for HPV copy number, invasive cancers frequently have lower copy numbers than dysplasias, which may be one reason for the lower sensitivity in our study. We conclude that cervicovaginal lavage samples are not ideal for molecular epidemiological studies of cervical cancer and that the validity of the correlation between HPV detection in lavage specimens and HPV within cervical lesions needs to be reexamined.

Treatment of cervical cancer did result in elimination of tumor HPV from the genital tract. For the longitudinal analysis of the DX cohort, proportions of samples with tumor HPV decreased from 54% at DX to 3% during CR (table 3). The type of therapy or stage of disease did not influence these findings. Similar results were found in the cross-sectional analysis including enrollment lavage for all patients with HPV-positive tumors (table 4). The strength of the observation that elimination of tumor is associated with elimination of tumor HPV is weakened by the limits in sensitivity of lavage sampling. As expected, detection of nontumor HPV did not significantly vary with disease state. HPV detection in lavage specimens from patients with HPV-negative primary tumors also showed no variation with disease status.

We conclude that cervicovaginal lavage is not the optimal sampling method for molecular epidemiological analysis for HPV in cervical tumors. Within the limits of the sampling and assay methods used in this study, tumor HPV was significantly less likely to be found in the genital tract when the patients achieved complete clinical response to curative therapy, regardless of the type of therapy. Lavage samples did detect local recurrence of tumor, but only at the time when recurrences were detected clinically. Improved sampling methods need to be evaluated before molecular detection of tumor HPV could be incorporated into clinical management of cervical cancer.

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