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Association of Oral Human Papillomavirus DNA Persistence With Cancer Progression After Primary Treatment for Oral Cavity and Oropharyngeal Squamous Cell Carcinoma

Carole Fakhry, MD^{1,2}; Amanda L. Blackford, ScM³; Geoff Neuner, MD⁴; et al
WeiHong Xiao, MD⁵; Bo Jiang, BS⁵; Amit Agrawal, MD⁶; Maura L. Gillison, MD, PhD⁵

Author Affiliations [Article Information](#)

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Key Points

Question Is persistent detection of oral human papillomavirus (HPV) DNA after completion of primary therapy associated with recurrence and survival among patients with HPV-positive oral or oropharyngeal cancer?

Findings In this cohort study of 396 patients, HPV DNA identical in type to that found in the tumor was detectable in oral rinses at diagnosis in 80% of patients with HPV-positive oropharyngeal cancer. Persistent detection of HPV DNA after completion of primary therapy was significantly associated with the increased risk of cancer recurrence and death.

Meaning The findings suggest that presence of HPV DNA is associated with increased risk of recurrence and death from HPV-positive oral or oropharyngeal cancer.

Abstract

Importance Detection of persistent oral human papillomavirus (HPV) DNA may be associated with recurrence of HPV-positive head and neck squamous cell carcinoma (HNSCC).

Objective To evaluate the dynamics of oral HPV DNA detection and associations with disease outcomes in patients with HPV-positive and HPV-negative HNSCC.

Design, Setting, and Participants This prospective, 2-institution, tertiary referral center study of 396 patients with newly diagnosed oral cavity or oropharyngeal HNSCC was performed from July 11, 2011, to May 7, 2016. Oral rinse samples were prospectively collected at diagnosis and at completion of primary therapy. Weekly oral rinse samples were collected during radiotherapy. Purified tumor and oral rinse sample DNA were evaluated for 37 HPV types, and viral load was quantified by type-specific real-time polymerase chain reaction. Cancers were stratified by tumor HPV status, and HPV was classified as tumor type if identical to that detected in the tumor or nontumor type.

Main Outcomes and Measures Prevalence of HPV DNA before, during, and after therapy. Associations between tumor-type and nontumor-type oral HPV DNA detection and recurrence-free and overall survival were evaluated.

Results Of the 396 patients (median age, 59 years [range, 19-96 years]; 295 [74.5%] men; and 354 [89.4%] white race/ethnicity), 217 had oropharyngeal cancer; 170, oral cavity cancer; and 9, unknown primary HNSCC. The prevalence of oral HPV detection at diagnosis was higher among patients with HPV-positive compared with HPV-negative HNSCC (24 of 194 [84.2%] vs 170 of 202 [12.4%]; $P < .001$). Oral HPV-16 DNA had an 81% sensitivity and 100% specificity for HPV-16-positive HNSCC. The prevalence and load of tumor-type HPV decreased significantly during primary therapy with odds ratio for probability of infection with each increasing month after diagnosis (0.41; 95% CI, 0.33-0.52; $P < .001$), whereas those of nontumor types did not (1.01; 95% CI, 0.97-1.06; $P = .62$). Current smoking was significantly associated with a reduced clearance of tumor-type HPV DNA (hazard ratio [HR], 0.54; 95% CI, 0.32-0.93). Two-year overall survival was significantly lower among the HPV-positive patients with persistent detection of tumor-type HPV after therapy than among those without detectable tumor-type DNA after therapy (68% vs 95%; adjusted HR, 6.61; 95% CI, 1.86-23.44; $P = .003$), as was recurrence-free survival (55% vs 88%; adjusted HR, 3.72; 95% CI, 1.71-8.09; $P < .001$). No associations were observed for nontumor type HPV DNA among patients with HPV-positive or HPV-negative HNSCC.

Conclusions and Relevance Prevalence and viral load of tumor-type HPV DNA decreased rapidly with therapy, and persistent detection was associated with increased risk of recurrence and death. Analysis of tumor type HPV DNA has considerable promise as a biomarker for treatment response and risk of progression.

Introduction

Human papillomavirus (HPV), predominantly high-risk type 16, is estimated to cause approximately 22% of oropharyngeal and 5% of oral cavity squamous cell carcinomas (SCCs) worldwide.¹ Survival rates are significantly higher among patients diagnosed with HPV-positive than HPV-negative oropharyngeal cancer.^{2,3} However, approximately 20% of

patients diagnosed with HPV-positive oropharyngeal cancer experience cancer progression or die within 5 years.⁴

National Comprehensive Cancer Network guidelines recommend that patients with head and neck SCC (HNSCC) undergo routine clinical surveillance for cancer recurrence.⁵ Early detection may provide patients the opportunity for long-term disease control through surgical salvage of locoregional recurrence or oligometastatic disease.^{6,7} Retrospective studies suggest that detection of oral HPV-16 DNA after completion of curative treatment may help identify patients at increased risk for recurrence of HPV-positive oropharyngeal cancer.⁸⁻¹⁰ In these studies, 8 of 9 patients with oral HPV-16 DNA detected after therapy developed recurrence.^{8,9} However, recurrence was also observed in the absence of detectable HPV DNA. These studies suggest that the presence of HPV DNA may be associated with detection of subclinical cancer.

The future clinical utility of treatment surveillance by oral HPV DNA detection requires further understanding of the dynamics of HPV DNA detection in patients diagnosed with HPV-positive HNSCC. Therefore, we designed a prospective cohort study to investigate the prevalence and dynamics of oral HPV DNA detection, as well as the association of HPV DNA presence with recurrence and survival among patients with HNSCC. To guide the interpretation of data, patients with HPV-negative HNSCC were included as a control population.

Methods

Patients 18 years and older with incident oral cavity, oropharyngeal, or unknown primary HNSCC who were seen at Ohio State University, Columbus, Ohio, or Greater Baltimore Medical Center, Baltimore, Maryland, from July 11, 2011, to May 12, 2016, were eligible. At enrollment, computer-assisted self-interview for collection of demographic and risk factor behaviors and venipuncture were completed. The protocol was approved by the institutional review board at Ohio State University, Greater Baltimore Medical Center, and MD Anderson Cancer Center. All patients provided written informed consent. All data and samples were deidentified.

Oral rinse samples were collected by a 30-second rinse and gargle with saline at diagnosis, after initial surgery (if applicable), and at 6 months. Among the subset of patients who received primary or adjuvant radiotherapy, oral rinse samples were collected weekly during radiotherapy. The DNA was purified from oral rinse samples after centrifugation and sequential digestion with DNase-free RNase A and proteinase K using the QIA-symphony Virus/Bacteria Midi Kit and Pathogen Complex 800 program on the QIA-symphony SP instrument (Qiagen Inc).¹¹

All tumors were classified as HPV positive or HPV negative based on detection of mRNA expression of E6 or E7 oncogenes for any of 13 high-risk HPV types, as previously described.¹² Tumor was snap-frozen in liquid nitrogen within 30 minutes of resection and macro-dissected to ensure more than 70% tumor. If fresh-frozen tumor was unavailable, DNA and RNA were extracted from formalin-fixed and paraffin-embedded tumor.¹² If tumor specimens were not available, HPV positivity was based on dual positivity for p16-expression by immunohistochemistry and HPV DNA in situ hybridization extracted from the clinical record, which has approximately 98% positive predictive value for E6/E7 mRNA expression.¹²

For DNA extracted from oral rinses, detection for 37 HPV types within the *Alphapapillomavirus* genera was performed by multiplex polymerase chain reaction (PCR) with PGMY primer pools and primers for β -globin, followed by HPV type specification by line blot hybridization using Roche Linear Array HPV Genotyping Test (Roche Molecular Systems, Inc). The β -globin-positive samples were considered to be evaluable.¹³ Samples were reported as HPV positive if any of the 37 HPV DNA types were detected, including high-risk (16/18/26/31/33/35/39/45/51/52/53/56/58/59/66/68/73/82) and low-risk (6/11/40/42/54/55/61/62/64/67/69/70/71/72/81/82 subtype [IS39]/83/84/89 [cp6108]) types.¹⁴ HPV load was measured in samples determined to be positive by Linear Array for any of 22 HPV types (6/11/16/18/26/31/33/35/39/45/51/52/53/55/56/58/59/62/66/68/73/82) by HPV type-specific TaqMan quantitative PCR assays targeted to the E6 or E7 regions.¹⁵ Samples were positive if HPV copy number was above the lower-limit of reproducibility (≥ 3) as derived from a reference standard curve.¹⁵ The number of human cells was estimated by TaqMan quantitative PCR targeted to a single-copy human gene, endogenous retrovirus-3. Type-specific HPV load measurements were normalized to number of endogenous retrovirus-3 copies and expressed as HPV load per 100 000 cells.¹⁴

Statistical Analysis

Patient characteristics were compared using Fisher exact test, 2-sided t tests, or Wilcoxon rank-sum tests. Changes in prevalence of oral HPV DNA and log-scale viral load were estimated using a generalized mixed effects regression modeling approach. For prevalence of oral HPV DNA, we used a logistic regression setting, and for log-scale viral load, given the large proportion of zeros, we used a negative-binomial model. Both modeling approaches included a random intercept for each patient to account for repeated measures during the course of the study. Time to clearance for type-specific HPV was defined as the date of a first positive test result to the date of the first negative test result (event) or last positive test result (censored) and estimated using the Kaplan-Meier method. Hazard ratios for clearance were estimated using Cox proportional hazards models with a robust variance estimation to account for clustering of nontumor-type infections within the same patient. Overall survival (OS) was estimated using the Kaplan-Meier method and analyzed with Cox proportional

hazards models adjusting for age, cigarette pack-years, node (N) stage, and tumor (T) stage. A landmark analysis of OS was performed when comparing patients with detectable tumor-type HPV after therapy, with a landmark of 6 months after diagnosis. Recurrence-free survival (RFS) was defined as the date of diagnosis to first recurrence (local, regional, and/or distant), estimated using cumulative incidence estimates with death as a competing event and compared between patient groups using proportional subdistribution hazards.¹⁶ We considered $P < .05$ to represent statistical significance and all P values were 2-sided.

Results

The study population consisted of 396 patients with oropharyngeal ($n = 217$), oral cavity ($n = 170$), and unknown primary ($n = 9$) HNSCCs (eTable 1 in the [Supplement](#)). Median age was 59 years (range, 19-96 years), and most patients were male (295 of 396; 74.5%) and white race/ethnicity (354; 89.4%).

Of the 396 patients, 202 had HPV-positive HNSCCs (51.0%), and 194 had HPV-negative HNSCCs (49.0%). Most oropharyngeal (187 of 217; 86.2%) and unknown primary (8 of 9; 88.9%) cancers were HPV positive, whereas only 7 of 170 oral cavity cancers (4.1%) were HPV positive. Human papillomavirus 16 was the most common type detected among the 195 of 202 HPV-positive tumors with evaluable mRNA (180; 92.3%) (eTable 2 in the [Supplement](#)). Additional HPV types included HPV-33 (6; 2.9%), HPV-35 (4; 1.9%), HPV-18 (2; 0.9%), and HPV-45/58/69 (1 each; 1.5%).

We compared the characteristics of the patients with HPV-positive and HPV-negative cancers. Patients with HPV-positive tumors were significantly more likely to be male (88.6% vs 59.8%), be younger (mean [SD] age, 57.4 [8.5] vs 60.8 [12.5] years), have never smoked (41.4% vs 31.4%), have used marijuana (64.6% vs 48.4%), and have a higher number of lifetime sexual partners (median, 15 vs 5) compared with patients with HPV-negative tumors (for each factor, $P < .002$) (eTable 1 in the [Supplement](#)).

Prevalence of HPV DNA Detection Before Initiation of Therapy

A total of 2922 oral rinse samples were tested for HPV: 2915 (99.8%) were β -globin positive, and 1197 (41.0%) were HPV positive. The HPV-type distribution detected during the baseline visit at the time of diagnosis is shown in eTable 2 in the [Supplement](#).

At diagnosis, HPV-positive patients were significantly more likely than HPV-negative patients to have detectable oral HPV DNA (24 of 194 [84.2%] vs 170 of 202 [12.4%]; $P < .001$). Detection of any oral HPV DNA had a sensitivity of 84% (95% CI, 78%-89%), specificity of 88% (95% CI, 82%-92%), positive predictive value of 88% (95% CI, 82%-92%), and negative predictive value of 84% (95% CI, 78%-89%) for a diagnosis of an HPV-positive tumor. Detection of oral HPV-16 DNA for an HPV-16-positive tumor had a

sensitivity of 80.6% (95% CI, 74.0%-86.1%), specificity of 100% (95% CI, 84.5%-100%), positive predictive value of 100% (95% CI, 97.5%-100%), and negative predictive value of 38.6% (95% CI, 26.0%-52.4%).

Factors associated with oral HPV DNA detection at diagnosis differed between patients with HPV-positive and HPV-negative cancers ([Table 1](#)). Among patients with HPV-positive cancers, stage T3 or T4 advanced tumor category (according to American Joint Cancer Committee staging)¹⁷ was associated with oral HPV detection. In contrast, for patients with HPV-negative cancers, factors associated with oral HPV infection were similar to those in the US population,¹⁸ including male sex and lifetime number of sexual partners.

Among the 202 patients with HPV-positive tumors, 161 patients had the same HPV DNA type detected in the oral rinse sample and in the tumor at baseline (hereafter referred to as tumor-type HPV DNA). The sensitivity of oral tumor-type HPV DNA for a diagnosis of HPV-positive HNSCC was 79.7% (95% CI, 73.5%-85.0%).

Of 202 patients, 45 (22.3%) with HPV-positive tumors had more than 1 HPV type detected at baseline. In addition, 23 of 202 (11.4%) had a high-risk HPV type detected that was not present in their tumor. Among patients with HPV-negative tumors, 12 of 194 (12.4%) had detectable HPV DNA, 19 of 194 (9.8%) had high-risk HPV DNA, and 9 of 194 (4.6%) had more than 1 HPV type detected at diagnosis.

Prevalence of Oral HPV DNA Before and After Therapy

We evaluated the association of cancer therapy with the prevalence of tumor-type HPVs among patients with HPV-positive tumors. After each primary treatment modality, the prevalence of tumor-type HPV decreased significantly. Specifically, the prevalence of tumor-type oral HPV DNA before and after primary surgical resection was 69.2% vs 13.7% ($P < .001$). In the subset of patients who required adjuvant radiotherapy, prevalence decreased from 70% to 38% after surgical resection and then decreased to 1% after radiotherapy ($P < .001$). Similarly, detection of tumor-type HPVs among patients treated with primary radiotherapy with or without chemotherapy decreased from 85% at baseline to 9% after completion of radiotherapy ($P < .001$). The prevalence of nontumor type infections was less than 1% both before and after radiotherapy. No decrease was observed for nontumor type HPV DNA (for adjuvant radiotherapy, $P = .79$; for primary radiotherapy, $P = .37$; and for surgery, $P = .58$).

Dynamics of HPV DNA Detection During Radiotherapy

Given the reduction in tumor-type HPV DNA prevalence before and after primary therapy, we investigated the dynamics of oral HPV DNA prevalence and viral load in the interval between diagnosis and completion of primary radiotherapy. [Figure 1A](#) shows the prevalence

of tumor-type HPV detection as a function of time. The prevalence of tumor-type HPV DNA detection decreased significantly among patients with HPV-positive tumors (odds ratio, 0.41; 95% CI, 0.33-0.52; $P < .001$). In contrast, prevalence of nontumor type infections remained unchanged (odds ratio, 1.01; 95% CI, 0.97-1.06; $P = .62$).

We investigated associations between clinical and demographic factors and time to clearance of tumor-type HPV DNA. Median time to clearance for the 168 tumor-type HPVs (161 detected at baseline and 7 detected during follow-up) was 42 days (95% CI, 37-49 days). The only factor significantly associated with reduced clearance was current smoking (hazard ratio, 0.49; 95% CI, 0.28-0.86; $P = .01$). For nontumor-type infection, smoking was not associated with clearance (hazard ratio, 0.76; 95% CI, 0.52-1.12; $P = .17$). Tumor and N stage at baseline were not associated with persistent infection.

To better visualize the association of therapy with tumor-type HPV DNA detection, we measured tumor-type HPV DNA load in HPV-positive patients treated with primary radiotherapy ([Figure 1B](#)). Samples were collected at baseline (visit 1), weekly during radiotherapy (visits 2-8), and 4 weeks after radiotherapy (visit 9). Tumor-type HPV DNA load decreased rapidly during primary radiotherapy for most patients (24% relative reduction per visit; $P < .001$). By contrast, there were no changes in viral load for nontumor-type HPV in patients with HPV-positive or HPV-negative tumors (0.5% relative increase per visit during primary radiotherapy; $P > .05$).

During radiotherapy, tumor-type HPV DNA became detectable for 7 patients with HPV-positive tumors. New, nontumor HPV types were also detected during radiotherapy in both patients with HPV-positive tumors and patients with HPV-negative tumors. The cumulative probability of detection of any new HPV infection during radiotherapy was 17% among HPV-positive patients and 14% among HPV-negative patients.

Detection of Oral HPV DNA After Therapy and Cancer Recurrence

We investigated associations between persistent detection of tumor-type HPV DNA after therapy and survival. Median follow-up time among the cohort by reverse Kaplan-Meier method was 2.6 years (range, 21 days to 5.5 years), and 11 patients (2.8%) were lost to follow-up. There were 79 deaths (20%) observed. Recurrence was observed in 38 of 199 HPV-positive patients (19.1%) and 59 of 186 HPV-negative patients (31.7%).

Overall survival and RFS were significantly higher for patients with HPV-positive tumors than for patients with HPV-negative tumors (2-year OS: 91% [95% CI, 87%-95%] vs 75% [95% CI, 69%-82%], $P < .001$; 2-year RFS: 83% [95% CI, 77%-88%] vs 71% [95% CI, 64%-78%], $P = .004$) ([Table 2](#)). Among the 202 patients with HPV-positive tumors, neither the presence of tumor-type oral HPV DNA nor high viral load (greater than median at baseline) at diagnosis were associated with prognosis.

Cancer outcomes associated with persistent HPV detection after treatment were evaluated among HPV-positive patients ([Figure 2](#) and [Table 2](#)). Tumor-type HPV was detectable after treatment in 14.3% HPV-positive patients for a median of 28 days (range, 0-300 days) after completion of primary therapy. Among these patients, the cumulative incidence of recurrence by 2 years was 45.3%. By contrast, 2-year incidence of recurrence among those without detectable tumor-type HPV DNA after treatment was 12.2%. Detection of tumor-type HPV DNA after completion of primary therapy was also associated with significantly increased risk of recurrence (adjusted HR, 3.72; 95% CI, 1.71-8.09; $P < .001$) ([Figure 2B](#)), including risk of local (adjusted HR, 9.81; 95% CI, 2.73-35.30; $P < .001$) and regional (adjusted HR, 5.75; 95% CI, 1.95-16.91; $P = .002$) recurrence, but not distant recurrence (adjusted HR, 2.25; 95% CI, 0.64-7.95; $P = .21$) ([Figure 2C](#) and [D](#)). In contrast, the presence of nontumor-type HPV DNA after treatment was not significantly associated with recurrence.

Detection of tumor-type HPV was significantly associated with an increased risk of death (adjusted HR, 6.61; 95% CI, 1.86-23.44; $P = .003$). Specifically, 2-year OS among patients with vs without detectable tumor-type DNA after therapy was 68% (95% CI, 47%-98%) vs 95% (95% CI, 90%-100%) ([Figure 2A](#)).

Discussion

Oral HPV DNA identical in type to that present in the tumor was detectable in most patients with HPV-positive HNSCC at diagnosis; both prevalence and viral load of tumor-type HPV DNA decreased rapidly during primary therapy. Persistence of tumor-type DNA was associated with risk of locoregional progression and death. In contrast, bystander nontumor type high-risk HPV DNA, which was detectable in approximately 10% of patients, was unaffected by therapy and not associated with survival. This finding is analogous to findings for high-grade cervical neoplasia, in which persistent tumor-type HPV is associated with recurrence whereas detection of synchronous HPV infection is not.¹⁹

Our data underscored the complexity of implementing oral HPV DNA-driven surveillance for p16-positive HNSCCs. Although p16 was an acceptable surrogate for HPV-positive oropharyngeal cancer,^{20,21} in the context of cancer surveillance, HPV type specification would be necessary. Although HPV-16 was the predominant type, it was inaccurate to follow it in all cases. Tumor HPV type specification can be performed for clinical diagnostic samples by using PCR, DNA, or RNA-based in site hybridization assays.^{12,22}

Tumor HPV positivity was associated with improved survival. Nevertheless, at least 20% of HPV-positive patients experienced recurrence, and 45% of recurrences were locoregional.²³ Of note, 2-year RFS and OS were worse for HPV-positive patients with HPV DNA persistence compared with HPV-negative patients. Our data suggest that persistent tumor-type oral HPV DNA identified a subset of HPV-positive patients with increased risk

of recurrence and consequently inferior OS. Oral HPV DNA detection after completion of primary therapy likely represented subclinical disease. Patients with persistent HPV DNA may benefit from close clinical surveillance or adjuvant therapy, for example, adjuvant immunotherapy that has been shown to improve outcomes for patients with lung cancer.²⁴ Furthermore, the high negative predictive value for recurrence of a negative test may be reassuring to patients. A limitation of oral HPV DNA detection is its weak association with distant recurrence. In this patient population, 55% of recurrences were distant; plasma HPV DNA may serve as a better biomarker.⁹

The association observed between HPV persistence and recurrence risk was analogous to that observed between plasma Epstein-Barr virus (EBV) DNA and nasopharyngeal cancer. However, associations between EBV DNA at diagnosis and survival are stronger.²⁵ At diagnosis, high plasma EBV DNA load is associated with risk of recurrence, distant metastases, and death.^{26,27} In contrast, we observed no associations with baseline oral HPV DNA load and clinical outcomes. Epstein-Barr virus DNA after treatment can identify subpopulations at high risk of recurrence and death who might benefit from adjuvant therapy, although improved survival remains elusive with this strategy.²⁸ An ongoing trial²⁹ is investigating whether patients with negative EBV DNA after treatment can be spared adjuvant cisplatin-fluorouracil without compromising OS.

Current smoking was the only factor associated with decreased clearance of tumor-type HPV. A history of cigarette smoking was associated with a modified prognosis of HPV-positive HNSCC, and smoking during radiotherapy was associated with an increased risk of recurrence and death regardless of HPV tumor status.^{30,31} Poor outcomes have been attributed to increased tumor hypoxia and radiotherapy resistance. Smoking is also associated with an increased risk of oral HPV infection and infection persistence in cancer-free individuals^{32,33} and thus a higher prevalence of infection among smokers.³⁴ All of these data are consistent with smoking-induced oral mucosal immunosuppression.

Our data have implications for patient counseling. Patients frequently asked whether they remained HPV infected after therapy. Tumor-type HPV cleared after completion of definitive therapy. However, nontumor-type HPV was detected in approximately 20% of patients, including 10% with high-risk HPV types, consistent with population-based estimates.¹⁸ Moreover, 14% had additional HPV types detected during radiotherapy, which may reflect epithelial repair of radiation-induced mucositis and possible reactivation of latent infection. Of note, even if nontumor-type HPV DNA was detected, it had no discernable association with cancer outcomes.

Recent data indicate that EBV DNA may have clinical utility for secondary prevention of EBV-positive nasopharyngeal cancer in areas of endemicity, leading to detection of subclinical disease at stages amenable to therapy associated with low morbidity and high

survival rates.³⁵⁻³⁷ Our data indicated that oral HPV-16 DNA had high sensitivity and specificity for a diagnosis of HPV-positive HNSCC. In the case of cervical cancer, persistent high-risk HPV infections are an established surrogate for risk of development of cervical neoplasia.³⁸ Secondary prevention strategies for HPV-positive HNSCC are constrained by the rarity of both oral HPV-16 DNA and cancer, factors that potentially could be overcome with risk-based models.

Strengths and Limitations

Strengths of this study include the prospective study design and large sample size. To our knowledge, this is the first study to perform comprehensive HPV type specification in both tumors and oral rinse samples, include patients with HPV-positive and HPV-negative tumors, classify tumor-type and nontumor-type infections, and evaluate the dynamics of oral HPV prevalence and viral load during and after therapy. Our study has several limitations. The median follow-up time (approximately 2 years) may have led to underestimation of associations between persistence and recurrence. However, 90% of cancer recurrences occurred within 2 years of follow-up.^{6,7}

Conclusions

Our data suggest that a subset of patients with HPV-positive HNSCC at high-risk for locoregional recurrence can be identified by detection of persistent, oral HPV after treatment. However, the clinical utility may be constrained by a need to identify the tumor-type infection, a low-moderate positive predictive value for recurrence, and weak associations with risk of distant metastases. Ongoing studies will evaluate whether multiplexed detection of plasma HPV DNA can improve these limitations.

[Back to top](#)

Article Information

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Corresponding Author: Maura L. Gillison, MD, PhD, Department of Thoracic Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Unit 432, Houston, TX 77030 (mgillison@mdanderson.org).

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Drafting of the manuscript: Fakhry, Blackford, Xiao, Jiang, Gillison.

Critical revision of the manuscript for important intellectual content: Fakhry, Neuner, Agrawal, Gillison.

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