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Presence of HPV DNA in convalescent salivary rinses is an adverse prognostic marker in head and neck squamous cell carcinoma

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Abstract

Human papillomavirus (HPV) 16 is present in up to 60% of patients with head and neck squamous cell carcinoma (HNSCC) and confers a favorable prognosis in terms of recurrence and mortality. Previous reports demonstrated that HPV-16 DNA can be detected in the initial salivary rinses from these patients. In this study, we assessed the feasibility of post-treatment HPV DNA shed from the oral mucosa as a prognostic marker for persistent/recurrent head and neck cancer. Fresh tumor samples and pre- and post-treatment salivary rinses were collected from 59 patients with HNSCC. HPV-16 *E6* and *E7* DNA copy number in these samples were quantified by real time PCR. Twenty of 59 patients (33.9%) were HPV-16 positive in their tumors before treatment. Four of 20 HPV tumor positive patients ultimately developed recurrence, and 2 of these 4 patients were HPV-16 positive in surveillance salivary rinses (sensitivity = 50%). Of the 39 (66.1%) HPV-16 negative patients on initial clinical presentation and the 16 HPV-16 positive patients who did not recur, none were HPV-16 positive in salivary rinses after treatment (specificity = 100%). HPV-16 presence in follow-up salivary rinses preceded clinical detection of disease recurrence by an average of 3.5 months. Patients with presence of HPV-16 DNA in surveillance salivary rinses are at significant risk for recurrence. Quantitative measurement of salivary HPV-16 DNA has promise for surveillance and early detection of recurrence.

Keywords

Human Papilloma Virus (HPV); Head and Neck Squamous Cell Carcinoma (HNSCC); quantitative PCR

INTRODUCTION

Each year, head and neck cancer represents 650,000 new cases and 350,000 deaths worldwide.[1] Approximately 90% of these cancers are squamous cell carcinomas. Both

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genetic and environmental factors contribute to the development of head and neck cancers. Known environmental risk factors leading to HNSCC include smoking, alcohol consumption[2] and viral pathogens, including HPV-16.[3,4] Using molecular biomarkers that provide early detection of disease recurrence or metastasis may afford more potential treatment options with better functional outcomes.

HPVs are a group of 7.9 kb double-stranded circular DNA viruses that are classified into high risk (e.g., types 16, 18, and 31) or low risk (e.g., types 11, 36, and 42) groups.[5] High risk HPVs have been demonstrated to be involved in epithelial carcinogenesis. HPV is found in both premalignant and malignant tumors.[6] Substantial molecular evidence has suggested that HPV type-16 is a high risk genotype that plays an important role in the pathogenesis of a subset of HNSCC.[7,8] Malignant transformation caused by HPV-16 is mediated through the expression of E6 and E7, and to a less extent, E5 proteins. E6 and E7 proteins alter tumor suppressor pathways by inactivating *p53* and *Rb* respectively[9,10] while E5 protein modulates proliferation.[11,12] Previous studies have shown that HPV-16 is identified in 90% of HPV associated HNSCC[3] and in 50% of oropharyngeal SCC.[3,13,14] We, therefore, selected HPV-16 *E6* and *E7* for our study for these reasons.

The existing body of literature has confirmed an independent, favorable outcome for HNSCC patients who have tumor positive HPV-16 status on initial evaluation.[3,15] However, the role of HPV-16 detection in saliva obtained in follow-up visits remains unknown. The goal of our study was to determine the significance of HPV-16 *E6* and *E7* DNA presence in post-treatment salivary rinses. We hypothesized that patients who have detectable levels of HPV-16 DNA in post-treatment salivary rinses may have an elevated risk of recurrence.

MATERIALS AND METHODS

Study Patients

This study was nested within a longitudinal cohort study of 59 patients who presented with histopathologically confirmed HSNCC (this includes patients who presented for treatment of a recurrence after primary treatment at an outside hospital) to the outpatient clinic of the Johns Hopkins Hospital in Baltimore, Maryland from 1999 to 2005. Patients were included if they had at least one post-treatment salivary sample and consented for the study. All patients had undergone treatment with curative intent. The study protocol was approved by the institutional review board of the Johns Hopkins Hospital. Written informed consent was obtained from all patients.

Tissue and Saliva

Tumor samples and salivary rinses from 59 HNSCC patients were obtained. The oral rinses were performed with 20 ml of normal saline gargled twice, for 20 and 10 seconds respectively. Pre-operative tumor and saliva specimens were collected prior to commencement of any therapy at the Johns Hopkins Hospital. In 24 cases where fresh frozen tumor DNA was not available, archival paraffin embedded tissue sections were obtained and *in situ* hybridization was performed as described previously[16] to assess the initial HPV-16 status. In total, we analyzed 53 cases with matched pairs of tumor and pre-treatment saliva DNA and 6 cases with tumor DNA only as pre-treatment saliva DNA was not available. Surveillance salivary rinses were obtained in the follow-up clinic visits from all the patients. Our protocol states that the first post-treatment rinse was collected 3 months after the completion of all treatment and then every 3 months for two years total. However we were not able to obtain follow-up salivary rinses every three months for all patients so we included patients with at least one post-treatment salivary sample in this analysis.

DNA Extraction

Microdissected tissues and saliva were extracted using techniques described previously.[17] Briefly, microdissected fresh tissues and saliva were extracted with phenol-chloroform, precipitated in 100% ethanol, centrifuged at 5100 rpm for 45 minutes, washed in 70% ethanol twice, dissolved DNA in LoTE buffer, and stored at −20°C. The samples were diluted to 50 ng in each reaction and then analyzed by quantitative PCR (Taqman HT 7900, Applied Biosystem).

Quantitative PCR

The 7900HT real time PCR system was used to perform quantitative PCR for HPV-16 *E6* and *E7* and *B*-actin. Specific primers and probes have been designed to amplify the *E6* and *E7* regions of HPV type 16: HPV-16 *E6* forward primer, 5'-TCAGGACCCACAGGAGCG-3'; HPV-16 *E6* reverse primer, 5'-CCTCACGTCGCAGTAACTGTTG-3', HPV-16 *E6* TaqMan probe, 5'-(FAM)-CCCAGAAAGTTACCACAGTTATGCACAGAGCT-(TAMRA)-3', HPV-16 *E7* forward primer, 5'-CCGGACAGAGCCCATTACAA-3', HPV-16 *E7* reverse primer, 5'-CGAATGTCTACGTGTGTGCTTTG-3', HPV-16 *E7* TaqMan probe, 5'-(FAM)-CGCACAACCGAAGCGTAGAGTCACACT-(TAMRA)-3'. A housekeeping gene (*B-globin*) were run in parallel with HPV-16 *E6* and *E7* to standardize the input DNA: *B*-actin forward primer, 5'-TCACCCACACTGTGCCCATCTACGA-3', *B*-actin reverse primer, 5'-CAGCGGAACCGCTCATTGCCAATGG-3', *B*-actin TaqMan probe, 5'-(FAM)-ATGCCCTCCCCCATGCCATCCTGCGT-(TAMRA)-3'. All samples were run in triplicate.

Data Analysis

The CaSki (American Type Culture Collection, Manassas, VA) cell line was used to develop standard curves for the HPV viral copy number as it is known to have 600 copies/genome equivalent. Standard curves for HPV-16 *E6* and *E7* were developed by using DNA extracted from CaSki cells, serially diluted into 50 ng, 5 ng, 0.5 ng, 0.05 ng, and 0.005 ng. A standard curve was also developed for the housekeeping gene *B-actin* (2 copies/genome), using the same serial dilutions of CaSki DNA, as described previously.[6] Tumor samples with ≥ 0.1 copy/genome and salivary samples with > 0 copy/genome were considered as HPV positive. Simple sensitivity and specificity analyses were performed on the cases with local recurrence. No statistical correlation was attempted due to modest sample size.

RESULTS

We examined HNSCC tumors and paired pre- and post-treatment salivary rinse samples from 59 patients for HPV-16 using quantitative PCR. Table 1 summarizes the demographic data and risk factors of our HNSCC patients by HPV-16 status. There were 20/59 patients who were determined to be HPV-16 positive on initial presentation (6 were determined by *in situ* hybridization and 14 were determined by quantitative PCR). As reported in prior studies, the HPV-16 positive HNSCC were located in the oropharynx ($p < 0.001$). Most of the HPV-16 positive patients presented with advanced disease ($p = 0.003$), and there was a higher rate of lymph node metastasis in the HPV-16 positive patients ($p = 0.001$), which was in agreement with prior published studies.[18,19] There was no difference in the size of the primary tumor between HPV-16 positive and negative groups ($p = 0.644$), suggesting that the high stage was due to early lymph node metastasis. Other risk factors for HNSCC such as smoking and alcohol consumption did not show statistically significant differences between the HPV-16 positive and negative patients.

Treatment outcomes were evaluated and correlated with the HPV-16 status. Figure 1 shows the breakdown of patient population by pre-treatment and surveillance HPV-16 status and disease recurrence. Overall, 10/59 (16.95%) of patients developed a recurrence after completion of their treatment, with 4 of these 10 patients demonstrating HPV-16 positivity in their initial tumors prior to treatment. Four of 20 (20.0%) patients with HPV-16 positive tumors developed loco-regional with or without distant metastasis, and 2 of these patients were HPV-16 positive in surveillance salivary rinses (sensitivity = 50%). The other 2 patients, however, had no detectable HPV-16 in their surveillance saliva despite disease recurrence. One of these two patients did have positive HPV-16 *in-situ* hybridization in the biopsy of recurrent tumor. Of note, there were no false negative results, all of the patients who did not recur remained HPV negative in their surveillance saliva to date (specificity = 100%). Table 2 details the statistical analysis of these patients.

Table 3 shows the relevant clinical data in the 4 HPV-16 positive patients who developed recurrences. All these patients had stage IV HNSCC with significant regional lymph node metastasis, and they all developed recurrence within 24 months after completion of treatment. We found an average of 3.5 months of lead time in detection of recurrence of HNSCC when compared to standard clinical evaluation. In this study, there did not appear to be any correlation between local, regional, or distant recurrence and our ability to detect HPV-16 in the surveillance saliva.

DISCUSSION

HNSCC is commonly associated with smoking and alcohol use, although other factors such as HPV infection, diet, and poor oral hygiene may also play a role in tumorigenesis. HPV-16 is most frequently present in a subset of HNSCC patients who have lesions localized in the oropharynx. The aim of this study was to determine the correlation between post-therapy HPV-16 status in the saliva and recurrence in HNSCC patients by using quantitative PCR to investigate the feasibility of early detection of recurrence using this technique.

In this study, 20/59 (33.90%) patients were HPV-16 positive on presentation, similar to the prevalence rates reported previously.[14,20–22] We also confirmed that HPV-16 was most commonly found in oropharyngeal cancers, and these patients tended to present with advanced stage disease.[23] However we did not find an association of HPV status with alcohol use ($p = 0.572$) and smoking ($p = 0.225$), perhaps due to a relatively small sample size as compared to prior studies.

Previous studies have shown the presence of HPV-16 in oral exfoliated cells of HNSCC patients, using either quantitative[20] or nonquantitative PCR methods.[24] Here we have confirmed the feasibility of correlating the HPV-16 DNA presence in surveillance salivary rinses and disease progression. We detected 2/4 HPV positive tumor recurrences through salivary rinses, and there were no cases of false positive HPV-16 detection in this cohort. Moreover, the presence of HPV-16 DNA in surveillance saliva was 100% specific for tumor recurrence. We also found that the presence of HPV-16 in surveillance saliva was a marker for locoregional recurrence or distant metastasis. The presence of detectable HPV-16 DNA in surveillance saliva could represent microscopic residual cancer or micro-metastasis that escaped detection by conventional pathology or radiological evaluations. Alternatively, the presence of HPV-16 DNA in salivary rinses may reflect an inability to eliminate HPV-16 DNA containing cells in local, regional and distant sites. Additional investigation, would, of course, be necessary to confirm this hypothesis.

In this limited cohort, we found that patients with HPV-16 positive surveillance salivary rinses are at high risk for development of recurrence and distant metastasis. This finding

may represent an immunologic impairment that contributes to cancer development and subsequent recurrence and metastasis. Therefore the presence of HPV in saliva may be used as a screening/surveillance tool and as a prognostic biomarker for disease progression. However, a larger scale, prospective study specifically directed at this issue would be necessary to confirm the feasibility of early detection of recurrence using quantitative HPV-16 PCR in follow up salivary rinses.

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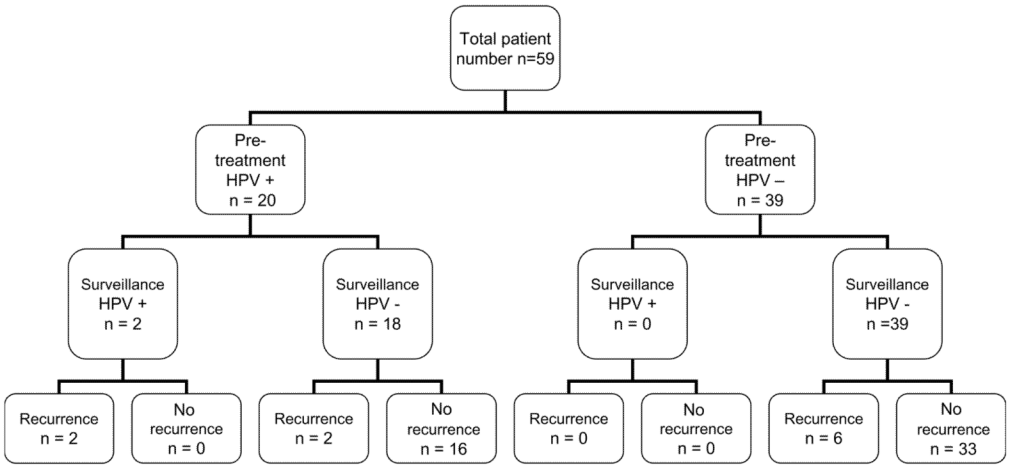

Figure 1.
Layout of patient groups by HPV status pre-treatment and post-treatment and recurrence.

Table 1

Demographic and Risk Factors By HPV-16 Status

Variables	HPV-16negative	HPV-16positive	*p*-value
Gender			
Male	30 (76.9)	18 (90.0)	0.222
Female	9 (23.1)	2 (10.0)	
Age			
<50yr	10 (25.6)	7 (35.0)	0.621
50–64yr	22 (56.4)	11 (55.0)	
≥65yr	7 (18.0)	2 (10.0)	
Race			
Caucasian	27 (69.2)	20 (100.0)	0.021
African American	10 (25.7)	0 (0.0)	
Other	2 (5.1)	0 (0.0)	
Smoking status			
Never	12 (30.8)	7 (35.0)	0.257
Former	6 (15.4)	7 (35.0)	
Current	17 (43.6)	5 (25.0)	
Unknown	4 (10.2)	1 (5.0)	
Drinking status			
Never	8 (20.5)	2 (10.0)	0.585
Former	4 (10.3)	1 (5.0)	
Current	19 (48.7)	13 (65.0)	
Unknown	8 (20.5)	4 (20.0)	
Stage			
Early (I–II)	17 (44.7)	1 (5.26)	0.003
Advanced (III–IV)	21 (55.3)	18 (94.74)	
TNM			
Tumor			
1	16 (42.1)	7 (36.84)	0.574
2	11 (28.9)	7 (36.84)	
3	8 (21.1)	5 (26.32)	
4	3 (7.9)	0 (0.0)	
Lymph Node			
N−	22 (56.4)	2 (10.0)	0.001
N+	17 (43.6)	18 (90.0)	
Tumor Site			
Oropharynx	4 (10.2)	19 (95.0)	<0.001
Oral cavity	23 (59.0)	0	
Larynx	9 (23.1)	0	
Hypopharynx	2 (5.1)	0	
Unknown primary	1 (2.6)	1 (5.0)	

Table 2

Sensitivity, specificity and predictive values of surveillance HPV-16 status

	Surveillance salivary HPV-16 status		
Recurrence	**positive**	**negative**	
Positive	2	2	Sensitivity = 2/4 = 50%
Negative	0	16	Specificity = 16/16 = 100%
	PPV = 2/2 = 100%	NPV = 16/18 =88.89%	

PPV = positive predictive value; NPV = negative predictive value

Table 3

Clinical course on pre-treatment HPV-16 positive patients who developed recurrence.

Post-treatment HPV-16 Status	Patient number	Initial Stage	T	N	Site of Tumor	Site of Recurrence	Disease Free Survival (months)	Lead Time to Clinical Diagnosis Using Saliva HPV DNA (months)
salivary rinse HPV-16 positive	68	4	1	2b	OP	right clavicle, left thyroid, bilateral lungs, chest wall, and multiple tracheal, carinal, bronchiolar and hilar LN	16.5	3.5
	141	4	2	2c	OC	tongue base, R axilla	12	3.5
salivary rinse HPV-16 negative	66	4	2	2c	OP	contralateral neck, superior nasopharyngeal margin; lung	21	not applicable
	17	4	3	2c	OP	neck recurrence, with circumferentially involved carotid artery	9	not applicable

OP = oropharynx; OC = oral cavity; LN = lymph node