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AGE, SEXUAL BEHAVIOR AND HUMAN PAPILLOMAVIRUS INFECTION IN ORAL CAVITY AND OROPHARYNGEAL CANCERS

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There are few well-established patient risk factors associated with human papillomavirus (HPV) infection in cancers of the oral cavity and oropharynx. The purpose of this study was to determine if there were significant different risk factors and tumor characteristics between HPV-positive and HPV-negative cancer cases. HPV was evaluated in cancer tissue and exfoliated oral cells of 193 oral cavity/oropharynx cancer patients using PCR and direct DNA sequencing. A patient questionnaire collected information about risk factors, sexual practices and medical history. The prevalence of HPV high-risk (HR) types was 20% in cancer cases. Three types were identified: HPV-16 (87%), HPV-18 (3%) and HPV-33 (11%). Risk factors for HPV-HR included younger age (≤ 55 years *vs.* > 55 years; adjusted OR = 3.4; 95% CI = 1.6–7.3) and younger-age cases who had more lifetime sex partners (adjusted OR = 3.8; 95% CI = 1.4–10.1), practiced oral-genital sex (adjusted OR = 4.3; 95% CI = 1.8–10.4) or oral-anal sex (adjusted OR = 19.5; 95% CI = 3.4–113). Compared to HPV-negative cancers, HPV-HR cancers were more likely to have a positive HPV-HR exfoliated oral cytology test (adjusted OR = 7.8; 95% CI = 3.4–18.4), later stage (adjusted OR = 3.0), nodal involvement (adjusted OR = 4.1) and advanced grade (adjusted OR = 3.0). This study shows new evidence that the prevalence of oncogenic mucosal HPV is higher in younger-age oral cavity/oropharynx cancer cases whose sexual practices are typically associated with sexual transmission of the virus. HPV detection also appears to be an indicator of advanced disease characteristics that may require different clinical treatment for this subset of patients. An exfoliated oral cytology test for HPV was a significant predictor of HR types in the cancers, suggesting that an oral rinse may provide an early biomarker of infected tumors.

Key words: *human papillomavirus; oral cancer; head and neck carcinoma; risk factors*

The causal link between human papillomavirus (HPV) infection and the development of head and neck carcinomas (HNCs) particularly in the oropharynx are becoming more firmly established.[1–3] A number of unanswered questions remain, however. Unlike cervical cancer, high-risk HPVs in HNC are neither a necessary nor a sufficient cause of cancer and only about 20% of these malignancies are associated with viral infection.[4] As yet limited evidence suggests that HPV can infect the oral cavity and upper respiratory tract through sexual transmission.[1,5] Whether tobacco and alcohol, the major risk factors for HNC, are less frequent exposures among those detected with HPV in HNC also needs clarification as the data are inconsistent.[6,7] Although poorly differentiated grade and nodal involvement have been more frequently reported in HPV-positive cancers, these findings usually are presented without adjustment for other risks and confounders associated with the disease.[8,9] If these findings are accurate, the information could lead to different treatment decisions and follow-up for recurrence in this subset of HNC. Finally, there is a need to determine whether a test based on oral exfoliated cells can be used to provide a sensitive method for predicting oncogenic mucosal HPV types in head and neck tissue.[10]

The purpose of this study was to determine if HPV-positive and HPV-negative oral cavity/oropharynx cancer cases were different in risk factors or tumor characteristics. Furthermore, we examined whether HPV DNA detected in exfoliated cells from an oral rinse might predict patients who have HPV-positive HNC, thus potentially providing a biomarker for identifying those at risk of cancer at these sites.

MATERIAL AND METHODS

Participants

Between 1994 and 1997, patients who presented with oral cavity or oropharyngeal cancer seen at the University of Iowa Health Care and the Veterans Affairs Medical Center in Iowa City, Iowa, were recruited into the study. Exclusion criteria included mental incapacity or language barrier, incomplete questionnaire, no oral exfoliated rinse and refusals (5%). Among the 202 enrolled head and neck cancer cases, an oral rinse of exfoliated cells was collected to test for HPV DNA. Only patients with biopsy material were included in this study. Reasons for not having biopsy material included patients who chose not to have treatment, had too little tumor tissue available for research purposes, or had radiation treatment prior to biopsy collection.

One of the purposes of this study was to determine whether HPV could be detected at any site in the oral cavity and oropharynx; thus all subsites were examined. Based on the American Joint Committee on Cancer (AJCC),[11] the combined oral cavity sites include cancers from lip vermilion and inner mucosa, tongue (excluding base of tongue), gingiva, floor of mouth, hard palate, other oral mucosa, parotid gland and submandibular gland. The oropharyngeal sites comprise base of the tongue, soft palate, uvula, palatine tonsil fossa, pillar and overlapping lesions, oropharynx and other unspecified oropharynx lesions. Staging also was based on the AJCC criteria.[11] To clarify the risk associated with histologic types and HPV infection, all head and neck carcinomas other than squamous cell carcinoma (SCC) diagnosed in this patient

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population were included ($n = 14$): acinic cell carcinoma, adenocarcinoma, adenoid cystic carcinoma, mucoepidermoid carcinoma and verrucous carcinoma.

A university-approved institutional review board form was administered prior to enrollment in the study. Patients completed a risk factor questionnaire that included information about sociodemographics, medical history, sexual practices, smoking, alcohol use and history of HPV-related diseases and oral lesions. Medical information was collected using the patient record and included prior cancer history, tumor site, treatments and staging information from the pathology reports. Prior to diagnosis, an exfoliated oral cell rinse was collected in 10 cc of normal saline. All specimens were reviewed prior to freezing at −80°C using a hemocytometer to verify and estimate the number of nucleated squamous cells. This information was used to ensure a minimum number of cells and sufficient DNA was included in each PCR reaction.

Sample preparation, DNA extraction, HPV typing

Hematoxylin and eosin-stained archived glass slides for all tissue blocks were examined to verify the presence of malignant tissue and diagnosis and to determine cancer grade. The block with the highest percentage of cancer was selected for DNA analysis. In a random 10% of cases, a section was cut from the block and stained, and all verified that the slide accurately represented the sections.

Procedures for DNA preparation and extraction from exfoliated cells have been described elsewhere.[12] Extraction of DNA from deparaffinized tissue sections was performed with a Qiagen DNA Tissue Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The DNA was eluted from the column with 100 μl of QIAamp buffer AE and stored at −20°C. HPV detection methods and reagents used were previously reported in Summersgill *et al*.[12] The DNA concentration was determined spectrophotometrically. Two percent of the DNA (typically 50–200 ng) extracted from biopsy specimens was PCR-amplified with MY09 and MY11 primers[13] to detect HPV and with a primer (HMB01) designed to amplify HPV-51 to improve detection.[14] Also included in the PCR reaction were primers that amplify a portion of the β-globin gene,[15] which was used to verify the presence of intact DNA and the adequacy of PCR amplifications. Formalin-fixed, paraffin-embedded incisional or excisional biopsy specimens were available for 195 cases (96.1%). Two of these tumor specimens did not amplify using β-globin and were excluded from the analyses, giving a final study population of 193. An aliquot of the PCR product then was transferred to a nylon membrane and hybridized with ^{32}P-labeled probes for detection of HPV (dot blot hybridization). Those samples positive only after the membrane hybridization underwent heminested PCR-amplification with MY09 and GP5$^+$ primers.[16] PCR products from cancer tissue and exfoliated oral cells were sequenced directly and compared to a DNA database to determine HPV type.[17,18] HPV types were defined as high risk (HPV-HR) on the basis of their association with cervical cancer risk. All other types were considered low risk (HPV-LR).

Laser-assisted microdissection

Procedures have been described previously.[8] Briefly, 5 μm thick sections from formalin-fixed, paraffin-embedded tissue samples were mounted on glass slides covered with a 1.35 μm thick polyethylene membrane (Palm, Wolfratshausen, Germany) treated with 1% poly-L-lysine. Sections were deparaffinized and stained with hematoxylin. The Robot-MicroBeam system (Palm) was used for laser-assisted microdissection (LMD). Clusters of tumor cells were cut from the tissue sections. Dissected tissue was digested with proteinase K (20 mg/ml) for 3 hr at 55°C. Proteinase K activity was stopped at 95°C for 10 min. DNA from microdissected specimens was further analyzed with either PCR amplification using GP5$^+$/GP6$^+$ primers[16] in a hot-start PCR or with nested PCR using 7.5 μl of the DNA each for β-globin PCR and HPV PCR. Microdissection was performed in a separate laboratory (J.P.K.'s) on coded slides to ensure independent blinded results. As few as 20 microdissected cells routinely yielded sufficient DNA for analysis.[8] The purpose of LMD was to verify that viral DNA detected in the formalin-fixed, paraffin-embedded sections was in the cancer cells and not in the adjacent normal epithelium. Thus, a subset of the study cases ($n = 18$) was examined. Where the results were different, the microdissection findings were considered to be more accurate and were used in the analysis.

Statistical analysis

The Wilcoxon rank-sum test and Pearson chi-square or Fisher's exact test were used to test for group differences between HPV-HR cases and HPV-negative cases in quantitative and categorical variables, respectively. A 2-tailed test was used for each risk factor. Multivariate logistic regression was performed to assess the association between detection of HPV-HR in biopsies and demographic, sexual behaviors and clinicopathologic factors among cases, while controlling for potential confounders and risk factors for oral cavity/oropharynx cancer. Nonlinearity was assessed for tobacco pack-years and average drinks per week. Due to the lack of linearity, tobacco pack-years was included in the adjustment as a categorical covariate. Effect modification was evaluated on a multiplicative scale. Adjusted odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were calculated using the multivariate logistic regression coefficients and their standard errors. Analyses were performed using the SAS system for Windows, version 8.2 (SAS Institute, Cary, NC).

RESULTS

Cancer site and HPV infection

Table I shows the frequency of cancer location and HPV status in the biopsy sections. The most common sites in which HPV-HR was detected were the tonsils, the base of the tongue, gingiva and oral mucosa. The prevalence rate of HPV-HR in cancers was 20% ($n = 38$). Three HPV types, all high-risk oncogenic mucosal, were identified in the cancer specimens: HPV-16, -18 and -33. HPV-16 was the predominant type in those infected (87%). Among the sites detected with the virus, cancers in the oropharynx were limited to either the base of the tongue or the tonsils, whereas in the oral cavity HPV was detected in multiple tumor sites. As suspected but rarely evaluated in prior studies, no HPV DNA was detected in either the lip or major salivary glands. No low-risk nononcogenic HPV types were found in the cancer cells of any tumors. HPV-HR types were detected in 10% of the oral cavity tumors and over 37% of oropharynx carcinomas, which also were at significantly greater risk of harboring high-risk types than was the oral cavity (adjusted OR = 6.2; 95% CI = 2.7–14.5). Among those detected with the virus, HPV-16 was found in 92% of cancers in the oropharynx and 77% in the oral cavity. Cancers in the oral cavity were more likely to include HPV-33, but there was no predilection for specific cancer sites by this type. Six different HPV-16 cases had DNA sequence variation in the amplified region; all differed from the laboratory HPV-16[19,20] or the HPV-16W12E,[21] indicating that these results were not due to laboratory contamination. The samples that were HPV-positive contained an average of 112 mm^2 of cancer and an average total of 220 mm^2 of tissue, similar to the average cancer and an average total tissue of those that were HPV-negative (104 and 234 mm,^{2} respectively). This suggests that the amount of cancer in the specimen did not influence the HPV positivity of a case.

To confirm that viral DNA detected in the formalin-fixed, paraffin-embedded sections was in the cancer cells and not in the adjacent normal epithelium, LMD was performed on a subset of 18 cancers. Twelve of the LMD-tested tumors originally were detected with an HR type. Because HPV is detected less often in the oral cavity, cases at this site originally detected with HPV-HR were oversampled in proportion to their positivity rate to verify that the virus was in tumor cells. Ten of the 12 cases with an HPV-HR type identified in the full sections were found to harbor the same type in the tumor cells using LMD. Two cases originally

TABLE I – CANCER SITE AND HPV TYPES IN BIOPSY TISSUE

Cancer site	Cancers		HPV-HR	HPV types		
	n	%	%	16	18	33
Oral cavity						
Lip, vermilion	3	1.6	0.0			
Tongue	37	19.2	8.1	2		1
Gingiva	15	7.8	13.3	1		1
Floor of mouth	28	14.5	10.7	2		1
Hard palate	9	4.7	11.1	1		
Other oral mucosa	31	16.1	12.9	4		
Major salivary glands	3	1.6	0.0			
Oropharynx						
Base of tongue	17	8.8	41.1	7		
Soft palate/uvula	9	4.7	0.0			
Tonsils	31	16.1	58.1	16	1	1
Oropharynx	5	2.6	0.0			
Other and unspecified pharynx	5	2.6	0.0			
	193	100.0[a]		33	1	4
Combined oral cavity	126	65.3		10		3
Combined oropharynx	67	34.7		23	1	1

[a]Total not equal 100% due to rounding.

identified as HPV-16-positive in the paraffin-embedded block were found to be HPV-negative in the malignant cells from LMD. LMD-tested cases with HPV-negative cancer sections ($n = 2$) remained negative. All specimens originally detected with LR types ($n = 3$) in the sections were evaluated by LMD to substantiate that the virus was located outside of tumor cells. Based on LMD, the tumor tissue was found to be either HPV-negative (section: HPV-17, hard palate; AF042003, gingiva) or infected with HPV-16 and not a LR type (section: HPV-13, floor of mouth). Microdissection confirmed that another case whose primary cancer was HPV-negative had an HPV-16 lymph node metastasis. Thus, although 5 cases with cancers changed HPV status, the total number with HPV-HR remained the same.

Demographic and risk factor characteristics

Risk factors associated with HPV-HR detection in oral cavity/oropharyngeal cancer cases are shown in Table II. Similar results were seen for the major risk factors when limited to cases with SCC ($n = 179$) and these are not presented. The adjusted results controlled for age, tobacco pack-years (never, ≤ 30, > 30) and alcoholic drinks/week. HPV-HR cases were significantly younger with the median age of the HPV-HR cases at 52 years compared to 64 years in the HPV-negative cases ($p = 0.0003$).

Sexual behavior was a significant risk factor for detection of virus in this cancer patient population. High-risk detected cases had a higher mean number of sex partners than the HPV-negative group (19 *vs*. 13 partners; $p = 0.02$) and a higher risk of infection among those reporting oral-anal (adjusted OR = 4.6) but not oral-genital contact. Males had more sexual partners than females (median, 6 *vs*. 1; $p < 0.0001$) and a higher percentage who had engaged in oral-genital sex (45% *vs*. 31%; $p = 0.07$), but not in oral-anal practices ($p = 0.5$). Despite the higher frequency of behaviors associated with sexually transmitted diseases, there was no difference in the viral positivity rate of males compared to females (22% *vs*. 15%; $p = 0.2$). Among women overall, there were no differences in oral-genital or oral-anal sexual practices and detection of HPV-HR, but females with HR detected cancers had more lifetime sexual partners (mean, 5 *vs*. 2; $p = 0.003$). No significant differences in any sexual behaviors were seen in males based on HPV status.

When further examined by age, it was apparent that these risky sexual practices were more frequent in the younger-age group (Table III). The rate of HPV-HR was 36% in younger cases but only 13% in the older-age group. Additionally, the younger cases had a higher median number of sexual partners compared to older cases (6 *vs*. 3; $p = 0.001$) and those 55 years of age and younger with ≥ 4 sex partners had an almost 4-fold greater risk of detection with HPV-HR compared to those > 55 years of age with ≤ 3 partners, They also had a significantly higher probability of engaging in both oral-genital sex and oral-anal sex than did older-age cases (Table III). Furthermore, younger-age cases were significantly more likely to have these risky sexual behaviors regardless of gender: higher lifetime number of partners (men, $p = 0.001$; women, $p = 0.03$), oral-genital sex ($p < 0.0001$, both genders) and oral-anal practices (men, $p = 0.09$; women, $p = 0.005$). Younger-age cases who did not engage in oral-anal sex remained at increased risk of having an HPV-positive cancer, most likely due to other risky sexual behaviors associated with viral transmission (Table III). There was no difference in risk of viral detection among those $>$ age 55 based on any of the sexual behaviors. Among those $\leq$ age 55, HPV-HR cases reported significantly more partners (median, 10 *vs*. 6; $p = 0.05$) than did HPV-negative younger-age cases and a higher risk of detection if they engaged in oral-anal sex (adjusted OR = 11.3; 95% CI = 1.6–79). Thus, the only significant difference in risk of HPV detection associated with sexual practices was seen in the younger group. It is worth noting that younger-age cases had a higher mean education level than the older-age group (13.3 *vs*. 12.1 years; $p = 0.003$). Table III shows that the analyses limited to SCC mirrored the overall findings for age, sexual practices and HPV status.

We next examined the association between HPV and the other major risk factors for HNC, tobacco and alcohol. Eighteen percent of cases were never smokers/never drinkers and 65% were current users of either or both substances. Among those who had ever used tobacco, HPV-HR cases smoked on average fewer pack-years than did HPV-negative cases (42 *vs*. 57 pack-years; $p = 0.03$), which might be expected based on their younger age. Nonetheless, there was no difference in the average number of cigarette equivalents/week by age group ($p = 0.6$). Compared to never users, former tobacco users also had a higher risk of detection with viral infection (Table II), as did those whose dose-duration usage was low/moderate (< 30 pack-years). There was no difference in alcohol status or the average number of drinks/week between HPV-HR and HPV-negative patients (40 *vs*. 37 drinks/week; $p = 0.7$). Nor was there significant dose-duration interaction between alcohol and tobacco in oral cavity/oropharynx cancer cases overall ($p = 0.3$) or in those with SCC ($p = 0.6$). The younger-age group drank more on average (44 *vs*. 35 drinks/week; $p = 0.1$) and compared to the older-age never-drinker group, there was significant effect modification ($p = 0.05$) between younger age and heavy alcohol consumption (≤ 55 years/> 21 drinks/week) associated with cancer HPV-HR status (adjusted OR = 10.3; 95% CI = 1.8–59.5). The risk was similar, although somewhat lower when data were

TABLE II – DEMOGRAPHIC AND RISK FACTORS FOR CANCERS OF THE ORAL CAVITY AND OROPHARYNX BY HPV STATUS

Characteristic	HPV-HR (n = 38; %)	HPV-negative (n = 155; %)	Adjusted OR (95% CI)[a]
Gender			
Male	28 (73.7)	97 (62.6)	1.5 (0.6–3.5)
Female	10 (26.3)	58 (37.4)	1.0
Race			
White	35 (92.1)	152 (98.1)	0.3 (0.05–1.6)
Other	3 (7.9)	3 (1.9)	1.0
Age			
≤ 55	21 (55.3)	37 (23.9)	3.4 (1.6–7.3)
> 55	17 (44.7)	118 (76.1)	1.0
Education			
≤ 12 years	22 (57.9)	102 (66.2)	1.0
> 12 years	16 (42.1)	52 (33.8)	1.1 (0.5–2.5)
Lifetime partners of			
0–3	14 (37.8)	83 (55.0)	1.0
≥ 4	23 (62.2)	68 (45.0)	1.2 (0.5–2.9)
Oral-genital contact			
Yes	23 (60.5)	53 (34.9)	1.6 (0.6–3.9)
No	15 (39.5)	99 (65.1)	1.0
Oral-anal contact			
Yes	5 (13.2)	3 (2.0)	4.6 (0.98–22.0)
No	33 (86.8)	149 (98.0)	1.0
Tobacco			
Never	5 (13.2)	35 (22.6)	1.0
Former	13 (34.2)	39 (25.2)	3.3 (0.96–11.2)
Current	20 (52.6)	81 (52.3)	1.4 (0.4–4.5)
Pack-years			
Never	5 (13.2)	35 (22.7)	1.0
0–30	14 (36.8)	28 (18.2)	2.9 (0.9–9.3)
> 30	19 (50.0)	91 (59.1)	1.3 (0.4–4.5)
Alcohol			
Never	7 (18.4)	42 (27.1)	1.0
Former	16 (42.1)	39 (25.2)	2.1 (0.6–7.6)
Current	15 (39.5)	74 (47.7)	0.8 (0.2–2.7)
Drinks per week			
Never	7 (18.4)	42 (27.1)	1.0
1–21	14 (36.8)	53 (34.4)	1.2 (0.3–4.1)
≥ 22	17 (44.7)	59 (38.3)	1.3 (0.3–5.3)
Tobacco/alcohol			
Never, never	5 (13.2)	29 (19.0)	1.0
≤ 30, ≤ 21	7 (18.4)	17 (11.1)	1.8 (0.5–7.0)
≤ 30, > 21	6 (15.8)	4 (2.6)	6.8 (1.3–36.3)
> 30, ≤ 21	7 (18.4)	30 (19.6)	1.7 (0.5–6.3)
> 30, > 21	11 (29.0)	54 (35.3)	0.9 (0.3–3.0)

Percentages based on available data.– [a]Adjusted for age, tobacco pack-years (never, 0–30, *vs.* > 30), drinks/week.

limited to the SCC cases (adjusted OR = 8.4; 95% CI = 1.4–49.4).

The medical history indicated no difference in genital warts, an abnormal Pap smear, cervical dysplasia, or genital cancer by HPV-HR status. However, men with HPV-HR cancers were more likely to report that their partners had a history of an abnormal Pap smear (adjusted OR = 5.5; 95% CI = 1.3–24.0) and cervical dysplasia (adjusted OR = 8.9; 95% CI = 1.3–61.4). These associations were seen primarily in the younger men based on all histologic types (≤ 55 years; $p = 0.06$, both conditions) as well as when limited to SCC malignancies. No differences were reported either in current or past history of other medical conditions, including immune-related diseases, diabetes, alcoholism, or benign oral lesions (*i.e.*, canker sores, oral herpes, lichen planus, oral leukoplakia).

Cancer cytology, morphology, stage, grade and site by HPV status

Table IV shows the multivariate analyses performed to evaluate cancer characteristics associated with viral infection. No tumor specimens and only 5% of exfoliated oral cell specimens were detected with nononcogenic HPV types in cases overall regardless of histology and in those limited to SCC. All exfoliated cells detected with HPV-HR types showed only the same 3 types found in tumor tissue specimens, namely HPV-16, -18 and -33. HPV-HR positivity based on oral exfoliated cells indicated that this assessment was highly significantly associated with HPV status in tumor tissue of the cancers independent of tobacco and alcohol use. This was true for oral cavity/oropharynx cancer cases, including all histologic types and SCC only.

Several cancer characteristics were associated with HPV-HR biopsy status. Those whose cancers were detected with HR types had a 3-fold greater risk of later-staged cancer. Over 3/4 of HPV-HR cases compared to only about half of the HPV-negative patients were identified with advanced stage (III or IV) and they were more than twice as likely to have positive lymph node status. The presence of oncogenic HPV types was not significantly different between well- and moderately differentiated cancers (13% *vs.* 17%; $p = 0.5$), but compared to these grades combined, the poor/undifferentiated cancers had at least a 3-fold higher odds of detection with HPV. The risk of HPV detection was not different between SCC and other histologic types. Among the 5 other histologic types reported in oral cavity/oropharynx cancers, HPV-HR was found in cancer cells of one verrucous carcinoma and one mucoepidermoid carcinoma, both containing HPV-16.

TABLE III – RISK OF HPV-HR CANCER ASSOCIATED WITH AGE AND SEXUAL PRACTICES

Age/Sexual Practice	*n*	All carcinomas (*n* = 193)		*n*	SCC only (*n* = 179)	
		HPV-HR (%)	Adjusted OR (95% CI)[a]		HPV-HR (%)	Adjusted OR (95% CI)[a]
Age/of partners						
> 55/≤ 3	80	10 (12.5)	1.0	72	9 (12.5)	1.0
> 55/≥ 4	52	7 (13.5)	1.0 (0.3–3.0)	51	7 (13.7)	1.1 (0.4–3.3)
≤ 55/≤ 3	17	4 (23.5)	2.3 (0.6–9.0)	17	4 (23.5)	2.3 (0.6–9.2)
≤ 55/≥ 4	39	16 (41.0)	3.8 (1.4–10.1)	35	15 (42.9)	4.4 (1.5–12.5)
Age/oral-genital sex						
> 55/no	102	12 (11.8)	1.0	93	11 (11.8)	1.0
> 55/yes	31	5 (16.1)	1.5 (0.5–4.7)	30	5 (16.7)	1.6 (0.5–5.2)
≤ 55/no	12	3 (25.0)	2.5 (0.6–10.9)	12	3 (25.0)	2.5 (0.6–11.4)
≤ 55/yes	45	18 (40.0)	4.3 (1.8–10.4)	41	17 (41.5)	4.8 (1.9–12.1)
Age/oral-anal sex						
> 55/no	132	17 (12.9)	1.0	122	16 (13.1)	1.0
> 55/yes	1	0 (0.0)	ND[b]	1	0 (0.0)	ND[b]
≤ 55/no	50	16 (32.0)	2.6 (1.1–5.9)	46	15 (32.6)	2.7 (1.2–6.4)
≤ 55/yes	7	5 (71.4)	19.5 (3.4–113)	7	5 (71.4)	19.5 (3.3–114)

[a]Adjusted for pack-group (never, 0–30, *vs.* > 30), drinks/week.– [b]Not computable.

TABLE IV – CLINICOPATHOLOGIC CHARACTERISTICS OF CANCERS OF THE ORAL CAVITY AND OROPHARYNX BY HPV STATUS

Characteristic	All carcinomas[a] (*n* = 193)			SCC only (*n* = 179)		
	HPV-HR (*n* = 38; %)	HPV-negative (*n* = 155; %)	Adjusted OR (95% CI)[b]	HPV-HR (*n* = 36; %)	HPV-negative (*n* = 143; %)	Adjusted OR (95% CI)[b]
HPV exfoliated cytology						
HPV-LR/negative	16 (42.1)	129 (84.9)	1.0	14 (38.9)	119 (85.0)	1.0
HPV-HR	22 (57.9)	23 (15.1)	7.8 (3.4–18.4)	22 (61.1)	21 (15.0)	9.7 (3.9–24.0)
Cancer location						
Oral cavity	13 (34.2)	113 (72.9)	1.0	11 (30.6)	102 (71.3)	1.0
Oropharynx	25 (65.8)	42 (27.1)	6.2 (2.7–14.5)	25 (69.4)	41 (28.7)	6.6 (2.7–16.2)
Stage						
0, I, II	9 (23.7)	73 (47.1)	1.0	9 (25.0)	68 (47.6)	1.0
III, IV	29 (76.3)	82 (52.9)	3.0 (1.3–7.1)	27 (75.0)	75 (52.5)	2.9 (1.2–7.0)
Lymph node status						
Negative	17 (44.7)	120 (77.9)	1.0	16 (44.4)	109 (76.8)	1.0
Positive	21 (55.3)	34 (22.1)	4.1 (1.9–9.1)	20 (55.6)	33 (23.2)	3.9 (1.7–8.8)
Metastases						
No	35 (94.6)	148 (97.4)	1.0	33 (94.3)	137 (97.9)	1.0
Yes	2 (5.4)	4 (2.6)	4.6 (0.7–31.5)	2 (5.7)	3 (2.1)	6.0 (0.7–49.4)
Cancer grade						
Well/moderately	23 (62.2)	127 (82.5)	1.0	23 (63.9)	122 (85.3)	1.0
Poorly/undifferentiated	14 (37.8)	27 (17.5)	3.0 (1.3–7.0)	13 (36.1)	21 (14.7)	3.7 (1.5–9.2)
Histology type						
SCC	36 (94.7)	143 (92.3)	2.2 (0.4–12.1)			
Other[a]	2 (5.3)	12 (7.7)	1.0			

[a]Includes HPV-positive (1 verrucous carcinoma and 1 mucoepidermoid carcinoma) and HPV-negative (4 verrucous carcinomas, 4 mucoepidermoid carcinomas, 2 adenoid cystic carcinomas, 1 acinic cell carcinoma, 1 adenocarcinoma, not otherwise specified).– [b]Adjusted for age, tobacco pack-years (never, 0–30, *vs.* > 30), drinks/week.

Oropharynx and oral cavity sites

Oropharyngeal cancers were significantly more often detected with HPV DNA than were sites in the oral cavity (Table IV). Nonetheless, one of the potential risk factors for viral transmission, number of partners (≥ 4), was higher in cases with virus detected in cancers of the oral cavity compared to the oropharynx (85% *vs.* 50%; $p = 0.07$) and significantly so in those with SCC ($p = 0.03$). HPV DNA was more likely to be detected in younger-age cases (≤ 55 years) regardless of cancer site: oropharynx, adjusted OR = 3.9, 95% CI = 1.2–12.7; oral cavity, OR = 3.4, 95% CI = 1.0–12.0. There were no significant differences in other demographic characteristics, sexual practices, oral or genital lesions, or medical conditions between the site-specific HR cases and negative cases. On average, those detected with HPV-HR in oropharyngeal cancers used tobacco less than did HPV-negative oropharyngeal cases (mean, 35 pack-years *vs.* 52 pack-years; $p = 0.04$), but were similar in alcohol consumption. There were no apparent dose-duration effects for either tobacco or alcohol between HPV-positive and HPV-negative cases in the oral cavity.

DISCUSSION

Our data provide evidence that younger-age cases with cancers of the oral cavity/oropharynx may have a higher prevalence of HPV in tumors because of behaviors linked to sexually transmitted diseases. This observation is consistent with data on cancers of the cervix and anogenital areas that involve viral transmission through direct physical contact.[22] Our results show that HPV-HR types are significantly more likely to be detected in younger adults with these HNC, 36% compared to 13%. This observation is supported by other studies reporting 36–78% positivity in those less than age 50–60 years compared to 12–29% in those greater than age 60.[6,9,23,24]

Although much speculation has been made about sexual transmission of the virus in HPV-HR HNC, few large-scale investigations have been conducted. A study by Schwartz *et al.*[6] found a higher odds of HPV-16 in oral cancer patients with 15 or more sex partners (adjusted OR = 2.5; 95% CI = 1.1–5.6), but the data were not presented by age group. In addition to identifying an increased risk of HPV detection associated with more sex partners (≥ 4) among this group of HNC cases compared to HPV-negative con-

trols, we found that the average age at first oral-genital contact in those ≤ 55 years was significantly earlier compared to those > 55 years (all histologic types, 20 *vs*. 27 years, $p = 0.006$; SCC, 20 *vs*. 27 years, $p = 0.009$), whereas the actual number of years of practicing this sexual behavior was not different (all histologic types, 20 *vs*. 20 years, $p > 0.5$; SCC, 19 *vs*. 20 years, $p > 0.5$). The sexually transmitted disease-related risk pattern seen in our younger cases is similar to that found for cervical cancer, namely, the risk of developing a malignancy is higher among those with younger age at the onset of sexual activity. Another finding in this study, that the risk of HPV-HR was greater among men whose female partners had a history of either an abnormal Pap smear or a cervical dysplasia, is consistent with data from a large Swedish cancer database.[25] That study found that husbands of women with invasive cervical cancer subsequently had a significantly greater standardized incident rate (SIR) at several HNC sites (SIRs = 2.0–2.7). Thus, the results in our investigation are consistent with a role for high-risk HPVs as sexually transmitted pathogens in the etiology of a subset of HNC.

In a previous case-control study, we reported that detection of HPV was an independent risk factor for oral cancers based on exfoliated oral cells.[1] In the current assessment of HPV presence in cancer tissue, we again found little indication that viral status was linked to either tobacco or alcohol use in these HNC cases. We and others[8,9,26] have shown evidence that HPV-HR carcinomas were found more often in those who had lower alcohol and/or tobacco exposures. In this study, patients were at higher risk of detection with HPV-HR types if they were low to moderate smokers compared to heavy smokers. Klussmann *et al*.[8] found significantly less alcohol use among HPV-HR tonsillar carcinoma cases ($p = 0.03$) compared to a combined group of patients who were HPV-negative at other HNC sites. Although we did not find this independent effect of alcohol, we did observe a significant interaction effect between younger age and heavy alcohol use associated with HPV-HR cancer status. Whether the risk associated with HPV DNA in HNC is further altered by interaction with alcohol or tobacco is still unclear since few investigations have evaluated this issue. Whereas we showed no interaction effect between alcohol and tobacco based on several methods (total dose-duration: pack-years × drinks/week; categorization by ever/never status and by never/current/former group), Schwartz *et al*.[6] found significant interaction only between HPV type 16 capsid seropositivity and current smoking but not alcohol. The dissimilar findings regarding the relationships between tobacco, alcohol and HPV-HR infection in HNC may be due to differences in patient characteristics, the mode of HPV detection assays (by genome DNA amplification or seropositivity against capsid antigens), or the anatomic sites examined, which may vary in susceptibility to these risk factors. In addition, whether there is a different carcinogenic pathway for HNC patients detected with HPV-HR types compared to those cancers related to heavy use of tobacco and alcohol is unclear and warrants further investigation.

Several studies have reported that detection of HPV DNA is highly correlated with poorly differentiated cancer grade[6,8,9,27,28] or positive lymph nodes.[5,9] In addition to higher cancer grade, this investigation found that HPV-HR in oral cancers also were significantly associated with positive nodal status and late-stage disease. Furthermore, metastatic cancer was more frequent among the HPV-HR cancers in our investigation. Despite these findings, we found that overall survival was significantly higher in these HPV-HR compared to HPV-negative cases (hazards ratio = 0.3).[29] Future clinical management and treatment decisions may need to take into consideration differences in the extent of disease associated with HPV infection and possibly recognize virus-related tumors as a separate disease entity.

HPV infection has not been established to play a role in HNC other than SCC. In anogenital cancer, however, HPV-HR infection is implicated in the development of tumors other than SCC, including adenocarcinomas and small cell carcinomas of the cervix.[30,31] In our study, 2 of 14 non-SCC cancers (a verrucous and a mucoepidermoid carcinoma) were HPV-positive. It is intriguing that both HPV-associated malignancies were also of squamous epithelial cell origin. Verrucous carcinoma (VC) and SCC both stem from the epithelial cells that line the oral cavity and mucoepidermoid carcinomas are thought to arise from the squamous cells lining the salivary ducts. Others have reported cases of HPV-16- and -18-positive non-SCC HNC, including VC.[32,33] Although more definitive studies are needed, it is possible that HPV may play a role in other carcinomas of the head and neck, especially those with a squamous epithelial cell origin.

Our study is one of the few to use LMD to evaluate whether the viral genomes are actually located in the tumor cancer cells or only elsewhere in adjacent normal tissue. This evaluation is important in excluding potential false positive findings associated with low-risk HPV types that are not known to have oncogenic potential, as well as in finding high-risk types only in normal tissue. Our molecular pathology data (to be presented in detail elsewhere) indicated that all 10 oral cancer lesions positive for HPV-HR types 16, 18, or 33 examined by LMD harbored the viral genomes within the tumor, while the adjacent epithelium was HPV-negative. In addition, 2 of 2 HPV-negative cancers were confirmed as negative by using LMD. This is in agreement with the results of a previous study published by one of us in collaboration with others,[8] which also found HPV-16 sequences only in cancers and not in adjacent mucosal tissue. Taken together, these results strongly support the hypothesis that the continued presence and expression of HPV-HR genes are required in HPV-positive HNC, as has been shown in cervical carcinogenesis.

We also employed LMD to clarify the potential role of non-HR HPV types in HNC. Our initial DNA amplification data of whole tissue sections had suggested that 3 cancers harbored other HPV types: HPV-13, HPV-17, and an as yet unnamed cutaneous isolate, AF042003. However, as described in our results, none of these HPV types was detected by LMD within the tumor tissue: 2 tumors were HPV-negative and 1 harbored HPV-16. In addition, 2 other tumors that scored as HPV-16 on whole sections were HPV-negative using LMD, indicating that the virus was also located outside of the tumor tissue. Other studies have reported the presence of non-HPV-HR types (HPV-6, -11 and other types often found in skin) in sections containing both tumor and adjacent tissue.[5,6] Our results suggest that non-HPV-HR types were not present in the tumors but were elsewhere. Any possible involvement of other HPV types in HNC will thus need to be reexamined by LMD or *in situ* hybridization techniques that can determine whether low-risk HPV types reside in HNC or elsewhere in the specimen.

The current study also indicates that detection of HPV-HR types in exfoliated cytology specimens collected from oral rinses may predict HPV-HR infection in cancers of the oral cavity and oropharynx. To maximize HPV DNA detection, we carefully controlled the inclusion of an adequate number of nucleated squamous cells in each oral specimen prior to amplification and used DNA sequencing to maximize detection of oral HPV DNA. Based on the results of this large sample size, oral exfoliated cell DNA appears to be a promising source to evaluate the risk of HPV-HR HNC. It is not apparent at present whether the HPV-HR genomes detected in the exfoliated cell DNA come from HPV-HR-harboring cancer cells collected by the rinse. Although the HPV-HR types identified in the cytologic specimens were usually the same as those found in the tumor tissue, the virus may stem from adjacent mucosal cells persistently infected with the same HPV-HR type from which the tumor has evolved. In contrast to cancer cells, in which either integrated HPV fragments or replicating HPV plasmid genomes range from 1 up to 10–30 copies per cell, HPV DNA becomes amplified to over 10,000 copies per cell in a few differentiated mucosal cells (referred to as jackpot cells) in the productively infected epithelium. A cytologic specimen thus could register as positive due to a single jackpot cell. HPV-HR-positive cytology

could, in fact, also result from an independent HPV infection, possibly with a different HPV-HR type, yet may still reflect the individual's increased susceptibility to HPV infection due to, for example, an altered immune response. These questions will need to be addressed in future studies focusing on the analysis of HPV integration and the identification of specific gene mutations in HPV-HR HNC. Such genetic alterations could serve as clonal markers to compare in the tumors and exfoliated cells. We and others believe that additional research may lead to a highly sensitive laboratory test to detect HPV in exfoliated cells that predicts current viral tumor status.[10]

Future research on the role of HPV infection in HNC should emphasize the evaluation of HPV-HR cancers for integration and gene expression as well as the analysis of genetic changes associated with different cofactors needed for initiation and promotion of carcinogenesis.[10] Specific integration patterns and other genetic alterations will also serve as clonal markers to explore HNC clonal progression (within the lesion as well as in the primary tumor and lymph node metastases), to distinguish potential multiple primaries and to elucidate the relationship between findings in tumor tissue and oral rinse cytology specimens. Large prospective human studies are needed to establish the temporality of HPV exposure and subsequent HNC development. Sensitive assays using oral exfoliated cells may provide high predictive power for detecting microsatellite alterations significant in early development of malignancies since most HNCs are not clinically apparent or symptomatic as premalignant tumors. This information may also be used to predict different treatments and monitor relapse.

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