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HUMAN PAPILLOMAVIRUS INFECTION AS A PROGNOSTIC FACTOR IN CARCINOMAS OF THE ORAL CAVITY AND OROPHARYNX

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Although studies have established human papillomaviruses (HPVs) as a risk factor for oral and oropharyngeal cancer, it is not clear whether viral infection affects survival in head and neck malignancies. This investigation examined the relationship between HPV and survival in carcinomas of the oral cavity and oropharynx. Formalin-fixed, paraffin-embedded tumor specimens from 139 newly diagnosed cases were tested for HPVs by PCR and DNA sequencing. Patient and tumor characteristics were obtained from questionnaires, pathology reports and cancer registries. Odds ratios (ORs) and relative risks (RRs) were based on logistic and Cox regression models, respectively. HPVs were detected in 21% of the tumors; 83% were HPV-16. Greater risk of HPV infection was associated with males (OR = 2.9, 95% CI = 1.0–8.6), a history of oral-genital sex (OR = 4.2, 95% CI = 1.5–11.7), and oropharyngeal tumors (OR = 10.4, 95% CI = 3.5–31.2). As tobacco usage increased, the odds of HPV detection decreased (OR = 0.97/pack-year, 95% CI = 0.96–0.99). HPV infected patients had better overall survival (RR = 0.3, 95% CI = 0.1–0.8) than those with HPV-negative tumors. There was an interaction between gender and HPV for overall (p = 0.05) and disease-specific (p = 0.03) survival that suggested that HPV infected males had better prognosis than HPV-negative males, but this was not the case among females. HPV status was identified as an independent prognostic factor in oral and oropharyngeal cancers. This result appeared to be gender-specific, suggesting the need for further study of the interaction between HPV and gender on survival.

Key words: *human papillomavirus; survival; oral cancer; oral cavity; oropharynx*

The US incidence rate for cancers of the oral cavity and pharynx (ICD-0: C00–C14) is 11.9 per 100,000.[1] Age-adjusted incidence and mortality rates increase with age and are almost 3 times greater in males than females.[1] Major risk factors in Westernized countries are considered to be tobacco and alcohol and these risks have not changed over the decades.[2–4] A link between oncogenic types of the human papillomavirus (HPV) and risk of oral and oropharyngeal cancer also has been suggested in the literature.[5–8]

HPVs are icosahedron tumor viruses with 8 kb DNA genomes. Over 120 types of HPV have been identified. HPV types are often designated as high risk (oncogenic) or low risk (nononcogenic). The high-risk types include those initially identified in cervical dysplasia and cancer, as well as those types whose DNA sequences are most closely related to them.[9]

Several researchers have examined the association between survival and detection of high-risk HPV types in cancer specimens. Results from studies that involved head and neck cancers including carcinomas of the oral cavity and oropharynx have reached different conclusions. Some researchers have found that patients with HPV detected in their cancers, as compared to those without, have better prognosis,[5,10,11] while others have found no difference[12,13] or poorer[14] prognosis. Among studies that have focused on oral and oropharyngeal squamous cell carcinomas (SCCs), the results also have not been consistent. A few studies[15,16] have found that HPV-infected patients have better survival than those with HPV-negative tumors. Mellin *et al.*[17] also found this to be the case among tonsillar SCCs. However, another group[18] found no difference in survival by HPV status among stage I/II SCCs and slightly poorer prognosis in HPV positive tumors among stage III/IV oral SCCs. Most recently, Lindel *et al.*[19] did not find a significant association between HPV infection and overall survival among SCCs of the oropharynx after adjusting for other prognostic factors.

The objectives of our study were to examine 1) the association between patient survival and the presence of HPV DNA in tumors among oral cavity and oropharyngeal cancer cases, while adjusting for other prognostic factors or potential confounders, and 2) possible interaction effects on survival between other prognostic factors and HPV DNA status.

MATERIAL AND METHODS

Patient characteristics and data collection

Patients were newly diagnosed oral and oropharyngeal cancer cases enrolled between March 1994 and September 1997 as part of a larger case-control study conducted at the University of Iowa Hospitals and Clinics (UIHC) and the Iowa City Veterans Affairs Medical Center.[6,20] All participants signed a Human Subjects consent form. Data regarding demographics, risk factors for oral

Abbreviations: AJCC, American Joint Committee on Cancer; CI, confidence interval; DNA, deoxyribonucleic acid; HPV, human papillomavirus; ICD, International Classification of Disease for Oncology (ICD-O); LMD, laser-assisted microdissection; NCI, National Cancer Institute; PCR, polymerase chain reaction; OR, odds ratio; RR, relative risk; SCC, squamous cell carcinoma; SEER, Surveillance, Epidemiology and End Results; UIHC, University of Iowa Hospital and Clinics; XRT, radiation therapy

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and oropharyngeal cancer, and risks related to HPV exposure were collected by a self-administered questionnaire. TNM staging based on the American Joint Committee on Cancer (AJCC)[21] and treatment were obtained from the UIHC Tumor Registry and from chart reviews. Tumor site, grade and histology were based on pathology reports. Dates of death or last known to be alive were obtained from the NCI Iowa SEER Cancer and the UIHC Tumor Registries. Cause of death was based on ICD-9 (deaths before 1999) and ICD-10 (deaths after 1999) codes from death certificates for in-state patients. Cause of death for the few out-of-state patients ($n = 8$) was obtained through direct contact with the patient's family or physician or through the research nurse coordinator for head and neck cancer patients. Tissue samples were obtained from patients prior to any treatment.

Of the 148 newly diagnosed cases enrolled in the study, formalin-fixed, paraffin-embedded tumor tissue was available for DNA analysis for 141 cases. Two of these cases were excluded from further analyses due to inadequate DNA (β-globin negative). The mean age of patients was 61 years (range 20–93); 64% were males. Many of the patients had been exposed to tobacco (82% ever users and 50% current users) and alcohol (76% ever users and 47% current users) on a weekly basis for at least 1 year. More than 50% of the patients were initially diagnosed with advanced stages of disease (2% stage 0, 14% stage I, 21% stage II, 19% stage III, and 44% stage IV). Almost all tumors (92%) were SCCs and 76% were well or moderately differentiated. In cervical cancer, HPV has been detected in a wide range of different histologies including adenocarcinomas[22] and therefore strongly suggested other histologies should be examined in carcinomas of the oral cavity and oropharynx. Tumor sites included tongue ($n=30$), base of tongue ($n=11$), gingiva ($n=11$), floor of mouth ($n=23$), palate ($n=13$), lip ($n=1$), other or unspecified parts of the mouth ($n=20$), tonsils ($n=22$), other or unspecified parts of oropharynx ($n=4$), pharynx ($n=3$) and parotid gland ($n=1$), and were dichotomized into oral cavity *vs*. oropharynx groupings as suggested by the AJCC[21] with the exception that the tumor located in the parotid gland was placed in the oral cavity group. Forty-two percent of the patients were treated with both surgery and radiation, 35% were treated with surgery only, 16% were treated with radiation and no surgery and the remaining cases received neither surgery or radiation with the exception of one case for which the treatment was unknown.

Tumor specimens

The hematoxylin and eosin-stained glass slides for all tissue blocks were evaluated to verify the presence of malignant tissue, diagnosis and tumor grade. For each case, the block with the highest percentage of tumor was selected for DNA analysis. On average 44% of the tissue in the selected blocks was tumor, with a median value of 40%. The assumption was made that the sections taken from the block were adjacent to those on the archived stained slides and that the slide accurately represented the sections taken from the block. This was verified in 10% of the cases. Sections that were 17 μm thick from each block were made on a Model 820 microtome (American Optical Spencer, NY). To decrease the variation in total amount of tumor obtained from the specimens, the number of sections taken from the blocks varied, depending on the amount of tumor in the block, as established by multiplying the area of tissue on the section by the percent of malignant cells in the section.[23] The goal was to obtain 400–500 mm^2 of tumor while not exhausting the tissue in the block. To prevent cross-contamination and to allow detection of such, the microtome blade was cleaned with a disposable tissue soaked with 70% ethanol before and after each block was sectioned. The sections were transferred to a sterile 1.5 ml Eppendorf tube with tweezers, which were then cleaned with a disposable tissue soaked in 70% ethanol. After each tenth tumor block, 5 sections were taken from a negative control block of a polypoid fibrous hyperplasia from the oral cavity.

DNA extraction and HPV detection

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 were essentially those previously reported in Summersgill *et al*.[20] Briefly, 2% of the DNA extracted from biopsy specimens were PCR-amplified with MY09 and MY11 primers[24] to detect HPV. Included in the same PCR reaction was a primer (HMB01) designed to amplify HPV-51.[25] Also included in the PCR reaction were primers that amplify a portion of the β-globin gene,[26] which was used to verify the presence of intact DNA and the adequacy of PCR amplifications. Twenty percent of the PCR reaction was examined after electrophoresis in agarose gel for the presence of HPV and β-globin PCR products. When a sample was negative for β-globin, additional sections were taken from the block and reanalyzed. An aliquot of the PCR product was transferred to a nylon membrane and hybridized with ^{32}P-labeled probes for a more sensitive detection of HPV (dot blot hybridization). The samples positive only after the membrane hybridization underwent hemi-nested PCR-amplification with MY09 and GP5+[27] primers. PCR products of the expected size were sequenced to determine HPV type. These techniques allowed us to identify low copy numbers of a broad range of low-risk and high-risk HPV types.

Laser-assisted microdissection (LMD)

Because it can be argued that the source of the virus in tumor sections was not the tumor cells, but connective tissue, normal epithelium, inflammatory cells or contamination, laser-assisted microdissection (LMD) was performed on a subset of oral cavity ($n=8$) and oropharyngeal ($n=8$) tumors. LMD was performed as previously described.[28] 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) and coated with 1% poly-L-lysine for increased tissue adhesion. Sections were deparaffinized and stained with hematoxylin. Laser-assisted microdissection was performed using the Robot-MicroBeam system (PALM). Under microscopic control, tumor cells, normal and dysplastic mucosa and metastatic tumor cells were laser-dissected and then digested in 15 μl PCR-buffer containing proteinase K (400 μg/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[27] in a hot-start PCR or with nested PCR,[29] using 7.5 μl of the DNA each for β-globin PCR and HPV PCR. To assure independent, blinded results, microdissection was performed in a separate laboratory (Cologne, Germany) on coded slides.

Statistical analysis

Total cumulative tobacco exposure was expressed in pack-years, where 1 pack-year is equivalent to smoking 1 pack of cigarettes a day for 1 year with 20 cigarettes/pack. Alcohol exposure was defined as the average number of drinks per week. The Wilcoxon rank sum test was used to compare continuous variables between groups of patients. The chi-square test was used to compare categorical variables between groups. Multiple logistic regression was used to estimate the odds ratios (ORs) for HPV DNA detection. Backward stepwise modeling with significance set to $p < 0.10$ was used to determine the adjustment variables for the ORs of HPV detection. HPV DNA results were based on the full-section paraffin-embedded results unless LMD results were available, in which case the LMD results were used. Analyses also were conducted based on paraffin-embedded results only, but the conclusions were similar to those presented.

Survival was measured in years from the date of diagnosis until death or until the date the patient was last known to be alive. Patients who died of other causes than oral and oropharyngeal

cancer were considered censored observations in the disease-specific survival analyses. Time-to-event measures were estimated by the Kaplan-Meier method.[30] Relative risks (RRs) were estimated from the Cox proportional hazards models.[31] The proportional hazards assumption was assessed as discussed by Allison.[32] In the final Cox regression models, interaction terms between HPV status and the other prognostic factors and confounders were examined. Ninety-five percent confidence intervals (CIs) for ORs and RRs were based on normal approximations.

Two models are presented for the multivariate survival analyses. The first, labeled Model 1, was based on a backward stepwise selection procedure that was used to assess the association between HPV infection and survival, while adjusting for other prognostic factors and confounders. The significance level used in the Cox models was set to $p < 0.20$ to allow for assessment of confounding. HPV status was included in the modeling process at each step. Variables considered in the modeling process were age, gender, education, number of partners, oral-genital sex history, tumor location (oropharynx *vs*. oral cavity), tumor histology (SCC *vs*. other carcinomas), tumor grade, stage of disease at diagnosis, pack-years, tobacco status, average number of drinks per week, alcohol status and treatment modality. Continuous and categorical scales for age, tobacco pack-years, alcoholic drinks and number of partners were examined in the models. The second, labeled Model 2, adds tobacco usage, alcohol usage and tumor histology to the first model derived from the statistical modeling criteria. In addition, to assure that inclusion of the other histologies did not obscure any of the reported results, analyses were conducted among SCC cases only. Some of the results from analyses that involved SCCs only are presented; all results were similar to those from analyses that involved all carcinomas. The same factors were controlled for in the analyses among SCCs only with the exception that tumor histology was not included in Model 2. All reported *p* values were 2-sided. All statistical analyses were performed using SAS version 8.0.[33] Survival curves were created using SPlus 2000.[34]

RESULTS

HPV DNA

Twenty-one percent of the tumors were detected with oncogenic types of HPV (24 HPV-16, 1 HPV-18 and 4 HPV-33; 95% CI = 14–28%). No low-risk types were detected. LMD demonstrated, without exception, that the viral DNA could be detected in cancer tissue but not in normal-appearing epithelium or in connective tissue. Demographics, tobacco and alcohol exposure, tumor descriptors and sexual behavioral characteristics by HPV DNA status are presented in Table I. Based on the multivariate analyses, the odds of HPV infection among those reporting a history of oral genital sex was over 4 times that of those not reporting such a history. Other significant risks for HPV infected tumors included male gender, oropharyngeal tumors and fewer tobacco pack-years.

TABLE I – DEMOGRAPHIC CHARACTERISTICS AND RISK FACTORS FOR HPV-INFECTED TUMORS

Characteristic	HPV-pos[1] (N = 29)	HPV-neg[1] (N = 110)	OR(95% CI)[2]	Adj-OR(95% CI)[3]
Age[2]	52 (20–78)	63 (26–93)	0.96 (0.93–0.99)	0.98 (0.93–1.03)
Age				
≤ 60	20 (69)	45 (41)	3.2 (1.3–7.7)	1.4 (0.4–5.1)
> 60	9 (31)	65 (59)	1.0	1.0
Gender				
Female	8 (28)	42 (38)	1.0	1.0
Male	21 (72)	68 (62)	1.6 (0.7–4.0)	2.9 (1.0–8.6)
Education				
≤ 12	16 (55)	73 (66)	0.6 (0.3–1.4)	1.3 (0.4–4.0)
> 12	13 (45)	37 (34)	1.0	1.0
Number of sex partners				
0–1	3 (10)	40 (36)	1.0	1.0
2–6	12 (41)	38 (35)	4.2 (1.1–16.1)	3.3 (0.7–15.3)
>6	14 (48)	29 (26)	6.4 (1.7–24.5)	3.9 (0.6–26.3)
Oral-genital sex				
No	9 (31)	69 (64)	1.0	1.0
Yes	20 (69)	39 (36)	3.9 (1.6–9.5)	4.2 (1.5–11.7)
Tumor location				
Oral cavity	10 (35)	84 (76)	1.0	1.0
Oropharynx	19 (65)	26 (24)	6.1 (2.5–14.8)	10.4 (3.5–31.2)
Tumor histology				
SCC	28 (96)	100 (91)	2.8 (0.3–22.8)	3.3 (0.3–33.8)
Other carcinomas[4]	1 (4)	10 (9)	1.0	1.0
Tumor grade				
Well/Moderate	17 (59)	89 (81)	1.0	1.0
Poor	12 (41)	21 (19)	3.0 (1.2–7.2)	2.1 (0.7–6.1)
Stage				
0/I	4 (14)	19 (17)	1.0	1.0
II	3 (10)	26 (24)	0.5 (0.1–2.7)	0.3 (0.1–2.1)
III	4 (14)	22 (20)	0.9 (0.2–3.9)	0.3 (0.04–1.9)
IV	18 (62)	43 (39)	2.0 (0.6–6.7)	0.9 (0.2–4.5)
Tobacco status				
Never	3 (10)	22 (20)	1.0	1.0
Ever	26 (90)	88 (80)	2.2 (0.6–7.8)	3.0 (0.6–15.8)
Tobacco pack-years[2]	27 (0–82)	43 (0–150)	0.99 (0.97–1.00)	0.97 (0.96–0.99)
Alcohol status				
Never	5 (17)	28 (26)	1.0	1.0
Ever	24 (83)	82 (74)	1.6 (0.6–4.7)	1.6 (0.4–6.5)
Drinks/week[2]	12 (0–114)	12 (0–210)	1.00 (0.99–1.01)	1.00 (0.99–1.02)

[1]Frequencies (percentages) are reported for categorical variables and medians (minimum-maximum) are reported for continuous variables;–[2]For continuous variables, odds ratios based on a one-unit increment.–[3]Adjusted for gender ($p = 0.058$), history of oral-genital sex ($p = 0.006$), tumor location ($p < 0.0001$) and tobacco pack-years as a continuous covariate ($p = 0.003$);–[4]includes 4 mucoepidermoid, 3 verrucous, 2 adenoid cystic, 1 acinar cell carcinoma and 1 adenocarcinoma; HPV-positive case in a male with a mucoepidermoid carcinoma.

These same risk factors for HPV detection were identified among analyses including SCCs only [oral-genital sex OR=4.4 (1.5–12.7); male gender OR = 3.3 (1.1–10.2); oropharyngeal tumors OR = 9.7 (3.2–29.4) and tobacco pack-years OR = 0.97 (0.95–0.99)]. The differences between the crude and adjusted ORs from Table I for age, education and number of partners are most likely due to the fact that those with a history of oral-genital sex were younger on average (50.7 *vs.* 68.5 years, $p < 0.001$), had more years of education (13.5 *vs.* 11.6, $p < 0.001$) and had more sexual partners over their lifetime (median 10 *vs.* 2, $p < 0.001$) than those without this history. Interestingly, among the 11 tumors with a histology type other than SCC, 1, which was a mucoepidermoid carcinoma, was detected with HPV-16.

Gender was not significantly associated with HPV-positive tumors in unadjusted analyses but was in multivariate analyses. Males tended to have riskier behaviors. They reported more tobacco (median pack-years: 45 *vs.* 25, $p < 0.01$) and alcohol usage (median drinks/week: 24 *vs.* 2, $p < 0.001$), and more sexual partners (median: 6 *vs.* 1, $p < 0.001$) but were not significantly more likely to report having engaged in oral-genital sex (47% vs. 37%, $p = 0.26$) than females. Males also were slightly younger than females (median: 60 *vs.* 65, $p = 0.07$).

Survival

Among the 63 deaths, 59% were due to oral or oropharyngeal cancer. Seventeen percent of all cases were never disease free. Of those last known to be alive, the median follow-up period was 4.8 years with a maximum of 6.6 years; 96% were followed for at least 2 years. Eighteen percent of those last known to be alive were treated for recurrence of the disease with minimum follow-up of 3.8 years.

Curves for overall and disease-specific survival by HPV DNA status are presented in Figure 1. Patients with HPV-positive tumors had better overall survival than those with HPV-negative tumors (RR=0.5, 95% CI = 0.3–1.1, $p = 0.07$). The 5-year survival rate for those with HPV-positive tumors was 71% *vs.* 49% for those with HPV-negative tumors. There was not a significant difference in the disease-specific survival curves by HPV DNA tumor status (Fig. 1*b*, $p = 0.62$).

Table II presents the adjusted relative risks and their corresponding 95% confidence intervals for overall and disease-specific survival. Multivariate analyses suggested that age, gender, tumor grade, stage of disease at initial diagnosis and treatment should be included in the model (see Model 1 in Table II). Surprisingly, neither tobacco nor alcohol usage was found to be prognostically significant. A second model (labeled Model 2 in Table II) is presented that adds tobacco pack-years, average number of drinks per week and tumor histology to Model 1. Inclusion of tobacco and alcohol usage, 2 major risk factors for oral cancer as well as for other diseases, seemed necessary to clarify their possible impact on the association between HPV infection and survival, especially given lesser tobacco usage was associated with HPV DNA detection. Other researchers[5,15,19] also have adjusted for these factors in their multivariate survival analyses. Tumor histology was added to the model to account for the fact that most studies have only included SCC cases. All covariates in the models satisfied the proportional hazards assumption.

Similar to the unadjusted analyses, results from the multivariate analyses suggested that patients with HPV-infected tumors had significantly better overall survival than those with HPV-negative tumors (Model 1: RR=0.46, $p = 0.05$; Model 2: RR=0.33, $p = 0.02$). Among SCCs only, results were similar (Model 1: RR = 0.50, $p = 0.09$; Model 2: RR = 0.39, $p = 0.04$). Although not statistically significant, patients with HPV-positive tumors also tended to have better disease-specific survival than those with HPV-negative tumors among all carcinomas (Model 1: RR = 0.59, $p = 0.25$; Model 2: RR = 0.45, $p = 0.13$) and among SCCs only (Model 1: RR = 0.74, $p = 0.53$; Model 2: RR = 0.55, $p = 0.27$).

Males had significantly worse overall and disease-specific survival than females. This might be explained by the result that males had significantly higher tobacco pack-years and average drinks per week than females but also might explain the lack of significant association found between tobacco and alcohol usage with survival. To study this further, gender was excluded from the modeling process. Without gender, the same prognostic factors were selected. In addition, tobacco and alcohol usage remained highly non-significant ($p > 0.60$). Based on model 2 from Table II, we also examined 2-way interactions between gender, pack-years and average drinks per week. None was significant (all $p > 0.10$).

For the interactions with HPV infection, the interaction between gender and HPV status was the only one that approached statistical significance for both overall (Model 1: $p = 0.09$; Model 2: $p = 0.05$) and disease-specific (Model 1: $p = 0.07$; Model 2: $p = 0.03$)

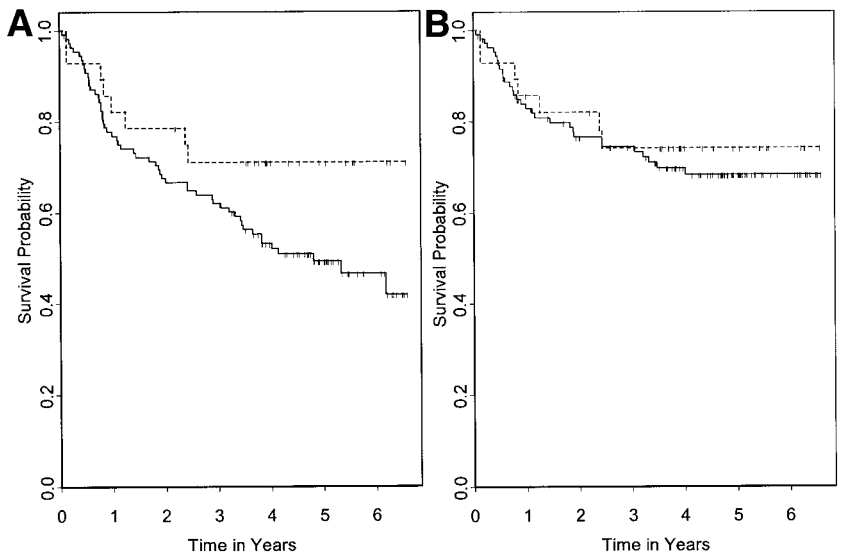

FIGURE 1 – Survival by HPV tumor status. Overall survival (*a*) and disease-specific survival (*b*) curves based on the Kaplan-Meier method were analyzed. The solid line is for those with HPV-negative tumors ($n=110$) and the dashed line is for those with HPV-positive tumors ($n=29$). Cause of death was unknown for one case with an HPV-negative tumor. Vertical tick marks on curves indicate censored observations.

survival. This was also observed among analyses involving SCC cases only; p values were the same. Figure 2 presents the overall and disease-specific survival curves by HPV DNA status and gender. Males with HPV-positive tumors tended to have the best overall and disease-specific survival. Table III presents the relative risk estimates by HPV status and gender using males with tumors that were HPV-negative as the reference group. Males infected with HPV had significantly better overall (Model 1: RR=0.29, p = 0.02; Model 2: RR=0.19, p <0.01) and disease-specific survival (Model 1: RR=0.33, p = 0.08; Model 2: RR=0.20, p=0.03) than males with HPV-negative tumors. In contrast, there was not a significant difference in overall and disease-specific survival between the HPV groups among females (all $p > 0.25$). To further describe the interaction, HPV-negative females tended to have significantly better prognosis than HPV-negative males, whereas males with HPV-positive tumors appeared to have better survival than females with HPV-positive tumors. The latter comparison was not statistically significant. Analyses among SCC cases only also suggested that HPV was an independent prognostic factor for overall survival (Model 1: RR = 0.31, p = 0.03; Model 2: RR = 0.22, p = 0.01) and disease-specific survival (Model 1: RR = 0.41, p = 0.17; Model 2: RR = 0.23, p = 0.05) among males but not among females (data shown in Table III).

To determine if other factors were explaining the interaction between HPV and gender, the available data among the 4 HPV and gender strata were compared. All of the women detected with HPV in their tumors had advanced stage of disease at initial diagnosis (25% stage III and 75% stage IV). In contrast, less than 70% of the patients from the other groups were initially diagnosed with advanced stage of disease (male/HPV-negative 60%; male/HPV-positive 67%; and female/HPV-negative 57%). The adjusted rel-

TABLE II – ADJUSTED RELATIVE RISK ESTIMATES WITH 95% CONFIDENCE INTERVALS FOR SURVIVAL[1]

Characteristic	Overall survival		Disease-specific survival	
	Model 1[2]	Model 2[3]	Model 1[2]	Model 2[3]
HPV-positive	0.5 (0.2–1.0)*	0.3 (0.1–0.8)	0.6 (0.2–1.5)***	0.5 (0.2–1.4)**
HPV-positive SCCs only[4]	0.5 (0.2–1.1)*	0.4 (0.2–1.0)	0.7 (0.3–1.9)***	0.5 (0.2–1.6)***
Age[5]	1.04 (1.02–1.07)	1.04 (1.02–1.07)	1.03 (1.00–1.06)*	1.03 (1.00–1.06)*
Male	1.9 (1.1–3.4)	2.6 (1.3–5.1)	2.2 (1.0–4.8)	3.0 (1.2–7.5)
Poorly differentiated	1.8 (0.9–3.3)*	2.1 (1.1–4.0)	1.7 (0.8–3.5)**	1.8 (0.8–4.1)**
Stage				
II	1.4 (0.5–4.2)***	1.4 (0.5–4.1)***	1.5 (0.2–15.3)	1.4 (0.1–14.1)***
III	2.4 (0.8–7.5)**	2.5 (0.8–7.6)**	6.0 (0.7–55.0)**	6.3 (0.7–57.8)**
IV	4.3 (1.5–12.8)	5.1 (1.7–15.6)	13.2 (1.5–113.7)	14.0 (1.6–122.4)
Treatment[6]				
Surgery, No XRT	2.2 (1.0–4.7)*	2.8 (1.2–6.4)	1.6 (0.5–5.1)***	1.8 (0.5–6.3)***
No surgery, XRT	3.2 (1.5–6.6)	3.9 (1.8–8.5)	3.6 (1.5–8.6)	4.2 (1.6–10.7)
No surgery, No XRT	6.8 (2.4–19.0)	8.0 (2.7–23.6)	7.5 (2.3–24.4)	9.2 (2.6–33.0)
Tobacco pack-years[5]	—	0.99 (0.98–1.00)**	—	0.99 (0.98–1.01)***
Drinks/Week[5]	—	1.00 (0.99–1.01)***	—	1.00 (0.99–1.01)***
SCC histology	—	2.7 (0.9–8.7)*	—	1.5 (0.4–5.7)***

[1]*$0.05 < p < 0.10$–**$0.10 < p < 0.20$–***$p > 0.20$. Results from analyses involving all carcinomas, except for second row labeled "HPV-positive SCCs only";–[2]Model 1 based on backward selection procedure using $p < 0.20$ and includes the following; HPV status, age, gender, tumor grade, stage of disease at initial diagnosis, and treatment;–[3]Model 2 includes the factors from Model 1 plus tobacco pack-years, average number of drinks per week, and tumor histology (SCC *vs.* other);–[4]Analyses among SCCs only; adjustment made for same factors presented except tumor histology was not included in Model 2;–[5]Relative risk estimates on a per-unit incremental effect (e.g., years or drinks);–[6]XRT, radiation therapy.

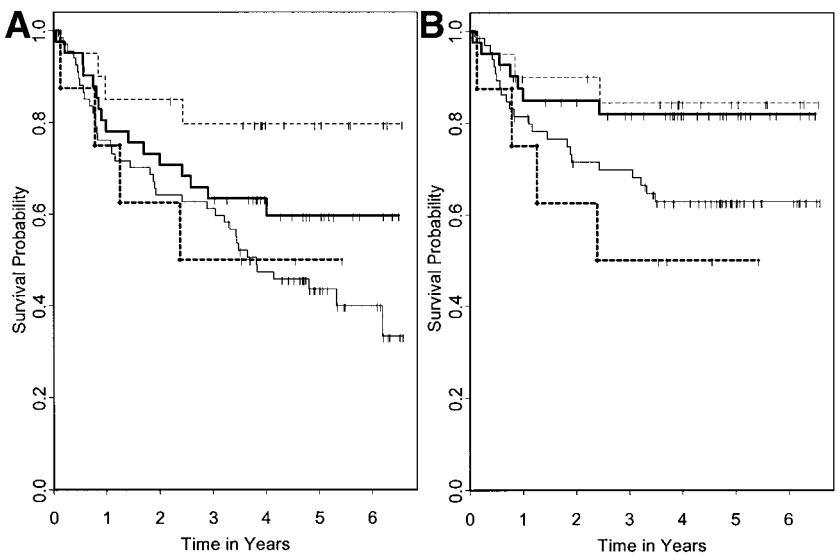

FIGURE 2 – Survival by gender and HPV tumor status. Overall survival (*a*) and disease-specific survival (*b*) curves based on the Kaplan-Meier method were analyzed. The solid line is for males with HPV-negative tumors (n=68), the dashed line is for males with HPV-positive tumors (n=21), the bold solid line is for females with HPV-negative tumors (n=42) and the bold dashed line is for females with HPV-positive tumors (n=8). Cause of death was unknown for 1 male with an HPV-negative tumor. Vertical tick marks on curves indicate censored observations.

TABLE III – RELATIVE RISK ESTIMATES FOR SURVIVAL BY GENDER AND HPV DNA STATUS[1]

Group	All carcinomas							
	Overall survival				Disease-specific survival			
	n	*Unadjusted*	*Model 1*	*Model 2*	*n*	*Unadjusted*	*Model 1*	*Model 2*
Male/HPV-neg	68	1.0	1.0	1.0	67	1.0	1.0	1.0
Male/HPV-pos	21	**0.3 (0.1–0.8)**	**0.3 (0.1–0.8)**	**0.2 (0.1–0.6)**	21	0.4 (0.1–1.3)	0.3 (0.1–1.1)	**0.2 (0.1–0.8)**
Female/HPV-neg	42	0.6 (0.4–1.1)	**0.4 (0.2–0.8)**	**0.3 (0.1–0.6)**	42	0.5 (0.2–1.1)	**0.3 (0.1–0.8)**	**0.2 (0.1–0.6)**
Female/HPV-pos	8	1.0 (0.3–2.7)	0.5 (0.2–1.6)	0.3 (0.1–1.1)	8	1.5 (0.5–4.4)	0.6 (0.2–2.1)	0.3 (0.1–1.5)
Group	SCC cases only							
	Overall survival				Disease-specific survival			
	n	Unadjusted	Model 1	Model 2	n	Unadjusted	Model 1	Model 2
Male/HPV-neg	60	1.0	1.0	1.0	59	1.0	1.0	1.0
Male/HPV-pos	20	**0.3 (0.1–0.8)**	**0.3 (0.1–0.9)**	**0.2 (0.1–0.7)**	20	0.4 (0.1–1.3)	0.4 (0.1–1.5)	**0.2 (0.1–1.0)**
Female/HPV-neg	40	0.6 (0.3–1.1)	**0.4 (0.2–0.8)**	**0.3 (0.1–0.6)**	40	0.4 (0.2–1.0)	**0.3 (0.1–0.9)**	**0.2 (0.1–0.6)**
Female/HPV-pos	8	0.9 (0.3–2.6)	0.5 (0.2–1.8)	0.4 (0.1–1.4)	8	1.5 (0.5–4.5)	0.8 (0.2–2.8)	0.4 (0.1–1.9)

[1]Model 1 includes age, tumor grade, stage of disease at initial diagnosis, and treatment; Model 2 includes the same as those in Model 1 plus tobacco pack-years, average drinks per week, and tumor histology (SCC *vs.* other). Model 2 did not include tumor histology in analyses among SCCs only. Data in boldface are for statistically significant comparisons (i.e., $p < 0.05$).

ative risks among stage III and IV patients only were similar to those already presented in Table III, suggesting that the observed gender effects were not primarily due to advanced stage of disease. A greater difference in median tobacco pack-years between the HPV groups among males (HPV-positive 27.9 *vs*. HPV-negative 50.0) than among females (HPV-positive 17.5 *vs*. HPV-negative 28.7) was also observed. Even though these differences by gender are in agreement with the overall inverse relationship found between tobacco usage and HPV detection, after adjusting for tobacco usage in the analyses, similar conclusions regarding the interaction between gender and HPV were found as noted in model 2. No other differences in patient or tumor characteristics were observed among the HPV and gender strata.

Although tumor location (oropharynx *vs*. oral cavity) was not a significant prognostic factor, since the HPV positivity rate was higher among oropharyngeal tumors than among oral cavity tumors, location was added to the multivariate survival models reported in Table II (data not shown; $p > 0.80$). The inclusion of tumor location did not change the relative risk estimates for HPV DNA status and the other prognostic factors and the interaction between HPV infection and gender still approached statistical significance. Moreover, the interaction between HPV detection and tumor location on survival was non-significant ($p > 0.15$).

DISCUSSION

Our study examined the association between HPV infection and survival in carcinomas of the oral cavity and oropharynx. A significant favorable association between the detection of oncogenic HPV types in tumors and overall survival was found after adjusting for other prognostic factors. The interaction between HPV status and gender approached statistical significance for overall as well as disease-specific survival. This interaction suggested that males detected with HPV had better prognosis than males with HPV-negative tumors for both overall and disease-specific survival but that this was not the case among females. This is the first study to explore and describe a possible interaction effect between HPV infection and gender on survival in oral and oropharyngeal cancers. Whether the association between HPV status and survival depends on gender is a valid finding requires further investigation for verification.

The HPV positivity rate of 21% in our study is similar to those reported by others for oral and oropharyngeal cancers[15,16,18] and head and neck[5,10,12,28] cancers. The HPV positivity rates in these studies ranged from 16 to 26%. Although our methods allowed for sensitive detection of a wide range of low- and high-risk HPV types, most of the tumors in our study contained HPV-16; a result also noted in other studies. Because it can be argued that the detection of HPVs in the sections may represent superficial or transient infection, and not the actual presence of the virus in the tumor cells, LMD was performed on a subset of tumors ($n = 16$). Other studies have used techniques such as real-time quantitative PCR[35] and *in situ* hybridization[5] to address this issue. Based on the fact that LMD demonstrated, without exception, that the viral DNA could be detected in cancer tissue but not in normal-appearing epithelium, we believe that the number of HPV-positive cases that may not contain virus in the tumors is very small and should not adversely influence the statistical results of our study. Oncogene expression of the HPV-16 positive cases with available frozen material has been evaluated with the results to be published elsewhere.

Although most (92%) of the tumors were SCCs, other histology types were included. In cervical cancer, HPV infection has been detected in a wide range of different histologies and therefore supports the examination of HPV in the other histology types among carcinomas of the oral cavity and oropharynx. There are few data reported in the literature on HPV in other histologic tumor types among oral and oropharyngeal cancers. A recent meta-analysis[36] suggested that verrucous carcinomas may be HPV-related in oral cancers. Another study[37] included 2 mucoepidermoid carcinomas from the head and neck region but neither was HPV-positive. While the number of the other histology types is small ($n=11$), it is interesting to note that 1 of the HPV positive carcinomas in our study was a mucoepidermoid carcinoma. To ensure that inclusion of the other types of carcinomas did not obscure the results, histology type was adjusted for in the multivariate survival models (Model 2). In addition, analyses were performed among SCC cases only but ORs, RRs and conclusions were similar.

Three of the 4 risk factors that were significantly associated with an increase in HPV detection also have been discussed elsewhere (male gender,[11,18] oropharyngeal tumors[5,8,10,11,28,35,38] and less tobacco usage[10,11,15,19,28,38]). The study by Koch and colleagues[11] suggested that there is a difference in the mechanism of tumorigenesis between smokers and non-smokers. The authors further pointed out that the small number of non-smokers with the disease makes the search for the possible different genetic alterations between the 2 groups difficult. It has been argued that the protective effect of smoking on HPV infection in the oral cavity and oropharynx may be due to increased keratinization of normally parakeratinized mucosal surfaces.[39] The increased keratin may make the mucosa more resistant to minor trauma, one proposed mechanism for the necessary infection of the basal layer cells by HPVs.

The fourth risk factor that was found to be significantly associated with HPV infection was oral-genital contact. The odds of HPV detection among those reporting a history of oral genital sex was 4 times that of those without this history. This result remained true even after adjustment for other significant factors and represents an interesting, novel finding as oral-genital contact has been hypothesized as one possible mode of transmission in the head and neck.[7] Like others,[12,15,17] we found a higher HPV positivity rate to be associated with younger age in unadjusted analyses. However, when a history of oral-genital sex was included in the multivariate analyses, age was no longer significantly associated with HPV status. This is most likely due to the fact that those with a history of oral-genital sex were about 18 years younger on average than those without this history ($p < 0.001$).

Our results regarding the significant association between overall survival and HPV infection are in accord with other studies.[5,10,11,15,16] However, unlike others[5,15,17] who examined disease-specific survival, HPV infection was not significantly associated with disease-specific survival in our study. The differences in the disease-specific survival results between our study and others could be due to differences in sample sizes, in percentages of deaths due to disease or in the other prognostic factors included in the analyses. However, another plausible explanation for the differences in the studies is that the association between HPV detection and survival is gender-specific, as suggested by our study, and not evaluated in the other studies.

In our study, 59% of the deaths were disease specific, that is, due to oral and oropharyngeal cancer. In the study by Gillison *et al*.,[5] 71% of the deaths were due to head and neck cancers. They not only included 144 oral and oropharyngeal cancers but also a large number of cases from the hypopharynx and larynx. Because somewhat different populations were studied, comparisons between the 2 investigations are difficult. The percentage of disease-specific deaths was not stated in another large study with a more similar population among oral and oropharyngeal cancers.[15]

Results for 2 different models were presented. The first included the following significant prognostic factors or confounders: age, gender, tumor grade, stage of disease at initial diagnosis and treatment modality. Although not statistically significant in the multivariate results, tobacco and alcohol usage and tumor histology were added to the first model to compare our results to other studies[5,15,19] that adjusted for these factors. The factors included in our analyses were similar to those of others, with the exception of gender. In some investigations,[10,17] gender was not significantly associated with survival in multivariate analyses. Another study[15] did not discuss the relationship between gender and survival. Other studies[5,12,19] did not find gender to be prognostically significant in unadjusted analyses and as such did not consider gender as a potential factor in their multivariate survival analyses. From our unadjusted analyses, gender also did not appear to be associated with overall or disease-specific survival. However, based on multivariate analyses in the absence of the interactions, females had approximately 2 times better prognosis than males after adjusting for stage of disease, tumor grade, patient age and treatment. Another study[40] also found females to have significantly better disease-specific survival than males among oral cancers after adjusting for age, race, stage and treatment. Unfortunately, their study did not include HPV findings. The significant difference in survival by gender from our study might be explained by the fact that males reported significantly more tobacco and alcohol usage than females. However, this prognostic finding remained after including tobacco and alcohol usage in the multivariate survival models (Table II).

Interestingly, our data suggested an interaction between gender and HPV DNA status for both overall and disease-specific survival. Among males, the differences in survival between the HPV groups were significant or approached statistical significance, and the hazards of death overall or due to disease for HPV-positive tumors were estimated to be about one third times that for HPV-negative tumors. Among females, the overall survival curves were fairly similar between the HPV groups, whereas for disease-specific survival, females with HPV-positive tumors seemed to have worse prognosis compared to females with HPV-negative tumors. However, the results among females were not statistically significant. None of the other interactions with HPV status was statistically significant. Schwartz and colleagues[15] also examined interactions between HPV-16 positivity and stage, smoking, alcohol, radiation, and surgery among oral and oropharyngeal cancers and found none to be statistically significant. Unfortunately, they did not examine the interaction between HPV and gender.

In our study, since oropharyngeal tumors were more likely to be HPV positive than tumors from the oral cavity, tumor location was added to the multivariate survival analyses. However, no significant differences were detected in survival between oral cavity *vs*. oropharyngeal carcinomas. Two other large studies[5,15] that examined the association between HPV infection and survival also did not include primary tumor site in their multivariate survival analyses. Gillison *et al*.[5] reported unadjusted hazard ratios of disease-specific survival by HPV DNA status for oropharyngeal and non-oropharyngeal tumors separately. However, in our study, from multivariate analyses, there was not a significant interaction on survival between HPV and tumor location, suggesting that the association between HPV and survival was not dependent on tumor site. Moreover, inclusion of tumor location in the multivariate survival models did not change the results from those presented.

Major strengths of our study include the detailed data about tobacco and alcohol exposure and sexual behavior characteristics, especially regarding a history of oral-genital sex. In addition, we had extensive follow-up on patients last known to be alive; median follow-up was 4.8 years with 96% followed for at least 2 years.

One limitation to our study was the relatively small number of women detected with HPV in their tumors ($n = 8$). Thus, the generalizations of the HPV comparisons for survival by gender are unclear at this time. Thirty-six percent of the newly diagnosed cases in our study were females. This rate is consistent with other research, which suggested that the incidence rates were 3 times greater in males than females.[1] In addition, in our investigation, the HPV-positivity rate for females was lower than that for males (16% *vs*. 24%). Most other studies also included more males than females and had a small number of women with HPV positive tumors. We tried to identify possible reasons why the disease-specific prognosis seemed worse for these 8 women in relation to the other HPV and gender strata but found none. As such, we believe further investigation into the association between HPV and survival among females is warranted.

Another limitation to our study was the lack of comorbidity data. The cases in our study were part of a larger case-control study[6] that was designed to examine the association between HPV detection and cancers of the oral cavity and oropharynx. As a result, data regarding other cardiovascular, respiratory and most other comorbid diseases were not documented. However, information about diabetes, immunodeficient diseases and alcoholism or other alcohol-related diseases were recorded. None of the patients in the study was documented as having an immunodeficient disease. Similar to the results for alcohol usage, alcoholism and other alcohol-related diseases were not associated with survival. There was a significant association between diabetes and survival in the patients in our study. However, even after including this comorbid condition in the multivariate analyses reported in Table II, the relative risk estimates for HPV status and the other factors remained the same. The investigation by Schwartz *et al*.[15] is the only other study to include comorbidity data in their survival models. The comorbid illnesses included hepatitis, diabetes and kidney disease. They compared survival with and without the presence of these comorbid diseases. The adjusted hazard ratios for HPV detection were similar, suggesting these conditions did not explain the association between HPV and survival in oral and oropharyn-

geal cancers. They also lacked data on cardiovascular, respiratory or other comorbid illnesses. Therefore, whether the association between HPV status and survival remains significant after adjusting for other types of comorbidities such as cardiovascular or respiratory diseases is still unanswered. In addition, it is unclear whether these other types of comorbidities are explaining the gender differences and interactions observed in our study.

To try to explain the positive relationship found between HPV infection and survival, several researchers have examined p53 mutations and HPV detection in the same head and neck cancer patients. Studies have found that patients with HPV-positive tumors are less likely to have p53 mutations[5,10,11,19] and that smokers are more likely to have p53 mutations.[11] However, many studies have been unable to find an association between p53 mutations and survival in head and neck cancers.[5,10,13] Furthermore, the study by Gillison *et al*.[5] demonstrated that HPV infection was an independent prognostic factor for survival after adjusting for the presence of a p53 mutation. Interestingly, Koch and colleagues[11] found that females were more likely to have a p53 mutation (OR=2.6, $p = 0.01$) than males after adjusting for current smoking status and heavy alcohol usage. Similar to our study, this result suggests gender differences. Unfortunately, at this time, we lacked data on p53 mutations.

It has also been hypothesized that HPV-positive tumors may have an increased sensitivity to radiation[5,15,19] and that this may explain the direction of the association found between HPV infection and survival. By considering separate effects for surgery and radiation, we examined this relationship. Similar to other researchers,[15] we did not find a significant interaction between radiation therapy and HPV detection for overall (all p values > 0.80) and disease-specific (all p values > 0.50) survival. Based on model 2 for overall survival, the adjusted relative risk estimate by HPV status was 0.34 (95% CI = 0.12–0.98) among those who had received radiation therapy and was 0.34 (95% CI = 0.09–1.29) among those who had not received radiation therapy. Conclusions for disease-specific survival were similar suggesting that the association between HPV infection and survival is independent of being treated with radiation therapy.

Gillison and colleagues[5] also suggested that field cancerization may be less applicable in patients with HPV infection due to the fact that they tend to use less tobacco and alcohol. Although most investigations, including our own, adjust for tobacco and alcohol in the multivariate survival analyses, a comparison in survival by HPV status among non-smokers would further support the notion that the favorable prognosis found among patients with HPV infection is independent of them tending to use less tobacco. Unfortunately, we were unable to make this comparison in our study due to the small number of non-smokers; a sample size issue also seen in other studies.

Although it remains unknown as to why HPV infection appears to be protective, our study did find a significant favorable association between oncogenic types of HPV and better overall survival. Males detected with HPV had better overall and disease-specific prognosis than males with HPV-negative tumors, but this was not observed among females. Our study also observed other gender differences (*e.g.*, differences in the percentage of cases enrolled, in the HPV positivity rate and in tobacco and alcohol usage), suggesting that larger studies will be required to verify whether the association between HPV detection and survival depends on gender.

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