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Human papillomavirus 6 seropositivity is associated with risk of head and neck squamous cell carcinoma, independent of tobacco and alcohol use

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Background: The risk of head and neck squamous cell carcinoma (HNSCC) associated with common human papillomavirus types has not been well defined.

Methods: We conducted a case–control study of 1034 individuals (486 incident cases diagnosed with HNSCC and 548 population-based controls matched to cases by age, gender, and town of residence) in Greater Boston, MA. Sera were tested for antibodies to human papillomavirus (HPV)6, HPV11, HPV16, and HPV18 L1.

Results: HPV6 antibodies were associated with an increased risk of pharyngeal cancer [odds ratio (OR) = 1.6, 1.0–2.5], controlling for smoking, drinking, and HPV16 seropositivity. In HPV16-seronegative subjects, high HPV6 titer was associated with an increased risk of pharyngeal cancer (OR = 2.3, 1.1–4.8) and oral cancer (OR = 1.9, 1.0–3.6), suggesting that the cancer risk associated with HPV6 is independent of HPV16. There was no association between smoking and alcohol use and HPV6 serostatus. Further, the risk of pharyngeal cancer associated with heavy smoking was different among HPV6-seronegative (OR 3.1, 2.0–4.8) and HPV6-seropositive subjects (OR = 1.6, 0.7–3.5), while heavy drinking also appears to confer differing risk among HPV6-negative (OR 2.3, 1.5–3.7) and -positive subjects (OR = 1.3, 0.6–2.9).

Conclusions: There may be interactions between positive serology and drinking and smoking, suggesting that the pathogenesis of human papillomavirus in HNSCC involves complex interactions with tobacco and alcohol exposure.

Key words: epidemiology, head and neck squamous cell carcinoma, human papillomavirus, risk factors, serology

introduction

Heavy alcohol and tobacco use are thought to account for the majority of the estimated 40 000 head and neck squamous cell carcinoma (HNSCC) cases diagnosed per year in the United States [1], with the well-described synergistic interaction between these exposures responsible for induction of much of this disease. Recent studies have shown that infection with high-risk human papillomavirus (HPV) is also a risk factor for HNSCC [2, 3]. Approximately 25% of HNSCC cases have detectable HPV DNA in tumor tissue [2]. Positive HPV16 L1 serology reflects exposure to HPV16 virus and is also associated with increased risk for HNSCC [4]. The majority of HPV DNA-positive HNSCC is positive for HPV16 [2, 4–7], and large studies of HPV serology have focused on detecting HPV16 antibodies [7–10]. Other HPV types, including high-risk type HPV18, however, also have been detected in HNSCC [2, 3, 11], but few studies of HPV serology in HNSCC have examined the seroprevalence of multiple types.

Unlike cervical cancer, where HPV is the necessary cause of disease [12, 13], the contribution of HPV to the development of HNSCC may be more complex, as this disease is primarily associated with other carcinogenic exposures, including alcohol and tobacco. Data suggest that the neoplastic transformation that occurs in the upper aerodigestive tract as a result of the carcinogenic action of alcohol and tobacco may be intensified or somehow altered by HPV infection [12, 14].

Case–control studies that have evaluated cumulative exposure to HPV16 by measuring serum anti-HPV16 L1 antibodies have been inconsistent with regard to the interaction between smoking and HPV16 serology in predicting risk for HNSCC [7–9]. Studies examining the role of HPV infection in HNSCC have found that viral presence (a marker of ongoing infection) is inversely associated with heavy alcohol and tobacco exposure [2, 6, 15]. Thus, exposure to alcohol and

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tobacco may play a complex role in HPV pathogenesis, potentially altering the overall immune response, including the antibody response and/or influencing tumor progression in infected individuals in a different fashion from those who have no HPV exposure or infection.

We, and others, have previously observed that detectable HPV16 antibody is associated with an increased risk for HNSCC, with greatest increases in risk for tumors of the oropharynx [4–6, 8, 9]. Questions remain concerning the natural history of HPV. Race/ethnicity is associated with positive HPV16 serology [13, 16] and some have suggested that this may be explained by differences in genetic background (i.e. HLA type) [17]. Female gender is also associated with a higher seroprevalence to HPV16 [16], as is sexual behavior, including numbers of oral and lifetime sexual partners [13]. Less well characterized until recently [6, 7] is the relationship between positive serology to HPV and tobacco and alcohol exposure.

Here, we present work that was designed to determine whether the detection of antibodies to HPV6, HPV11, and HPV18 are associated with HNSCC risk, independent of serologic HPV16 positivity. Additionally, we hypothesized that, among controls, a positive serologic response to HPV would be more prevalent in females and in non-white individuals, consistent with prior observations.

methods

study population

We conducted a case-control study in Boston, MA, from December 1999 to December 2003. Cases and controls were drawn from the Greater Boston Metropolitan Area, a population of roughly 3.5 million people in 249 cities and towns within a 1-h drive of Boston. The institutional review boards at all participating institutions (listed below) approved this study, and all volunteer participants provided informed consent.

Incident cases of HNSCC were identified through the multidisciplinary head and neck clinics, otolaryngology, and radiation oncology departments at nine large teaching hospitals in Boston, MA (Beth Israel Deaconess Medical Center, Boston Medical Center, Boston Veterans Administration, Brigham and Women's Hospital, Dana-Farber Cancer Institute, Harvard Vanguard Medical Associates, Massachusetts Eye and Ear Infirmary, Massachusetts General Hospital, and New England Medical Center).

Eligibility requirements included being a resident of the study area, at least 18 years old, first diagnosis of HNSCC within the past 6 months, and being alive at the time of initial contact. We defined HNSCC using the *International Classification of Disease Codes, Ninth Revision* (ICD-9) codes 141, 143-6, 148, and 161. All patients with carcinoma *in situ*, lip, salivary gland, or nasopharyngeal cancer or recurrent cancer of the head and neck region were excluded. Histologic classification of malignancy was based upon that reported by pathology at the participating hospitals.

Population-based controls were frequency matched (1 : 1) to cases by age (±3 years), gender, and town of residence. These controls were identified through systematic random selection from the Massachusetts Resident Lists for the 249 cities and towns within the study area. These annually compiled Resident Lists are mandated by state law and include all residents >17 years of age. The resident lists include name, gender, year of birth, occupation, and last address [18]. Residents are listed in the book by street address and precincts and a potential control of the same gender and age as the case was sought from this list, alternating the search direction through the book starting at the case address.

A total of 823 eligible cases were invited to participate. Overall, 57 of these cases refused to participate. Of the 766 consented cases, another 44 did not complete the questionnaire, resulting in an 88% case participation rate. A total of 1643 subjects were identified and eligible for participation as controls. Of these subjects, 828 refused to participate, 815 subjects were consented, and 771 were ultimately enrolled in the study. Six of the controls were withdrawn as they were matched to a case that became ineligible, such that 765 controls were enrolled and completed. This represents an overall participation rate of 47% for controls. On the basis of the availability of serum samples, this analysis included a total of 486 cases and 548 controls.

questionnaire

Participating cases and controls were given a self-administered questionnaire to collect medical history and demographic information as well as information on tobacco and alcohol consumption. Each questionnaire was reviewed with each participant by a trained research coordinator. Smoking history was ascertained with a standardized instrument that assesses the number of years smoked, the number of cigarettes smoked per day, age at which an individual started smoking, number of years since quitting, and the duration of smoking. Similar information was obtained regarding lifetime consumption of beer, wine, and liquor. For cases, clinical and pathological information on the tumor including size, location, stage, differentiation, and treatment were collected by medical record review, with subsequent confirmatory review by a single pathologist.

HPV serology

Venous blood samples were obtained from cases and controls. Serum was separated within 12–24 h of blood drawing and samples were stored at −80°C. Frozen samples were shipped to Merck & Co., Inc. testing laboratory (West Point, PA) where samples were thawed under refrigeration and then heat inactivated for 30 ± 2 min at 56 ± 2°C before analysis.

The procedures for analyzing HPV6, HPV11, HPV16, and HPV18 serology via Competitive Luminex® Immunoassays have been described previously [19]. The L1 genes of HPV types 6, 11, 16, and 18 were expressed in the yeast *Saccharomyces cerevisiae*. The L1 proteins self-assemble into a virus-like particle (VLP) structure. The VLP products were then purified from lysates of the yeast as previously described [20–23] with modifications. The HPV VLPs for types 6, 11, 16, and 18 were each covalently conjugated to the free carboxyl groups on xMAP® Multi-Analyte COOH Microspheres (Luminex® Corporation, Austin, TX) numbered 6, 11, 16, and 18 as previously described [24]. The following mAbs were used to chemically couple to phycoerythrin and used to detect neutralizing epitopes on coupled HPV VLPs 6, 11, 16, and 18: H6.M48, K11.B2, H16.V5, and H18.J4. A quadriplex reference standard solution was prepared at a concentration of 1000 milli-Merck units per milliliter (mMU/ml) for each HPV type in Antibody Depleted Human Serum (ADHS), a prescreened assay matrix (Human Serum Special Stripped, Valley Biomedical, Winchester, VA). The four reference standards were serum samples from African Green Monkeys hyperimmunized with HPV monovalent L1 VLP types 6, 11, 16, or 18. The quadriplex reference standard was diluted in a two-fold serial dilution in ADHS to create a 12-point standard curve with final well concentrations ranging from 0.25 to 500 mMU/ml. The assay plate setup contained four controls, ADHS diluent, and titer controls (high, low, and negative). All standards, controls, and samples were tested in duplicate.

Baseline testing of serum samples was carried out at a 1 : 4 dilution; final assay conditions consisted of 25 μl of serum, 25 μl of ADHS, 25 μl of mAb-PE quadriplex (0.1 μg/ml for each mAb), and 25 μl of VLP-microspheres

(5000 VLP-microspheres per well per type). The plate was covered with a foil seal and placed on a shaker (600–800 rpm) at RT for 16–25 h. The contents of the assay plate were then transferred to a 1.2 μm Low Protein Binding Filter Plate (Millipore, Billerica, MA). The plates were washed three times with 200 μl of assay wash buffer and resuspended in 125 μl of assay wash buffer for analysis on a Bio-Plex Suspension Array System (Bio-Rad, Hercules, CA). Fluorescent units were read and averaged, dilution corrected mMU/ml serum values were computed based on four parameter fit logistics of the standard curve on each assay plate. Samples that exceeded the limits of quantitation for the standard curve were retested at higher dilution factors, diluted in ADHS.

statistical analysis

All statistical analyses were carried out with SAS software (version 9.1; SAS Institute, Cary, NC). HPV16 seropositivity was defined as ≥12 mMU while HPV6, HPV11, and HPV18 seropositivity was defined as ≥8 mMU, above the threshold considered background reactivity. Race was dichotomized as white and non-white (Asians, African-Americans, Native Americans, and Hispanics) due to the limited number of non-white individuals. Lifetime cigarette smoking was measured as pack-years (years smoked times packs per day) and drinking was measured by average alcohol consumption over a lifetime, i.e. average drinks per week. Light drinking was defined as <6.2 drinks/week, the median among controls who consumed alcohol, while heavy drinking was defined as ≥6.2 drinks/week. Light smoking was defined as <25.4 pack-years, the median among controls who smoked, and heavy smoking was defined as ≥25.4 pack-years. For multivariate models, drinking and smoking behavior was dichotomized as non/light versus heavy to facilitate interpretation of odds ratios (ORs).

To facilitate analysis of disease by site, tumors were classified as oral cavity (ICD-9 codes 143, 144, 145), pharynx (ICD-9 codes 146, 148 and 149), or larynx (ICD-9 code 161) in accordance with the recommendations of the American Joint Committee on Cancer. ICD-9 141 was classified as oral cavity if the tumor was located at the anterior of the tongue, but was classified as pharynx if the tumor was located at the base of the tongue.

Unconditional logistic regression was carried out to calculate ORs and 95% confidence intervals (CIs) for HNSCC risk associated with HPV serology (HPV6, HPV11, and HPV18) while adjusting for smoking, drinking, and HPV16. We also evaluated the associations between HPV serology and demographics in the controls alone. All tests were two sided and a *P* value of 0.05 was considered statistically significant.tpb -2pt

results

We studied 486 HNSCC cases and 548 controls (Table 1). Approximately three quarters of our study population was male (74% of cases, 73% of controls). The mean age was 60 years of age for cases and 61 years of age for controls with the majority of the population being white (92% of cases, 91% of controls).

Both alcohol and tobacco consumption were associated with increased risk for HNSCC, with dose–response relationships observed between these exposures and risk for HNSCC (Table 1). Compared with participants who drank less than five or five drinks/week, individuals who consumed 15–30 drinks/week had a two-fold increased risk for HNSCC (all tumor sites) and those who consumed >30 drinks/week had a 3.5-fold increased risk for HNSCC. Compared with nonsmokers, individuals who had a lifetime exposure of 21–45 pack-years had a 1.7-fold increased risk for HNSCC and those with >45 pack-years had a 3.2-fold increased risk for HNSCC. Smoking was most strongly associated with risk of laryngeal cancer; individuals with a lifetime exposure of 21–45 pack-years had

Table 1. Characteristics of HNSCC cases and controls

	Controls	All cases (*n* = 486)		Pharynx (*n* = 204)		Oral	(*n* = 189)	Larynx (*n* = 93)	
	(*n* = 548) *n* (%)	*n* (%)	OR (95% CI)[a]	*n* (%)	OR (95% CI)[a]	*n* (%)	OR (95% CI)[a]	*n* (%)	OR (95% CI)[a]
Gender									
Female	147 (27%)	125 (26%)		41 (20%)		67 (35%)		17 (18%)	
Male	401 (73%)	361 (74%)		163 (80%)		122 (65%)		76 (82%)	
Age (years)[b]									
Mean (SD)	61 (11)	60 (12)		59 (10)		59 (14)		61 (11)	
Range	25–89	21–91		29–86		21–87		34–91	
Race									
White	500 (91%)	445 (92%)	1.0	187 (92%)	1.0	174 (92%)	1.0	84 (90%)	1.0
Non-white	48 (9%)	41 (8%)	0.8 (0.5–1.3)	17 (8%)	0.8 (0.4–1.5)	15 (8%)	0.8 (0.4–1.4)	9 (10%)	0.9 (0.4–2.1)
Drinking (drinks/week)									
<5	241 (44%)	133 (27%)	1.0	53 (26%)	1.0	55 (29%)	1.0	25 (27%)	1.0
>5–15	170 (31%)	109 (22%)	1.1 (0.8–1.6)	51 (25%)	1.3 (0.8–2.0)	37 (20%)	1.2 (0.7–1.9)	21 (23%)	1.0 (0.5–1.9)
>15–30	76 (14%)	95 (20%)	2.0 (1.4–3.1)	35 (17%)	1.7 (1.0–2.9)	36 (19%)	2.7 (1.6–4.8)	24 (26%)	1.8 (0.9–3.6)
>30	61 (11%)	149 (31%)	3.5 (2.3–5.2)	65 (32%)	3.3 (2.0–5.6)	61 (32%)	5.6 (3.2–9.9)	23 (25%)	1.9 (0.9–3.8)
Smoking (pack-years)									
0	182 (33%)	90 (19%)	1.0	41 (20%)	1.0	44 (23%)	1.0	5 (5%)	1.0
>0–20	154 (28%)	95 (20%)	1.2 (0.8–1.7)	41 (20%)	1.1 (0.7–1.8)	39 (21%)	0.9 (0.6–1.6)	15 (16%)	3.5 (1.2–10)
>20–45	125 (23%)	131 (27%)	1.7 (1.2–2.5)	52 (25%)	1.5 (0.9–2.5)	54 (29%)	1.4 (0.8–2.4)	25 (27%)	6.9 (2.5–19)
>45	87 (16%)	170 (35%)	3.2 (2.1–4.7)	70 (34%)	2.9 (1.7–4.9)	52 (28%)	1.9 (1.1–3.3)	48 (52%)	19.7 (7.2–54)

[a]Models include all listed variables; odds ratios not calculated for matching variables (gender and age).
[b]For age, summary statistics are presented instead of counts and percentiles.

Table 2. Spearman correlation between serology types[a] among controls (n = 548)

	HPV6	HPV11	HPV18	HPV16
HPV6	1.00	0.19; $P \le 0.0001$	0.17; $P = 0.0001$	0.13; $P = 0.003$
HPV11		1.00	0.08; $P = 0.06$	0.15; $P = 0.0004$
HPV18			1.00	0.12; $P = 0.007$
HPV16				1.00

[a]Correlations were evaluated using titer values.

a 6.9-fold increased laryngeal cancer risk and those with >45 pack-years had a 19.7-fold increased cancer risk.

Table 2 presents the correlation between serology types among controls. HPV titer of each type was significantly (P < 0.01) but weakly correlated to HPV titer of all other types (Spearman correlation coefficients ranged from 0.08 to 0.19), though the correlation between HPV11 and HPV18 was marginally significant (P = 0.06). Among controls, HPV6 seropositivity was most common (14%) followed by HPV16 seropositivity (11%) and HPV18 seropositivity (8%). As shown in Table 3, controls with HPV16 seropositivity were significantly more likely to be seropositive for HPV6 (OR = 2.2, 95% CI 1.1–4.3), HPV11 (OR = 3.4, 1.4–8.0), and HPV18 (OR = 2.1, 1.0–4.8). Non-white controls were significantly more likely to be seropositive for HPV6 (OR = 2.5, 1.2–5.0) and HPV11 (OR = 3.2, 1.2–8.3), while heavy smokers were more likely to be seropositive for HPV6 (OR = 2.1, 1.2–3.5). Controls who were 60 years or older were less likely to be HPV11 seropositive compared with those under 60 years of age (OR = 0.5, 0.2–1.0).

Table 4 presents the crude and adjusted ORs for HPV6, HPV11, and HPV18 serology. The adjusted ORs controlled for smoking, drinking, and HPV16, though confounding by HPV16 serology, account for the majority of the difference between the crude and adjusted risk estimates. HPV6 seropositivity was associated with an increased risk of pharyngeal cancer (OR = 1.6, 1.0–2.5) while controlling for smoking, drinking, and HPV16. In fact, assuming a more robust titer is associated with a more biologically important exposure, the lower risk of pharyngeal cancer associated with low HPV6 titer (OR = 1.5, 0.8–2.6) compared with a higher HPV6 titer (OR = 1.8, 1.0–3.2) is consistent with HPV6 being the etiologic agent. Similarly, an increased risk of oral cancer was associated with high HPV6 titer (OR = 1.9, 1.1–3.4) but not low HPV6 titer, while controlling for smoking, drinking, and HPV16. Additionally, we investigated a possible synergistic effect of HPV6 and HPV16 seropositivity on HNSCC risk but found that the interaction was not significant (P = 0.8). HPV11 and HPV18 seropositivity were not associated with risk for pharyngeal or oral cancer, although the unadjusted models suggested some elevated risk for pharyngeal cancer that was not evident after adjustment for smoking, drinking, and HPV16. Including race, age, and gender in the models did not appreciably alter the point estimates for these ORs.

To control for potential residual confounding of HPV16 seropositivity, given that HPV6 and HPV16 titer were significantly correlated, we next restricted our analysis to individuals with negative HPV16 serology. In this analysis (Table 5), high HPV6 titer remained significantly associated with both pharyngeal (OR = 2.3, 1.1–4.8) and oral cancers (OR = 1.9, 1.0–3.6), while controlling for smoking and drinking. The analyses of the other HPV types likewise remained unchanged. Finally, since this analysis could still have residual confounding as a result of differential loss of immune markers, we also examined the association of HPV6 with pharyngeal cancer restricted to HPV16 positive cases and controls. While these numbers were small (for HPV6 there were 43 negative controls and 66 negative cases, with 15 and 34 HPV6-seropositive controls and cases), the magnitude of the risk estimates remained similar; the OR was 1.5 (0.7–3.0) in the crude data and 1.4 (0.7–3.0) after adjusting for age, gender, smoking, and drinking.

Finally, as we have previously noted that the association of HPV16 seropositivity with HNSCC risk was significantly modified by drinking and smoking [7], we examined whether there was evidence of effect modification of the HPV6 association with HNSCC by drinking and smoking. For risk of pharyngeal cancer, we found that the effect of HPV6 seropositivity was lower in heavy smokers (OR = 1.5, 0.7–3.4) than in non/light smokers (OR = 3.1, 2.0–4.8) and lower in heavy drinkers (OR = 1.2, 0.6–2.7) than in non/light drinkers (OR = 2.4, 1.5–3.8), although the interaction terms were not statistically significant (P = 0.1 for interactions of HPV6 seropositivity with smoking and drinking). Similarly for risk of laryngeal cancer, the effect of HPV6 seropositivity was lower in heavy smokers (OR = 2.2, 0.7–6.6) than in non/light smokers (OR = 6.1, 3.4–10.7) and lower in heavy drinkers (OR = 0.6, 0.2–1.7) than in non/light drinkers (OR = 1.8, 1.0–3.1), although the interaction terms were not statistically significant (P = 0.1 for interaction of HPV6 seropositivity with smoking and P = 0.07 for interaction of HPV6 seropositivity with drinking).

discussion

In the present study, approximately one-third of the healthy population was HPV6, HPV11, HPV16, or HPV18 seropositive, agreeing with a previous study of seroprevalence to high-risk types [25]. While most studies of HPV serology in healthy individuals have focused on the detection of antibodies to high-risk type HPV16 [16, 25, 26], the highest seroprevalence in our study was to HPV6. The prevalences of positive serology to HPV11 and HPV18 were lower than that of HPV6 (and HPV16) and likely reflect a lower prevalence of these HPV types in the general population [27, 28].

Among healthy individuals, seropositivity to one type was correlated with seropositivity to other types. The overall risk of HPV infection and the risk for multiple HPV infection increases substantially with lifetime number of sex partners [16, 26]. Thus, the association of seropositivity for one HPV type and seropositivity for a second type was likely a reflection of sexual behavior. Genetic susceptibility to HPV infections, such as HLA type, may also contribute to HPV seropositivity [15].

The higher HPV seroprevalence among healthy women compared with men is in agreement with previous studies of HPV serology [16, 29], likely reflecting more efficient

Table 3. Predictors of positive HPV6, 11, and 18 serology among controls (n = 548)

Variable	HPV6				HPV11				HPV18			
	Positive (%)	Negative (%)	Crude OR (95% CI)	Adjusted OR (95% CI)[a]	Positive (%)	Negative (%)	Crude OR (95% CI)	Adjusted OR (95% CI)[a]	Positive (%)	Negative (%)	Crude OR (95% CI)	Adjusted OR (95% CI)[a]
Age												
<60	35 (46%)	210 (44%)	1.0	1.0	18 (58%)	227 (44%)	1.0	1.0	19 (44%)	226 (45%)	1.0	1.0
>60	41 (54%)	262 (56%)	0.9 (0.6–1.5)	0.8 (0.5–1.3)	13 (42%)	290 (56%)	0.6 (0.3–1.2)	**0.5 (0.2–1.0)**	24 (56%)	279 (55%)	1.0 (0.5–1.9)	1.0 (0.5–1.9)
Gender												
Male	54 (71%)	347 (74%)	1.0	1.0	20 (65%)	381 (74%)	1.0	1.0	28 (65%)	373 (74%)	1.0	1.0
Female	22 (29%)	125 (26%)	1.1 (0.7–1.9)	1.3 (0.7–2.4)	11 (35%)	136 (26%)	1.5 (0.7–3.3)	1.7 (0.7–4.0)	15 (35%)	132 (26%)	1.5 (0.8–2.9)	1.7 (0.8–3.5)
Race												
White	63 (83%)	437 (93%)	1.0	1.0	24 (77%)	476 (92%)	1.0	1.0	36 (84%)	464 (92%)	1.0	1.0
Non-white	13 (17%)	35 (7%)	**2.6 (1.3–5.1)**	**2.5 (1.2–5.0)**	7 (23%)	41 (8%)	**3.4 (1.4–8.3)**	**3.2 (1.2–8.3)**	7 (16%)	41 (8%)	2.2 (0.9–5.3)	2.1 (0.9–5.1)
Drinking												
No/low	34 (45%)	252 (53%)	1.0	1.0	13 (42%)	273 (53%)	1.0	1.0	19 (44%)	267 (53%)	1.0	1.0
High	42 (55%)	220 (47%)	1.4 (0.9–2.3)	1.3 (0.8–2.2)	18 (58%)	244 (47%)	1.5 (0.7–3.2)	1.6 (0.7–3.7)	24 (56%)	238 (47%)	1.4 (0.8–2.7)	1.6 (0.8–3.1)
Smoking												
No/low	40 (53%)	325 (69%)	1.0	1.0	17 (55%)	348 (67%)	1.0	1.0	27 (63%)	338 (67%)	1.0	1.0
High	36 (47%)	147 (31%)	**2.0 (1.2–3.3)**	**2.1 (1.2–3.5)**	14 (45%)	169 (33%)	1.7 (0.8–3.5)	1.9 (0.9–4.4)	16 (37%)	167 (33%)	1.2 (0.6–2.3)	1.2 (0.6–2.3)
HPV16												
Negative	61 (80%)	429 (91%)	1.0	1.0	22 (71%)	468 (91%)	1.0	1.0	34 (79%)	456 (90%)	1.0	1.0
Positive	15 (20%)	43 (9%)	**2.5 (1.3–4.7)**	**2.2 (1.1–4.3)**	9 (29%)	49 (9%)	**3.9 (1.7–9.0)**	**3.4 (1.4–8.0)**	9 (21%)	49 (10%)	**2.5 (1.1–5.4)**	**2.1 (1.0–4.8)**

Bold typeface indicates $P < 0.05$.

[a]The adjusted models include age, gender, race, drinking, smoking, and HPV16.

Table 4. Positive HPV6, 11, and 18 serology and HNSCC risk

Serology	Controls, *n* (%)	Pharynx			Oral			Larynx		
		Cases, *n* (%)	Crude OR (95% CI)	Adjusted OR (95% CI)[a]	Cases, *n* (%)	Crude OR (95% CI)	Adjusted OR (95% CI)[a]	Cases, *n* (%)	Crude OR (95% CI)	Adjusted OR (95% CI)[a]
HPV6										
Negative	472 (86%)	149 (73%)	1.0	1.0	150 (79%)	1.0	1.0	76 (82%)	1.0	1.0
Positive	76 (14%)	55 (27%)	**2.3 (1.5–3.4)**	**1.6 (1.0–2.5)**	39 (21%)	1.6 (1.1–2.5)	1.4 (0.9–2.1)	17 (18%)	1.4 (0.8–2.5)	1.1 (0.6–2.0)
HPV6[b]										
Negative	472 (86%)	149 (73%)	1.0	1.0	150 (79%)	1.0	1.0	76 (82%)	1.0	1.0
Low titer	43 (8%)	27 (13%)	**2.0 (1.2–3.3)**	1.5 (0.8–2.6)	13 (7%)	1.0 (0.5–1.8)	0.9 (0.4–1.7)	9 (10%)	1.3 (0.6–2.8)	1.1 (0.5–2.5)
High titer	33 (6%)	28 (14%)	**2.7 (1.6–4.6)**	**1.8 (1.0–3.2)**	26 (14%)	**2.5 (1.4–4.3)**	**1.9 (1.1–3.4)**	8 (9%)	1.5 (0.7–3.4)	1.0 (0.4–2.3)
HPV11										
Negative	517 (94%)	186 (91%)	1.0	1.0	176 (93%)	1.0	1.0	88 (95%)	1.0	1.0
Positive	31 (6%)	18 (9%)	1.6 (0.9–3.0)	0.9 (0.5–1.9)	13 (7%)	1.2 (0.6–2.4)	1.0 (0.5–2.0)	5 (5%)	0.9 (0.4–2.5)	0.7 (0.2–2.0)
HPV11[b]										
Negative	517 (94%)	186 (91%)	1.0	1.0	176 (93%)	1.0	1.0	88 (95%)	1.0	1.0
Low titer	16 (3%)	8 (4%)	1.4 (0.6–3.3)	0.8 (0.3–2.2)	6 (3%)	1.1 (0.4–2.9)	0.8 (0.3–2.2)	2 (2%)	0.7 (0.2–3.3)	0.5 (0.1–2.3)
High titer	15 (3%)	10 (5%)	1.9 (0.8–4.2)	1.0 (0.4–2.7)	7 (4%)	1.4 (0.6–3.4)	1.2 (0.5–3.0)	3 (3%)	1.2 (0.3–4.1)	1.0 (0.3–3.7)
HPV18										
Negative	505 (92%)	175 (86%)	1.0	1.0	178 (94%)	1.0	1.0	86 (92%)	1.0	1.0
Positive	43 (8%)	29 (14%)	**1.9 (1.2–3.2)**	1.3 (0.7–2.3)	11 (6%)	0.7 (0.4–1.4)	0.7 (0.3–1.4)	7 (8%)	1.0 (0.4–2.2)	0.8 (0.3–2.0)
HPV18[b]										
Negative	505 (92%)	175 (86%)	1.0	1.0	178 (94%)	1.0	1.0	86 (92%)	1.0	1.0
Low titer	22 (4%)	15 (7%)	**2.0 (1.0–3.9)**	1.5 (0.7–3.4)	5 (3%)	0.6 (0.2–1.7)	0.6 (0.2–1.6)	3 (3%)	0.8 (0.2–2.7)	0.6 (0.2–2.2)
High titer	21 (4%)	14 (7%)	**1.9 (1.0–3.9)**	1.1 (0.5–2.5)	6 (3%)	0.8 (0.3–2.0)	0.8 (0.3–2.1)	4 (4%)	1.1 (0.4–3.3)	1.1 (0.4–3.5)

Bold typeface indicates $P < 0.05$.

[a]Cases and controls matched for age and gender; adjusted models controlled for smoking, drinking, and HPV16.

[b]Low and high titer categories determined using median positive titer values as cut points (HPV6 = 20, HPV11 = 13, HPV18 = 14.5).

Table 5. Positive HPV6, 11, and 18 serology and HNSCC risk (restricted to HPV16-negative subjects)

Serology	Controls, *n* (%)	Pharynx			Oral		
		Cases, *n* (%)	Crude OR (95% CI)	Adjusted OR (95% CI)[a]	Cases, *n* (%)	Crude OR (95% CI)	Adjusted OR (95% CI)[a]
HPV6							
Negative	429 (88%)	83 (80%)	1.0	1.0	134 (83%)	1.0	1.0
Positive	61 (12%)	21 (20%)	**1.8 (1.0–3.1)**	1.4 (0.8–2.6)	28 (17%)	1.5 (0.9–2.4)	1.2 (0.7–2.0)
HPV6[b]							
Negative	429 (88%)	83 (80%)	1.0	1.0	134 (83%)	1.0	1.0
Low titer	35 (7%)	7 (7%)	1.0 (0.4–2.4)	0.8 (0.3–2.0)	9 (6%)	0.8 (0.4–1.8)	0.7 (0.3–1.5)
High titer	26 (5%)	14 (13%)	**2.8 (1.4–5.6)**	**2.3 (1.1–4.8)**	19 (12%)	**2.3 (1.3–4.4)**	**1.9 (1.0–3.6)**
HPV11							
Negative	468 (96%)	97 (93%)	1.0	1.0	154 (95%)	1.0	1.0
Positive	22 (4%)	7 (7%)	1.5 (0.6–3.7)	1.3 (0.5–3.4)	8 (5%)	1.1 (0.5–2.5)	0.9 (0.4–2.1)
HPV11[b]							
Negative	468 (96%)	97 (93%)	1.0	1.0	154 (95%)	1.0	1.0
Low titer	10 (2%)	5 (5%)	2.4 (0.8–7.2)	2.0 (0.6–6.6)	3 (2%)	0.9 (0.2–3.4)	0.7 (0.2–2.7)
High titer	12 (2%)	2 (2%)	0.8 (0.2–3.7)	0.7 (0.1–3.5)	5 (3%)	1.3 (0.4–3.7)	1.1 (0.4–3.2)
HPV18							
Negative	456 (93%)	94 (90%)	1.0	1.0	154 (95%)	1.0	1.0
Positive	34 (7%)	10 (10%)	1.4 (0.7–3.0)	1.3 (0.6–2.9)	8 (5%)	0.7 (0.3–1.5)	0.7 (0.3–1.5)
HPV18[b]							
Negative	456 (93%)	94 (90%)	1.0	1.0	154 (95%)	1.0	1.0
Low titer	17 (3%)	5 (5%)	1.4 (0.5–4.0)	1.3 (0.4–3.8)	4 (2%)	0.7 (0.2–2.1)	0.7 (0.2–2.2)
High titer	17 (3%)	5 (5%)	1.4 (0.5–4.0)	1.3 (0.5–4.0)	4 (2%)	0.7 (0.2–2.1)	0.6 (0.2–1.9)

Bold typeface indicates $P < 0.05$.

[a]Cases and controls matched for age and gender; adjusted models controlled for smoking and drinking.

[b]Low and high titer categories determined using median positive titer value as cut point.

presentation of HPV antigens to the immune system in the female genital tract compared with the male genital tract [29, 30]. Like other studies of HPV seroprevalence, we observed that non-white individuals were more likely to have positive HPV serology [13, 16] which again maybe explained by genetic differences, differences in exposure, or a combination of both.

A novel finding from our study was the association between HPV serostatus and heavy smoking. HPV seropositivity reflects prior or ongoing HPV infection. It is possible that smokers/drinkers have a higher susceptibility to HPV infection, perhaps as a result of local immunosuppression [31]. Smokers and drinkers may have HPV infections of longer duration, resulting in a higher rate of HPV seroconversion. Alternatively, heavy smoking and drinking may be a marker of a higher number of sexual partners. However, we previously observed that a higher number of sexual partners was associated with higher seroprevalence to HPV16 in healthy individuals, controlling for smoking and drinking behaviors [4]. These data suggest that there is, in fact, a biological effect of smoking on either the infectivity of HPV or on the host's ability to mount a serologic response.

We and others have previously observed that HPV16 seropositivity is associated with increased risk for HNSCC, with greatest risk present for pharyngeal cancer [2, 4, 6, 9]. A surprising finding from our current study was that risk for oral and pharyngeal cancer was also associated with HPV6 seropositivity, independent of HPV16. HPV6 is considered a low-risk type and studies have failed to find risk associated with cervical [32] or oral cancer [2, 5]. The observation of an elevated HNSCC risk associated with an immune response to HPV could reflect a different mechanism by which HPV6 leads to HNSCC. It is possible that HPV6 works through a 'hit and run' mechanism, explaining the lack of HPV6 DNA in HNSCC. Also, there is evidence of malignant progression in some cases of recurrent respiratory papillomatosis, a disease characterized by benign lesions associated with HPV6, particularly in subjects with long-standing disease, radiation therapy, and tobacco exposure [3]. As has been hypothesized to explain the greater risk associated with HPV16 and tumors of the pharynx, the tissue of the pharynx may be more susceptible to HPV infections in general. Indeed, a greater immune response may be associated with an infection in the pharyngeal region, a region in close proximity to lymphoid tissues.

We observed marginally significant interactions between HPV6 seropositivity and drinking and smoking in predicting risk for HNSCC. Consistent with previous findings for HPV16, positive serology for HPV6 was associated with a more marked cancer risk among non/light smoking compared with the heavy smoking [6, 7]. The interpretation of these interactions is complex; it is possible that heavy smokers and drinkers have relatively less risk associated with positive serology as a result of decreased infectivity of the virus associated with these exposures. There are data suggesting that increased keratinization may result from heavy tobacco and alcohol exposure [33, 34] which in turn could result in a lower prevalence of HPV in smokers/drinkers as keratinization may make the mucosa more resistant to minor trauma [15]. It is also possible that alcohol and/or tobacco affect the infectivity of the virus itself as alcohol has been shown to inhibit the infectivity of other viruses [35]. Alternatively, as positive serology was not associated with risk for HNSCC in heavy smokers and drinkers, these individuals may simply have less exposure or the virus may not act as effectively upon cells significantly altered by smoking/tobacco which are known to produce large fields of clonally altered cells [36].

In assessing risk for HNSCC associated with HPV L1 antibodies, there are clear limitations to the conclusions we can draw. As HPV is a localized infection, we cannot distinguish between oral/cervical/anal/penile infections based on serological responses and patients may be infected at multiple sites. Further, there are individual differences in the time between infection and seroconversion [37] and some individuals may fail to seroconvert. Therefore, HPV-seronegative subjects may nevertheless be HPV infected. In addition, in many cases, serum was drawn from case patients posttreatment. It is possible that patients who were treated with radiation and/or chemotherapy had dysregulated immune responses to HPV as a result of their treatment. Finally, there remains some possibility that there is still residual confounding of the HPV6 association with HNSCC by HPV16. Although our analysis employed multiple analytic strategies to control for HPV16 seropositivity, there is no absolute assurance that residual confounding is not present. The assay that we used for detecting a serologic response was designed specifically to measure an immune response to vaccination. Any bias in the nature of exposures that gives rise to this serologic response could bias our data. Hence, while our data are consistent with an increased cancer risk associated with HPV6, it cannot be considered definitive. Our observation, at a minimum, should be examined in other study groups to validate the finding of an increase in risk of HNSCC associated with HPV6.

It should be noted that there are advantages to studies of HPV serology. Case–control studies of HPV serology in HNSCC are feasible in serum while detecting HPV in tissue samples (such as buccal rinses) is problematic; these studies have yielded low levels of detectable HPV DNA and in cases do not reflect HPV DNA levels in biopsy samples [9]. While fresh tumor samples are difficult to obtain, serum can be easily obtained at relatively little burden to patients.

In our data, HPV6 seropositivity and HPV16 seropositivity are independently associated with increased risk for pharyngeal tumors. A role for HPV in HNSCC is becoming increasingly apparent, and our data suggest that the mechanism of HPV-associated HNSCC is likely to be complex, involving interactions with tobacco and alcohol. Finally, our data support the notion that the vaccines currently in progress for cervical cancer may prevent HNSCC associated with both HPV16 and HPV6.

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