ARTICLE

Lack of Association of Alcohol and Tobacco with HPV16-Associated Head and Neck Cancer

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Background Human papillomavirus type 16 (HPV16) seropositivity and alcohol and tobacco use have been associated with risk of head and neck squamous cell carcinoma (HNSCC). However, it is less clear whether HPV16 influences HNSCC risk associated with alcohol and tobacco use.

Methods Incident cases of HNSCC diagnosed between December 1999 and December 2003 were identified from nine medical facilities in Greater Boston, MA. Control subjects were frequency matched to case subjects on age, sex, and town of residence. A total of 485 case subjects and 549 control subjects reported information on lifetime smoking and alcohol consumption and provided sera, which was used to determine presence of HPV16 antibodies. Unconditional logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) of HNSCC risk by alcohol consumption (drinks per week: <3, 3 to <8, 8 to <25, ≥25) and smoking (pack-years: none, >0 to <20, 20 to <45, ≥45), adjusting for age, sex, race, education, and HPV16 serology. Polytomous logistic regression was used to estimate odds ratios and 95% confidence intervals for the association of HPV16 serology, alcohol consumption, and tobacco use in site-specific analyses. All statistical tests were two-sided.

Results The strongest risk factors by tumor site were smoking for laryngeal cancer, alcohol for cancer of the oral cavity, and HPV16 for pharyngeal cancer. For pharyngeal cancer, risk increased with increasing alcohol consumption ($OR_{\geq 25 \text{ versus } <3 \text{ drinks per week}} = 5.1$, 95% CI = 2.4 to 11.0) and smoking ($OR_{\geq 45 \text{ pack-years versus never smoker}} = 6.9$, 95% CI = 3.1 to 15.1) among HPV16-seronegative subjects but not among HPV16-seropositive subjects ($P_{\text{interaction, HPV16 serology and alcohol}} = .002$; $P_{\text{interaction, HPV16 serology and smoking}} = .007$). Among light drinkers or never smokers, HPV16 seropositivity was associated with a 30-fold increased risk of pharyngeal cancer.

Conclusions Alcohol or tobacco use does not further increase risk of HPV16-associated pharyngeal cancer. HNSCC risk associated with smoking, alcohol, and HPV16 differs by tumor site.

J Natl Cancer Inst 2007;99:1801–10

It is estimated that, in the United States in 2007, 45 000 patients will be diagnosed with head and neck squamous cell carcinoma (HNSCC), which includes cancers of the oral cavity, pharynx, and larynx, and that approximately 11 000 people are likely to die from HNSCC during that year (1). The majority of HNSCC patients present with advanced disease, and this often requires intensive treatment with chemotherapy, radiation therapy, and surgery with consequent long-term morbidity and attendant mortality. The annual cost of treatment of HNSCC in the United States has been estimated to be $3.2 billion (2).

Two modifiable risk factors, tobacco use and alcohol consumption, are thought to explain approximately 75% of HNSCC incidence (3). Epidemiologic studies have shown that the risk of HNSCC increases with cigarette smoking, regardless of whether smoking is analyzed as the amount smoked, duration of tobacco use, or cumulative smoking (3–8). Among people who do not smoke, alcohol has been found to be an independent risk factor for HNSCC (9–17). In addition, the risk for HNSCC associated with joint exposure to alcoholic beverages and tobacco smoke exceeds the sum of the individual risks (3,11,18–24).

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See "Funding" and "Notes" following "References."

DOI: 10.1093/jnci/djm233

CONTEXT AND CAVEATS

Prior knowledge

It was unclear how infection with human papillomavirus type 16 (HPV16) influences the association between smoking and drinking and the risk of head and neck squamous cell carcinoma.

Study design

Alcohol consumption and smoking habits among patients who were diagnosed with head and neck squamous cell carcinoma and matched control subjects were ascertained by a questionnaire, and the presence of HPV16 in the blood was determined using antibody tests. Logistic regression was used to examine the relationships between these risk factors.

Contribution

This study found that smoking and drinking was not associated with the risk of head and neck squamous cell carcinoma among those whose blood tested positive for HPV16.

Implications

Head and neck squamous cell carcinomas that are associated with viral infection and those associated with smoking and drinking may have different etiologies.

Limitations

The presence of serum antibodies to the virus may be a poor surrogate for viral infection at the cancer site, and the low participation rate of matched control subjects may have biased risk estimates.

Another risk factor for HNSCC is infection with human papillomavirus (HPV), particularly type 16 (HPV16). HPV16 seropositivity is associated with an approximately fourfold increased risk of HNSCC (7,25,26). The prevalence of HPV16 antibodies has been found to increase with the number of sexual partners in both HNSCC case and control subjects (25,27–29). Because systemic antibodies are generated by local HPV infection, the presence of HPV16 antibodies indicates HPV16 exposure but not necessarily infection in the head and neck region. However, HPV16 genomic DNA has been detected in HNSCC tumors of patients that were positive for HPV16 antibodies (25,26). In addition, epidemiologic research suggests that nonsmokers and light or nondrinkers are more likely to have tumors positive for HPV16 than are heavy smokers and drinkers (30). Thus, HPV16-related tumors may have an etiology that is distinct from alcohol- and tobacco-related disease.

There has been limited investigation of the relationship among HPV16, alcohol consumption, and smoking. In this analysis, we investigated whether HPV16 is a confounder or effect modifier for the relationship between alcohol and tobacco use and risk of HNSCC. We compared dose–response models for cancer risk and use of alcohol and tobacco for individuals with and without detectable antibodies against HPV16. We also examined the relationships among HPV16 seropositivity, alcohol consumption, tobacco use, and cancer risk by tumor site.

Methods

Study Population

Patients diagnosed with HNSCC in the Greater Boston, MA, area between December 1999 and December 2003 were identified from head and neck clinics and departments of otolaryngology or radiation oncology at nine medical facilities (New England Medical Center, Massachusetts General Hospital, Massachusetts Eye and Ear Infirmary, Dana-Farber Cancer Institute, Brigham and Women's Hospital, Boston Veterans Administration, Beth Israel Deaconess Medical Center, Boston Medical Center, and Harvard Vanguard Medical Associates). The study area included 249 cities and towns within 1 hour's drive of Boston.

Eligible patients had carcinoma located on the tongue, gum, floor of mouth, other location in the mouth, oropharynx, hypopharynx, ill-defined site within lip oral cavity or pharynx, and larynx (corresponding to International Classificiation of Disease, Ninth Revision (ICD-9) codes 141, 143, 144, 145, 146, 148, 149, and 161, respectively), as determined by pathology reports. Additional eligibility criteria included residence in the study area, age of 18 years or older, and a diagnosis of HNSCC no more than 6 months before the time of patient contact. Patients presenting with recurrent disease were excluded. Potential control subjects were identified from Massachusetts town books, which are required by state law to list all residents 17 years and older (31). Control subjects were matched to case subjects on sex, age (within 3 years), and town of residence using random selection. All study protocol and materials were approved by the Institutional Review Board at the nine medical facilities and the Harvard School of Public Health, and all study participants provided written informed consent.

Questionnaire

Study participants answered a self-administered questionnaire (Supplementary Fig. 1, available online) that was reviewed with study personnel. Case subjects received their questionnaires during an initial clinic visit. Control subjects were mailed their questionnaires, and a research coordinator reviewed their responses in person on a subsequent visit. The questionnaire included questions about demographic characteristics, medical history, diet, and detailed smoking and drinking habits. Specifically, subjects were asked to report their average consumption of beer, wine, and liquor in a typical week for 10 time periods in their life (when they were 10–14, 15–18, 19–23, 24–29, 30–39, 40–49, 50–59, 60–69, 70–79, and ≥80 years old). One drink of beer was defined as one bottle, can, or glass of beer; one drink of wine was defined as one glass of wine, champagne, or wine cooler; and one drink of liquor was defined as one shot, cocktail, or mixed drink. Subjects indicated how many drinks they consumed by selecting from multiple-choice answers (none or <1 per week, 1–2 per week, 3–6 per week, 1 per day, 2–4 per day, 5–7 per day, 8–10 per day, ≥11 per day). Lifetime average drinks per week were determined by summing across all drinking ages and dividing by the total number of years the subject reported drinking. If subjects refused to answer the questions about lifetime alcohol consumption (50 case subjects, but no control subjects, refused), their alcohol consumption was determined from an abbreviated section of the questionnaire in which they reported how many days of the week (none, 1–2, 2–3, 4–5, 6–7 days) they usually had at least one drink from three categories of drinks (beer, wine, liquor). For each of these beverage types, they also indicated the usual number of beverages they would have for the days they were drinking (0, 1–2, 3–5, 6–8, 9–11, 12–14, ≥15 drinks).

To estimate lifetime average drinks per week, the midpoint of each category of number of drinks was multiplied by the midpoint of the category for the number of days a week they drank for each of the three types of beverages, and the products were summed.

A detailed lifetime smoking history was also collected. Subjects were asked if they had ever smoked 100 cigarettes or more (five packs) in their lifetime. If they had not, they were considered to be never smokers. The questionnaire asked subjects about smoking habits during each decade of life (ages 10–19, 20–29, 30–39, 40–49, 50–59, 60–69, 70–79, ≥80 years old). Subjects were asked how many packs they would typically smoke each day within those ages (none, <½ pack, ½ pack, ½–1 pack, 1 pack, 1½–2 packs, 2 packs, 2½–3 packs, 3 packs, 3½–4 packs, 4 packs, ≥4 packs). The midpoint of the number of packs for each category was used to indicate the packs per day for each subject at a particular age, and then pack-years (i.e., pack/day-years) was calculated by totaling the number of cigarettes smoked per day over the subject's lifetime. If subjects refused to fill in their lifetime smoking history (43 case subjects and one control subject refused), they were asked to report their average cigarettes smoked per day when they were regular smokers and how many years they smoked. Pack-years was calculated by multiplying these two values.

HPV16 Serology

The HPV16 serologic status of case and control subjects was ascertained as described previously (25). Serum was separated from plasma within 24 hours of collection and stored at −80 °C. The HPV Competitive Luminex Immunoassay was used to determine presence of antibodies to the L1 protein of HPV16 (32). Positive and negative controls were used for quality control, and all samples were tested in duplicate.

Detection of HPV16 Viral DNA in HNSCC Tumors

Details regarding the collection of tumor specimens and the DNA extraction have been provided previously (25,33). HPV16 in tumor DNA was detected using the short fragment polymerase chain reaction assay, as described previously (25). We slightly modified the method of Kleter et al. (34), in which PCR primers were developed for universal detection of HPV, amplifying the beta-actin locus as a control. DNA from Siha and Ca33 cells was used for positive and negative controls. This assay was conducted blinded to HPV16 serologic status, sexual history, and other risk factors.

Statistical Analysis

We compared the dose–response for HNSCC risk and alcohol and tobacco use among those who were HPV16 seropositive with those who were HPV16 seronegative. We first created categories for alcohol and tobacco use based on the distribution of these variables in control subjects. However, for both of these risk factors, half of the case subjects were in the highest category, limiting our ability to analyze differences in the dose–response. Therefore, the cut points for alcohol and smoking were based on the joint distribution of case subjects and control subjects. For alcohol consumption, there were too few never drinkers to use them as a stable reference category. Therefore, quartiles of alcohol consumption were determined (rounding to the nearest whole drink), resulting in the following categories for average alcoholic drinks per week: <3, 3 to <8, 8 to <25, and ≥25. For smoking, never smokers served as the referent group and the remaining exposed subjects were divided into tertiles (>0 to <20, 20 to <45, and ≥45 pack-years). Unconditional logistic regression was used to generate odds ratios (ORs) and 95% confidence intervals (CIs) containing three indicator variables for the four categories of average drinks per week and three indicator variables for the four categories of smoking and controlling for age (continuous), sex, race (white or other), education (did not finish high school or high school diploma or more education), and HPV16 serologic status (negative or positive). Using a greater number of categories for education (e.g., no high school diploma, high school diploma, college, or advanced degrees) did not influence the observed associations (data not shown); therefore, the dichotomous variable for education was included in the model. Controlling for household income did not change the observed associations between alcohol, tobacco, HPV16 serology, and HNSCC risk; therefore, it was left out of the final model. Unconditional logistic regression is appropriate for frequency matching when matching variables are included in the model (35). Trend tests for quartiles of alcohol and tobacco were conducted using an ordinal term to represent categories of increasing drinking or smoking.

Joint effects odds ratios and 95% confidence intervals for alcohol and smoking were modeled using the lowest exposure category for the two risk factors combined (e.g., never smoker and lowest alcohol intake category) as the reference category and including indicator variables for the remaining combined categories. Tests for statistical interaction were conducted using ordinal terms for smoking and alcohol intake categories and a cross product of these terms (a one degree of freedom test). Joint effects models and tests for interaction were also conducted for HPV16 serologic status with smoking and HPV16 serologic status with drinking.

Differences in the dose–response between HNSCC risk and use of alcohol and tobacco by HPV16 status were also examined using the presence of HPV16 in tumor DNA as the measure of viral status. Models were generated separately for tumors positive for HPV16 DNA relative to control subjects and tumors without detectable HPV16 in their DNA relative to the control subjects.

We also examined the association between HPV16 serology, alcohol consumption, tobacco use, and HNSCC risk by tumor location. Tumors were classified as oral cavity, pharynx, or larynx based on recommendations by the American Joint Committee on Cancer (AJCC) (36). The AJCC recommends that tumors at the base of the tongue be classified as pharyngeal and those located at the anterior of the tongue be classified as of the oral cavity. Therefore, the pathology reports for subjects with carcinoma of the tongue were reviewed and further classified as located at the anterior or the base of the tongue. This review was conducted blinded to history of alcohol consumption, tobacco use, and HPV16 status. Oral cavity tumors corresponded to ICD-9 codes 143, 144, 145, and, if located at the anterior of the tongue, 141; pharyngeal tumors corresponded to ICD-9 codes 146, 148, 149, and, if at the base of the tongue, 141; and laryngeal tumors corresponded to ICD-9 code 161. Two cases of tongue cancer

Table 1. Selected descriptive characteristics for case subjects with head and neck squamous cell carcinoma and control subjects*

Characteristic	Case subjects, n = 485	Control subjects, n = 549	P†
Age, y			
Mean (SD)	59.5 (11.6)	61.0 (11.4)	
Sex			
Male	360 (74.2%)	402 (73.2%)	
Female	125 (25.8%)	147 (26.8%)	
Race			
White	444 (91.5%)	501 (91.3%)	.854
Other	41 (8.5%)	48 (8.7%)	
Education‡			
High school diploma or higher	361 (82.0%)	503 (91.8%)	<.001
Did not finish high school	79 (18.0%)	45 (8.2%)	
Smoking, pack-years			
None	90 (18.5%)	182 (33.1%)	<.001
>0 to <20	92 (19.0%)	152 (27.7%)	
20 to <45	126 (26.0%)	123 (22.4%)	
≥45	177 (35.5%)	92 (16.8%)	
Alcohol consumption, average drinks per wk			
<3	90 (18.5%)	154 (28.0%)	<.001
3 to <8	95 (19.6%)	175 (31.9%)	
8 to <25	121 (25.0%)	142 (25.9%)	
≥25	179 (36.9%)	78 (14.2%)	
HPV 16 serology			
Negative	340 (70.1%)	491 (89.4%)	<.001
Positive	145 (29.9%)	58 (10.6%)	

* SD = standard deviation; HPV16 = human papillomavirus type 16.

† Tests controlled for age and sex.

‡ Data missing on highest level of education for one control subject and 45 case subjects.

were excluded from the site-specific analyses because the pathology review did not allow for further classification. Polytomous logistic regression was used to estimate odds ratios and 95% confidence intervals for the association of HPV16 serology, alcohol, and tobacco with the three tumor locations compared with control subjects, controlling for age, sex, race, and education. Models were generated using SAS version 9.1. All tests were two-sided, and a P value of .05 was considered to be statistically significant.

Whereas the relationship between increasing smoking and increasing HNSCC risk is usually a linear one, the association between HNSCC and alcohol use is not as predictable. Odds ratios below the null have been reported with light consumption of alcoholic drinks (37–39). In addition, selected cut points for categoric models may obscure a nonlinear association (40). For these reasons, we used a semiparametric approach to further describe the dose–response between average drinks per week and risk of HNSCC. In a logistic regression model, the log odds ratio was modeled as a smoothed function of average drinks per week using restricted cubic splines based on truncated polynomials (41,42). In doing so, we modeled the association without an a priori assumption about the shape of the dose–response curve. Four knots were used, and the curve was not sensitive to changes in the number of knots. We also fitted restricted cubic splines for alcohol separately by HPV16 serologic status to determine if the dose–response for alcohol and HNSCC risk differed by HPV16. Spline models were generated using R 2.3.1.

Results

A total of 823 patients with incident HNSCC were identified as eligible for the study. Of these, 57 patients refused to participate and 44 did not complete their questionnaire, leaving 722 enrolled case subjects and a case participation rate of 88%. For population-based control subjects, 1643 members of the study communities were identified as eligible; 828 refused to participate, and 815 consented. Six control subjects were excluded when their corresponding case subject later became ineligible. Among the remaining control subjects who provided consent, 765 completed the questionnaire and were enrolled in the study, resulting in a 47% participation rate for control subjects. Beginning in 2001, we requested blood samples, which were obtained from 81% of the case subjects and 80% of the control subjects enrolled after that time. The presence of HPV16 antibodies was successfully determined for 485 case subjects and 549 control subjects, and these subjects were included in this analysis.

The distribution of selected characteristics for case and control subjects is shown in Table 1. For both groups, the mean age was approximately 60, and there was a preponderance of males and whites. Case subjects were less likely to have finished high school than control subjects (P<.001) after controlling for age and sex. They also smoked more (P<.001) and consumed more alcoholic drinks per week on average (P<.001). A greater percentage of case subjects (29.9%) tested positive for HPV16 antibodies than did control subjects (10.6%), and this difference was statistically significant (P<.001).

The associations between HNSCC and alcohol use and smoking are shown in Table 2, which compares models controlling and not controlling for HPV16 serology. In the model without HPV16 serology, the risk of HNSCC increased with increasing smoking ($OR_{\geq 45 \text{ pack-years versus never smokers}}$ = 3.0, 95% CI = 2.0 to 4.6) and alcohol consumption ($OR_{\geq 25 \text{ drinks per week versus } <3 \text{ drinks per week}}$ = 3.0, 95% CI = 1.9 to 4.6). In the model adjusting for HPV16 serology, the magnitude of the association between HNSCC and smoking remained strong ($OR_{\geq 45 \text{ pack-years versus never smokers}}$ = 3.4, 95% CI = 2.2 to 5.3), as did the association of HNSCC with alcohol use ($OR_{\geq 25 \text{ drinks per week versus } <3 \text{ drinks per week}}$ = 3.1, 95% CI = 2.0 to 4.9). Thus, HPV16 serology was not a strong confounder for associations of HNSCC risk and cigarette smoking and alcohol consumption, even though HPV16 seropositivity was itself a strong risk factor for HNSCC (OR = 4.5, 95% CI = 3.1 to 6.5).

We next fit a restricted cubic spline model to examine a possible nonlinear relationship between alcohol and HSNCC risk (Fig. 1). The plot was truncated at 50 drinks per week because there were few subjects who consumed in excess of this amount. Although the odds ratio between alcohol consumption and HNSCC risk was below the null for those who consumed less than 10 drinks per week, the confidence interval included the null in this range of exposure. Otherwise, the risk of HNSCC increased with increasing alcohol consumption.

We fit restricted cubic splines to examine the relationship between alcohol use and HSNCC after stratifying by HPV16

Table 2. Association of head and neck squamous cell carcinoma with alcohol, tobacco, and human papillomavirus type 16 (HPV16) serology*

Characteristic	Case subjects, n = 485 (%)	Control subjects, n = 549 (%)	Model 1 (without HPV16 serology) OR† (95% CI)	Model 2 (with HPV16 serology) OR† (95% CI)
Smoking, pack-years				
None	90 (18.5)	182 (33.2)	1.0 (Referent)	1.0 (Referent)
>0 to <20	92 (19.0)	152 (27.7)	1.2 (0.8 to 1.7)	1.1 (0.7 to 1.7)
20 to <45	126 (26.0)	123 (22.4)	1.7 (1.2 to 2.6)	1.8 (1.2 to 2.7)
≥45	177 (35.5)	92 (16.8)	3.0 (2.0 to 4.6)	3.4 (2.2 to 5.3)
P_{trend}			<.001	<.001
Alcohol consumption, average drinks per wk				
<3	90 (18.5)	154 (28.0)	1.0 (Referent)	1.0 (Referent)
3 to <8	95 (19.6)	175 (31.9)	1.0 (0.7 to 1.5)	1.0 (0.7 to 1.6)
8 to <25	121 (25.0)	142 (25.9)	1.5 (1.0 to 2.2)	1.4 (0.9 to 2.1)
≥25	179 (36.9)	78 (14.2)	3.0 (1.9 to 4.6)	3.1 (2.0 to 4.9)
P_{trend}			<.001	<.001
HPV16 serology				
Negative	340 (70.1)	491 (89.4)		1.0 (Referent)
Positive	145 (29.9)	58 (10.6)		4.5 (3.1 to 6.5)

* OR = odds ratio; CI = confidence interval.

† Models also controlled for sex, age (continuous), race (white, other), education (<high school graduate, ≥high school graduate).

serology. The risk of HNSCC increased with increasing consumption of alcoholic beverages among those who were HPV16 seronegative (Fig. 2). However, among the HPV16 seropositive subjects, there was no association between HNSCC and alcohol use. In a linear model with an interaction term for alcohol and HPV16 serology, these differences in the relationship between alcohol use and HSNCC by HPV16 status were statistically significant ($P_{interaction}$ = .035).

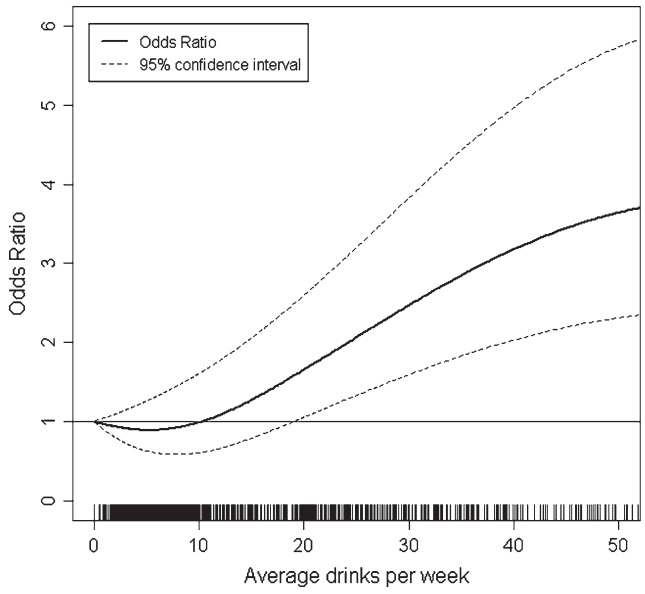

Fig. 1. Odds ratio and 95% confidence intervals for the association between average consumption of alcoholic drinks per week and head and neck squamous cell carcinoma. The logistic regression model controlled for age, sex, race, education, pack-years, and HPV16 serology (485 case subjects and 549 control subjects). The **rug plot** along the *x*-axis indicates the distribution of average alcoholic drinks per week.

Given the differences in the dose–response for alcohol consumption and HNSCC risk by HPV16 seropositivity, we investigated the interaction between alcohol and tobacco among those who were HPV16 seronegative (Table 3). The interaction between alcohol and smoking was statistically significant ($P_{interaction}$ = .014). The study lacked power to examine the interaction between

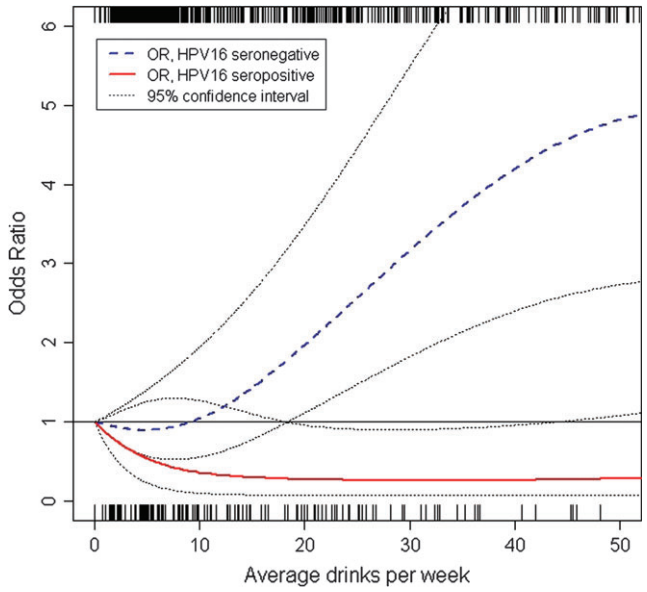

Fig. 2. Odds ratio for the association between average consumption of alcoholic drinks per week and head and neck squamous cell carcinoma stratified by human papillomavirus type 16 (HPV16) serology. Both models controlled for age, sex, race, education, and pack-years. The **rug plot** along the top horizontal axis indicates the distribution of average alcoholic drinks consumed among those who were HPV16 seronegative (340 case subjects and 491 control subjects). The **rug plot** along the bottom horizontal axis indicates the distribution of average alcoholic drinks consumed among those who are HPV16 seropositive (145 case subjects and 58 control subjects).

Table 3. Joint effects of smoking and alcohol consumption in a study of head and neck squamous cell carcinoma among those who were human papillomavirus type 16 (HPV16) seronegative*

	Smoking, pack-years			
Alcohol consumption, average drinks per wk	**None**	**>0 to <20**	**20 to <45**	**≥45**
<3				
Case subjects/control subjects, n	26/71	11/33	11/17	10/21
OR† (95%CI)	1.0 (Referent)	1.1 (0.5 to 2.6)	2.3 (0.9 to 5.9)	1.8 (0.7 to 4.8)
3 to <8				
Case subjects/control subjects, n	13/52	20/55	12/36	15/18
OR† (95%CI)	0.8 (0.4 to 1.8)	1.4 (0.7 to 2.9)	1.6 (0.7 to 3.7)	4.5 (1.9 to 10.8)
8 to <25				
Case subjects/control subjects, n	10/35	13/30	23/36	38/20
OR† (95%CI)	1.3 (0.5 to 3.2)	1.8 (0.8 to 4.3)	2.5 (1.2 to 5.4)	9.6 (4.4 to 21.2)
≥25				
Case subjects/control subjects, n	6/8	11/14	45/22	76/23
OR† (95%CI)	3.5 (1.0 to 12.2)	3.0 (1.1 to 8.4)	9.3 (4.4 to 19.9)	14.8 (7.1 to 30.8)

* OR = odds ratio; CI = confidence interval.

† This single model controlled for sex, age (continuous), race (white, other), and education (<high school graduate, ≥high school graduate); $P_{interaction}$ = .014.

alcohol and tobacco among the HPV16-seropositive individuals. However, an analysis that included all subjects, regardless of HPV16 serologic status, showed reduced odds ratios such that the interaction was no longer statistically significant (results not shown).

In addition to examining all HNSCC combined, we analyzed tumors by site. Among the case subjects, 203 subjects had pharyngeal tumors, 187 had tumors of the oral cavity, and 93 had laryngeal tumors. We used polytomous logistic regression to estimate the association between site-specific HNSCC risk and smoking, alcohol use, and HPV16 serology, controlling for age, sex, race, and education (Table 4). Laryngeal cancer was most strongly associated with smoking. Only five subjects with laryngeal cancer were never smokers, and there was a statistically significant trend of increasing risk with increasing smoking up to an odds ratio of 19.9 (95% CI = 7.2 to 54.9, P_{trend}<.001) among those with 45 pack-years or more. Only those in the highest category of alcohol consumption (≥25 drinks per week) had an elevated risk of laryngeal cancer, and the increase was not statistically significant. HPV16 seropositivity was also associated with a nearly threefold increased risk of cancer of the larynx (OR = 2.7, 95% CI = 1.5 to 5.1).

For cancer of the oral cavity, the strongest risk factor was alcohol consumption ($OR_{\geq 25 \text{ drinks per week versus } <3 \text{ drinks per week}}$ = 4.8, 95% CI = 2.6 to 8.9). The trend of increasing risk with increasing alcohol

Table 4. Site-specific head and neck squamous cell carcinoma and association with human papillomavirus type 16 (HPV16) serology, alcohol, and tobacco using polytomous logistic regression*

		Case subjects by tumor location					
Characteristic	**Control subjects, n = 549 (%)**	**Larynx, n = 93 (%)**	**OR† (95% CI)**	**Oral cavity, n = 187 (%)**	**OR† (95% CI)**	**Pharynx, n = 203 (%)**	**OR† (95% CI)**
Smoking, pack-years							
None	182 (33.2)	5 (5.4)	1.0 (Referent)	44 (23.5)	1.0 (Referent)	41 (20.2)	1.0 (Referent)
>0 to <20	152 (27.7)	15 (16.1)	3.7 (1.3 to 10.6)	38 (20.3)	0.9 (0.6 to 1.6)	38 (18.7)	0.9 (0.5 to 1.6)
20 to <45	123 (22.4)	22 (23.7)	6.5 (2.3 to 18.5)	52 (27.8)	1.4 (0.8 to 2.4)	52 (25.6)	1.6 (0.9 to 2.8)
≥45	92 (16.8)	51 (54.8)	19.9 (7.2 to 54.9)	53 (28.3)	1.8 (1.0 to 3.2)	72 (35.5)	3.2 (1.8 to 5.6)
P_{trend}			<.001		.027		<.001
Alcohol consumption, average drinks per wk							
<3	154 (28.0)	18 (19.4)	1.0 (Referent)	34 (18.2)	1.0 (Referent)	38 (18.7)	1.0 (Referent)
3 to <8	175 (31.9)	18 (19.4)	0.8 (0.4 to 1.7)	41 (21.9)	1.4 (0.8 to 2.4)	36 (17.7)	0.8 (0.5 to 1.4)
8 to <25	142 (25.9)	23 (24.7)	0.9 (0.4 to 1.8)	44 (23.5)	1.9 (1.1 to 3.3)	54 (26.6)	1.3 (0.8 to 2.3)
≥25	78 (14.2)	34 (36.6)	1.5 (0.7 to 3.1)	68 (36.4)	4.8 (2.6 to 8.9)	75 (37.0)	2.9 (1.6 to 5.2)
P_{trend}			.312		<.001		<.001
HPV16 serology							
Negative	491 (89.4)	75 (80.7)	1.0 (Referent)	160 (85.6)	1.0 (Referent)	103 (50.7)	1.0 (Referent)
Positive	58 (10.6)	18 (19.4)	2.7 (1.5 to 5.1)	27 (14.4)	1.7 (1.0 to 2.8)	100 (49.3)	10.0 (6.6 to 15.3)

* OR = odds ratio; CI = confidence interval.

† All odds ratios come from a single polytomous model that also controlled for sex, age (continuous), race (white, other), and education (<high school graduate, ≥high school graduate).

Table 5. Risk of pharyngeal cancer by alcohol consumption and human papillomavirus type 16 (HPV16) serology*

HPV16 serology	Alcohol consumption, average drinks per wk	Control subjects, n = 549 (%)	Pharyngeal cancer case subjects, n = 203 (%)	Joint effects OR† (95% CI)	Stratified OR† (95% CI)
Negative	<3	142 (25.9)	13 (6.4)	1.0 (Referent)	1.0 (Referent)
	3 to <8	161 (29.3)	14 (6.9)	1.1 (0.5 to 2.5)	1.1 (0.5 to 2.5)
	8 to <25	121 (22.0)	27 (13.3)	2.3 (1.1 to 4.9)	2.3 (1.1 to 4.9)
	≥25	67 (12.2)	49 (24.1)	5.1 (2.4 to 11.0)	5.1 (2.4 to 11.0)
Positive	<3	12 (2.2)	25 (12.3)	29.1 (11.3 to 74.9)	1.0 (Referent)
	3 to <8	14 (2.6)	22 (10.8)	19.8 (7.8 to 50.1)	0.7 (0.3 to 1.9)
	8 to <25	21 (3.8)	27 (13.3)	15.3 (6.5 to 36.3)	0.5 (0.2 to 1.4)
	≥25	11 (2.0)	26 (12.8)	16.2 (6.1 to 43.5)	0.6 (0.2 to 1.7)
$P_{interaction}$				.002	

* OR = odds ratio; CI = confidence interval.

† OR adjusted for sex, age (continuous), race (white, other), education (<high school graduate, ≥high school graduate), and smoking (none and 0 to <20, 20 to <45, ≥45 pack-years).

consumption was statistically significant (P_{trend}<.001). Smoking was also a risk factor for cancer of the oral cavity, although the association was not as strong as with alcohol. The risk of cancer of the oral cavity was also elevated with positive HPV16 serology (OR = 1.7, 95% CI = 1.0 to 2.8).

Pharyngeal cancer had the strongest association with HPV16 seropositivity (OR = 10.0, 95% CI = 6.6 to 15.3). However, smoking and alcohol were also risk factors for cancer of this site ($OR_{\geq 45 \text{ pack-years versus never smokers}}$ = 3.2, 95% CI = 1.8 to 5.6; $OR_{\geq 25 \text{ drinks per week versus } <3 \text{ drinks per week}}$ = 2.9, 95% CI = 1.6 to 5.2).

With case subjects of pharyngeal cancer approximately equally divided between HPV16 seronegative and seropositive, we were able to examine whether the relationship between alcohol consumption and risk of pharyngeal cancer differed by HPV16 antibody status (Table 5). Among those who were HPV16 seronegative, the risk of pharyngeal cancer increased with greater alcohol consumption ($OR_{\geq 25 \text{ drinks per week versus } <3 \text{ drinks per week}}$ = 5.1, 95% CI = 2.4 to 11.0, P_{trend}<.001). Among the low alcohol consumers (<3 drinks per week), the odds ratio comparing the risk of pharyngeal cancer for those who were HPV16 seropositive with those who were HPV16 seronegative was 29.1 (95% CI = 11.3 to 74.9). However, among those who were HPV16 seropositive, consumption of alcoholic beverages was not associated with increased risk of pharyngeal cancer. The interaction between HPV16 serologic status and alcohol for pharyngeal cancer was statistically significant ($P_{interaction}$ = .002).

We also examined whether the association between smoking and risk of pharyngeal cancer varied by HPV16 serologic status (Table 6). Risk of pharyngeal cancer increased with smoking among those who were HPV16 seronegative ($OR_{\geq 45 \text{ pack-years versus never smokers}}$ = 6.9, 95% CI = 3.1 to 15.1, P_{trend}<.001). Among never smokers, risk of pharyngeal cancer increased approximately 30-fold for the HPV16-seropositive subjects compared with the seronegative subjects (OR = 32.5, 95% CI = 13.3 to 79.5). However, among the HPV16-seropositive subjects, smoking cigarettes did not increase risk of pharyngeal cancer. The interaction between smoking and HPV16 status was statistically significant ($P_{interaction}$ = .007).

The relationships among pharyngeal cancer risk, HPV16 status, and alcohol and smoking were essentially unchanged when HPV16 DNA detection in tumors was used to determine HPV16 status. There was no dose–response relationship between either alcohol or tobacco use and pharyngeal cancer risk in case subjects with detectable HPV16 DNA in tumors compared with the control subjects. However, there was an increasing risk of pharyngeal

Table 6. Risk of pharyngeal cancer by smoking and human papillomavirus type 16 (HPV16) serology*

HPV16 serology	Cigarettes, pack-years	Control subjects, n = 549 (%)	Pharyngeal cancer case subjects, n = 203 (%)	Joint effects OR† (95% CI)	Stratified OR† (95% CI)
Negative	None	166 (30.2)	10 (4.9)	1.0 (Referent)	1.0 (Referent)
	>0 to <20	132 (24.0)	15 (7.4)	1.7 (0.7 to 4.0)	1.7 (0.7 to 4.0)
	20 to <45	111 (20.2)	30 (14.8)	3.4 (1.5 to 7.5)	3.4 (1.5 to 7.5)
	≥45	82 (14.9)	48 (23.7)	6.9 (3.1 to 15.1)	6.9 (3.1 to 15.1)
Positive	None	16 (2.9)	31 (15.3)	32.5 (13.3 to 79.5)	1.0 (Referent)
	>0 to <20	20 (3.6)	23 (11.3)	15.5 (6.3 to 37.9)	0.5 (0.2 to 1.2)
	20 to <45	12 (2.2)	22 (10.8)	22.4 (8.4 to 60.1)	0.7 (0.3 to 1.8)
	≥45	10 (1.8)	24 (11.8)	27.7 (9.8 to 77.9)	0.9 (0.3 to 2.4)
$P_{interaction}$				.007	

* OR = odds ratio; CI = confidence interval.

† OR adjusted for sex, age (continuous), race (white, other), education (<high school graduate, ≥high school graduate), and alcohol consumption (<3, 3 to <8, 8 to <25, ≥25 drinks per week).

cancer associated with increasing alcohol consumption and with higher pack-years for case subjects in whom HPV16 DNA was undetectable in the tumor (data not shown).

Finally, since blood was drawn after treatment began for approximately one-fifth of case subjects, we examined whether drawing blood before or after treatment commenced influenced antibody detection. We found that approximately the same proportion of case subjects was classified as HPV16 seropositive whether they had blood drawn before or after starting treatment for disease (26.7% or 28.3%, respectively).

Discussion

In this study, we found that HPV16 modified the associations between HNSCC and its major risk factors, alcohol and tobacco use. In particular, HPV16 seropositivity was associated with a 10-fold increased risk of pharyngeal cancer after controlling for alcohol and tobacco use. Among those who drank less than three drinks per week or were never smokers, there was an approximately 30-fold increased risk of pharyngeal cancer associated with being HPV16 seropositive. Furthermore, subjects who were HPV16 seropositive exhibited no increased risk of pharyngeal cancer with increasing drinking or smoking. HPV16 seropositivity was also a statistically significant risk factor for laryngeal cancer as well as cancers of the oral cavity, although laryngeal cancer was most strongly associated with smoking and alcohol consumption was the strongest risk factor for cancer of the oral cavity.

Our observation that there was no dose–response for drinking or smoking and HNSCC risk among those who were HPV16 seropositive is supported by previous studies in which HPV16 exposure was similarly determined through detection of antibodies against the HPV16 L1 protein (7,26,43). For example, D'Souza et al. (43) reported that, among HPV16-seronegative subjects, the risk of oropharyngeal cancer was associated with heavy drinking and heavy smoking, but neither heavy drinking nor heavy smoking was associated with increased risk among those who were HPV16 seropositive. Furthermore, in a multi-center study in Europe, Herrero et al. (26) found that the risk of oropharyngeal cancer associated with smoking or pan chewing was stronger for HPV16-seronegative subjects (OR = 9.2) than for HPV16-seropositive subjects (OR = 4.0: from $OR_{HPV+,\ ever\ smoker} \div OR_{HPV+,\ never\ smoker} = 26.6 \div 6.7$). Finally, in a population-based case–control study in Washington State, Schwartz et al. (7) analyzed the associations of pack-years of tobacco smoking and antibodies against HPV16 with cancers of the oropharynx and oral cavity combined. The association with smoking was not as strong among the HPV16-seropositive subjects (OR = 3.8: from $OR_{HPV+,\ \geq 20\ pack\text{-}years} \div OR_{HPV+,\ <20\ pack\text{-}years} = 10.8 \div 2.8$) as among the HPV16-seronegative subjects (OR = 5.6), although the authors also reported synergy between current smoking and HPV16 seropositivity. However, a limitation of that analysis was that the referent category combined never smokers and former smokers, even though never smokers have been found to have a different prevalence of HPV16 seropositivity than smokers in previous studies (25,26). In addition, former smokers could be considered misclassified if subjects quit smoking not long before diagnosis. For these reasons, the use of current smoking may not be the preferred metric for investigating the interaction of smoking and HPV16 seropositivity, and the results using pack-years are likely to be more reliable.

In contrast to the results of our analysis, a study that used oral exfoliated cells to determine infection with high-risk HPV types (including 16, 18, and others) reported synergy between HPV infection and high alcohol consumption as risk factors for HNSCC (44). However, the use of oral rinses or brushes as a means of detecting HPV in the head and neck has been questioned, in part because of weak sensitivity of the assay to the presence of HPV in the tumor (7,26,45,46). It has also been suggested that these methods may not detect subclinical HPV infections at the basal layer of normal tissue (47). Finally, exfoliated cells from the oral mucosa of smokers may differ from those of nonsmokers; hence, the target cells for assessing HPV16 presence may differ by smoking status (48).

The concept of field cancerization has long been proposed to explain the development of HNSCC (49). Exposure to tobacco or alcohol, which has been associated with inactivation of tumor suppressors, including TP53 and Rb (50–53), has been proposed to lead to the development of a field or clonal unit in which the daughter cells maintain the altered cellular phenotype (54). Subsequent genetic alterations in the field may contribute to the development of frank malignancy. Recently, the concept of field carcinogenesis for HNSCC has also been applied to HPV16-related disease (55,56). The development of a field may occur when the E6 viral protein targets p53 for ubiquitination and degradation and the E7 protein disrupts the function of pRb (57,58), thus inactivating tumor suppressor pathways. Our data showing no association between pharyngeal cancer and alcohol or tobacco use among the HPV16 seropositive are consistent with the idea that when these tumor suppressor pathways are inactivated by HPV16, any additional phenotypic clonal selection associated with alcohol and tobacco exposure will act on a limited number of pathways that are distinct from p53 and pRb. However, there may be other explanations for these observed interactions, including that perhaps HPV16 may protect against the effects of alcohol and tobacco in some way.

In the present analysis, alcohol consumption and tobacco use were determined based on the reporting of decade-specific exposures. This is an improvement over reporting of consumption of alcoholic beverages or tobacco use based only on subjects' usual exposure in adulthood, which may misrepresent their consumption habits during other time periods. We also examined other measures of exposure, such as cumulative alcoholic drinks per week over a subject's lifetime and average packs per day; however, the observed interactions with HPV16 persisted when these measures were used.

Self-reporting of alcohol and tobacco use was conducted retrospectively, thus recall bias may be of concern. Case subjects with greater alcohol intake may underreport their alcohol consumption more than control subjects, a tendency that appeared to attenuate an association between breast cancer and alcohol in a case–control study of breast cancer (59,60). In a study of head and neck cancer, for which alcohol is such a strong risk factor for disease, it is possible that there may be a greater bias in reporting. Thus, our results may underestimate the association between alcohol consumption and HNSCC. In fact, case subjects were more likely than control subjects to refuse to answer detailed lifetime smoking

and drinking histories and instead answered the abbreviated questionnaire on alcohol consumption and tobacco use. However, for recall bias to have influenced the main finding of a difference in the dose–response for subjects with HPV16 exposure compared with those without, case subjects would need to have reported differently based on their HPV16 antibody status. This is unlikely because case subjects were unaware of study hypotheses pertaining to HPV16 exposure or that it would be determined from serology.

The use of HPV16 serology to determine HPV exposure may have resulted in misclassification of HPV16 status. HPV16 antibody status is not specific to location of viral exposure; therefore, we cannot say with certainty whether subjects had HPV16 exposure in the upper respiratory tract as opposed to another location in the body. However, this uncertainty applies to both case subjects and control subjects. In addition, for case subjects, we do not know whether HPV16 infection was present during carcinogenesis or if infection occurred after the tumor developed. However, the latter is unlikely because we have previously shown that HPV16 seropositivity was associated with presence of HPV16 DNA in tumors (25).

Another limitation of the study was the participation rate of the control subjects because the representation of the major risk factors could be biased based on the tendency to choose to participate in the study. We were unable to determine whether participating control subjects differed from those who did not participate with respect to alcohol and tobacco use. The observed 10% prevalence of HPV16 seropositivity in control subjects was comparable to that reported by studies in the United States and Europe (26,61,62). For lower participation from control subjects to explain the interactions between HPV16 and alcohol or tobacco, their participation would need to differ concurrently by HPV16 status and alcohol or tobacco.

Our results strongly support the emerging view that the etiology of HPV16-related HNSCC is distinct from that of HNSCC tumors associated with smoking and drinking. This may have important implications for the treatment of HNSCC, both in terms of targeted therapies and the effect of smoking or drinking cessation on survival or recurrence of an HPV16-positive tumor. Our results need to be replicated in different populations, preferably using HPV16 serology to represent HPV16 exposure and having never smokers (or low drinkers) serve as the reference category. Our analysis also indicated that cancer risk associated with tobacco, alcohol, and HPV16 varies by tumor site, which is consistent with previous reports (26,37,63), and these findings of different patterns of risk at different sites may help elucidate the specific mechanisms responsible for the induction of cancers at particular locations.

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Funding

National Institutes of Health (CA100679, CA78609, and T32 ES07155).

Notes

The authors take full responsibility for the study design, data collection, analysis and interpretation of the data, the decision to submit the manuscript for publication, and the writing of the manuscript.

Manuscript received April 27, 2007; revised September 20, 2007; accepted October 22, 2007.