

Distinct Risk Factor Profiles for Human Papillomavirus Type 16–Positive and Human Papillomavirus Type 16–Negative Head and Neck Cancers

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Background

High-risk types of human papillomavirus (HPV), including HPV-16, cause a subgroup of head and neck squamous cell carcinomas (HNSCCs). We examined whether the risk factors for HPV-16–positive HNSCCs are similar to those for HPV-16–negative HNSCCs in a hospital-based case–control study.

Methods

Case subjects (n = 240) diagnosed with HNSCC at the Johns Hopkins Hospital from 2000 through 2006 were stratified by tumor HPV-16 status as determined by in situ hybridization. Two control subjects (n = 322) without cancer were individually matched by age and sex to each HPV-16–positive and HPV-16–negative case subject. Data on risk behaviors were obtained by use of audio computer-assisted self-interview technology. Multivariable conditional logistic regression models were used to estimate the odds ratios (ORs) for HPV-16–positive HNSCC and HPV-16–negative HNSCC associated with risk factors. All statistical tests were two-sided.

Results

HPV-16 was detected in 92 of 240 case subjects. HPV-16–positive HNSCC was independently associated with several measures of sexual behavior and exposure to marijuana but not with cumulative measures of tobacco smoking, alcohol drinking, or poor oral hygiene. Associations increased in strength with increasing number of oral sex partners ($P_{\text{trend}} = .01$) and with increasing intensity (joints per month, $P_{\text{trend}} = .007$), duration (in years, $P_{\text{trend}} = .01$), and cumulative joint-years ($P_{\text{trend}} = .003$) of marijuana use. By contrast, HPV-16–negative HNSCC was associated with measures of tobacco smoking, alcohol drinking, and poor oral hygiene but not with any measure of sexual behavior or marijuana use. Associations increased in strength with increasing intensity (cigarettes per day), duration, and cumulative pack-years of tobacco smoking (for all, $P_{\text{trend}} < .001$), increasing years of heavy alcohol drinking (≥ 15 years of 14 drinks per week; $P_{\text{trend}} = .03$), and increasing number of lost teeth ($P_{\text{trend}} = .001$). Compared with subjects who neither smoked tobacco nor drank alcohol, those with heavy use of tobacco (≥ 20 pack-years) and alcohol had an increased risk of HPV-16–negative HNSCC (OR = 4.8, 95% confidence interval [CI] = 1.8 to 12) but not of HPV-16–positive HNSCC (OR = 0.67, 95% CI = 0.29 to 1.9).

Conclusions

HPV-16–positive HNSCCs and HPV-16–negative HNSCCs have different risk factor profiles, indicating that they should be considered to be distinct cancers.

J Natl Cancer Inst 2008;100:1–14

Human papillomavirus (HPV) infection is now recognized to play a role in the pathogenesis of a subgroup of head and neck squamous cell carcinomas (HNSCCs). This subgroup is clinically characterized by the presence of high-risk HPV genomic DNA sequences in the tumors (approximately 95% contain HPV-16 DNA) and are predominantly oropharyngeal squamous cell carcinomas located in the lingual and palatine tonsillar regions with poorly differentiated, basaloid histopathology (1). In addition, patients with HPV-positive HNSCC have a better prognosis than patients with HPV-negative HNSCC (2–5), in part because of their better therapeutic response to chemoradiotherapy (6).

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See “Funding” and “Notes” following “References.”

DOI: 10.1093/jnci/djn025

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45 CONTEXT AND CAVEATS

Prior knowledge

High-risk types of human papillomavirus (HPV), including HPV-16, cause a subgroup of head and neck squamous cell carcinomas (HNSCCs), but it is unclear whether the risk factors for HPV-16-positive HNSCCs are similar to those for HPV-16-negative HNSCCs.

Study design

A hospital-based case-control study of HNSCC to compare the risk factor profiles for HPV-16-positive and HPV-16-negative HNSCCs.

Contribution

55 HPV-16-positive HNSCC was independently associated with several measures of sexual behavior and exposure to marijuana but not with cumulative measures of tobacco smoking, alcohol drinking, or poor oral hygiene. HPV-16-negative HNSCC was associated with measures of tobacco smoking, alcohol drinking, and poor oral hygiene but not with any measures of sexual behavior or marijuana use.

Implications

65 HNSCCs are a heterogeneous group of malignancies with at least two etiologically distinct pathways for HNSCC pathogenesis, one mediated by tobacco and alcohol and the other by HPV.

Limitations

70 The use of HPV-16 in situ hybridization alone to stratify cases as HPV-positive or HPV-negative may have misclassified tumor HPV status. The control population may not adequately represent the true prevalence of exposures of interest in the general population. Differential recall bias among case subjects, residual confounding by sexual behavior, or possible confounding by use of other substances could have influenced observed associations between marijuana use and HPV-16-positive HNSCC.

75 HPV-positive HNSCCs also have genetic alterations that are indicative of HPV oncoprotein function (1). High-risk types of HPV encode E6 and E7, two viral oncoproteins that promote tumor progression by inactivating the *TP53* and retinoblastoma tumor suppressor gene products, respectively. By inactivating p53 and Rb, E6 and E7 functionally disrupt the same cell cycle regulatory and DNA repair pathways that are frequently inactivated via genetic or epigenetic alterations during molecular progression of HNSCC (7). The fact that HPV-positive HNSCCs are characterized by wild-type p53 (3,8), wild-type p16 (5), and infrequent amplification of cyclin D (9,10), whereas the converse is true for HPV-negative HNSCC, is consistent with the functions of these viral oncoproteins. By contrast, p53 mutations in HNSCC are associated with tobacco and alcohol use (11). HPV-positive HNSCCs also differ from HPV-negative HNSCCs in their patterns of allelic (12) and chromosomal (13,14) loss and in their global gene expression profiles (9,15). These observations provide support for the existence of at least two separate pathways for the multistage carcinogenesis of HNSCC: one driven primarily by the mutagenic effects of tobacco and alcohol and the other driven by HPV-mediated transformation.

95 Although the clinical and molecular-genetic characteristics of HPV-positive HNSCC and HPV-negative HNSCC clearly differ, it has been unclear whether the risk factors for these cancers are

also distinct. We performed a case-control study of HNSCC in which the case subjects were stratified by tumor HPV status to compare the risk factor profiles for HPV-positive and HPV-negative HNSCC.

Participants and Methods

Participants

105 Eligible case subjects were identified from among consecutive patients who had been diagnosed with HNSCC at the outpatient otolaryngology clinic of the Johns Hopkins Hospital (Baltimore, MD) from May 2000 through June 2006. Patients were eligible for inclusion in this study if they were older than 17 years and were newly diagnosed with a histologically confirmed squamous cell carcinoma of the oral cavity, paranasal sinus, pharynx, or larynx or an unknown primary HNSCC. Anatomic site of origin was determined by a physical examination that was performed by the treating head and neck surgeon. Case subjects were prospectively classified as having a diagnosis of either HPV-16-positive or HPV-16-negative HNSCC by use of in situ hybridization for HPV-16 performed on formalin-fixed, paraffin-embedded tumor samples obtained during the diagnostic biopsy (see below).

120 Eligible control subjects included patients older than 17 years with no history of cancer who were evaluated as an outpatient for any benign condition between May 2000 and June 2006 at the same otolaryngology clinic where the case subjects were enrolled. This control population was considered to be representative of the referral population from which the case subjects were identified. After enrollment of a case subject and determination of his or her tumor HPV status, eligible control subjects in the same sex and age (5-year intervals) categories were invited to participate until two control subjects were individually matched to each HPV-16-positive and HPV-16-negative case subject. Control subjects were sampled with replacement between the two case groups (HPV-16-positive and HPV-16-negative case subjects). Therefore, a control subject could be matched to both an HPV-16-positive and an HPV-16-negative case subject in the same age and sex category. The study protocol was approved by the Institutional Review Board of the Johns Hopkins Hospital. Written informed consent was obtained from all study participants.

Procedures

140 Venous blood and oral rinse samples were collected from case subjects before the initiation of therapy and from control subjects at enrollment. Serum was separated from venous blood and oral exfoliated cells from oral rinse samples by centrifugation and stored at -80°C . For all case subjects, we also obtained the formalin-fixed, paraffin-embedded tumor sample and, when possible, a snap-frozen sample from the diagnostic biopsy specimen for HPV detection.

150 Detailed information regarding subject demographics, oral hygiene, medical history, family history, lifetime sexual behaviors, and lifetime history of marijuana, tobacco, and alcohol use was obtained from all participants with the use of audio computer-assisted self-interview (ACASI) technology, as previously described (16).

Laboratory Analyses

HPV DNA Detection in Tumor and Oral Rinse Samples.

155 16 DNA was detected in paraffin-embedded tumor samples by use
of the GenPoint catalyzed in situ hybridization signal amplification
system for biotinylated probes (Dako, Carpinteria, CA) (17). This
signal amplification system can detect a single integrated copy of
160 HPV-16 DNA. Briefly, tissue sections (5- μ m thick) that were
mounted onto duplicate slides were subjected to deparaffinization,
heat-induced target retrieval with the use of a steamer, and diges-
tion with proteinase K (20 μ g/mL; Roche Diagnostics, Indianapolis,
IN) at room temperature. One slide for each case subject was
hybridized at 37°C overnight with a biotinylated DNA probe that
165 was specific for HPV-16 (code Y1407, Dako). The second slide for
each case subject was hybridized at 37°C overnight with a biotinyl-
ated plasmid probe as a negative control (code OQ002; Dako).
Both slides were subjected to low- and high-stringency washes fol-
lowed by signal amplification with the use of a Tyramide Signal
170 Amplification System kit (code K0620; Dako). The signal was
developed by adding diaminobenzidine to the slide for 3–5 minutes
and monitoring color change by light microscopy. The sections
were counterstained with hematoxylin and mounted under cover-
slips. Positive control slides created from a single case of confirmed
175 HPV-16–positive head and neck cancer were processed with each
batch. Case subjects were considered to be positive for HPV-16 if
a punctate signal specific to tumor cell nuclei was detected, the cor-
responding negative control lacked a signal, and a strong nuclear
signal was present in the positive control.

180 DNA was purified from oral rinse samples obtained from case
and control subjects by standard proteinase K digestion, phenol-
chloroform extraction, and ethanol precipitation. DNA was puri-
fied from fresh-frozen tumor samples that had been macrodissected
to ensure that more than 70% of the sample evaluated was tumor
185 by proteinase K digestion, phenol–chloroform extraction, and eth-
anol precipitation.

Purified DNA from fresh-frozen tumors was screened for the
genomic DNA of 37 types of HPV (low-risk HPV types: 6, 11, 40,
42, 54, 61, 70, 72, 81, and CP6108; high-risk HPV types: 16, 18,
190 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82;
indeterminate-risk HPV types: 55, 62, 64, 67, 69, 71, 83, 84, and
IS39) by consensus multiplex polymerase chain reaction (PCR)
with the use of PGMY09/11 L1 primer pools and primers for
 β -globin, as previously described (18). The PCR products were
195 denatured and hybridized to a prototype HPV probe array (Roche
Molecular Systems, Inc., Alameda, CA). Samples that were positive
for β -globin were considered to be evaluable, and HPV type was
reported for β -globin–positive and HPV-positive samples.

200 HPV-16 DNA was detected in oral rinse DNA samples from
case and control subjects with the use of a TaqMan real-time PCR
method (Applied Biosystems, Foster City, CA) that was designed
to amplify the E6 region of the viral genome, as previously
described (19). Samples that had at least one copy of viral DNA
were considered to be positive (lower limit of detection for assay
205 was one DNA copy per reaction).

We measured HPV viral load in fresh-frozen tumors that were
positive for HPV types 31, 33, or 35 (which, along with HPV types
16 and 18, account for approximately 99% of the HPV detected
in approximately 800 tumors that we have examined in the past

10 years) with the use of a real-time consensus PCR assay that
210 was designed to amplify the E6 and/or E7 regions of these viral
genomes. The primer (forward and reverse) and probe sequences
for amplification were as follows: HPV-31 E6 (bp 479–554,
GenBank accession no. J04353): 5'-ATTCCACAACATAGGAGG
AAGGTG-3', 5'-CACTTGGGTTTCAGTACGAGGTCT-3',
215 and 5'-ACAGGACGTTGCATAGCATGTTGGA-3'; HPV-33
E6 and E7 (bp 553–688, GenBank accession no. M12732): 5'-
CTGTGACGTGTAAAAACGCC-3', 5'-TCCAAGCCTTCAT
CCTCATC-3', and 5'-ACACAAGCCAACGTTAAAGGAA-3';
and HPV-35 E7 (bp 745–840, GenBank accession no. M74117):
220 5'-TGTGAGGCGACACTACGT-3', 5'-GGGGCACACTATT
CCAAATG-3', and 5'-AGAGCACACACATTGACATACG-3'.
The primers and probes were synthesized by Integrated DNA
Technologies (Coralville, IA). Probes were labeled with 6-
carboxy-fluorescein at the 5' end and with Black Hole Quencher-1
225 at the 3' end. Standard curves for amplification reactions were
generated by use of a fivefold dilution series of pUC57 vector
(GenScript, Piscataway, NJ) containing the E6 and/or E7 target
region for HPV-31 (bp 361–751), HPV-33 (bp 331–721), or
HPV-35 (bp 421–903) in a background of human placental DNA
230 (5 ng/ μ L). Each PCR reaction contained 2 \times TaqMan universal
PCR master mix (Applied Biosystems), 0.1 μ mol of probe, 0.2
 μ mol of each primer, and 2 μ L of purified tumor DNA. Amplification
conditions included a 10-minute incubation at 95°C, followed by
50 cycles of 15 seconds at 95°C and 60 seconds at 60°C (HPV-33
235 and HPV-35) or 59.3°C (HPV-31). The cycle threshold (CT) of
unknown samples was determined from an equation derived from
a linear regression through the log CT of the standard curve. Each
sample, including the standards, was assayed in duplicate. Reactions
were performed in an Applied Biosystems 7300 PCR system. Viral
load was standardized to human diploid genome equivalents as
240 measured by a TaqMan real-time PCR assay targeted to a single-
copy human gene, human endogenous retrovirus-3 (*ERV3*) (19).

Measurement of Serum Antibodies to HPV Types 16, 18, 31, and 35 Capsid Proteins.

245 Serum samples from case and control
subjects were tested for immunoglobulin G (IgG) antibodies to the
L1 proteins of HPV types 16, 18, 31, and 35 with the use of type-
specific, virus-like particle (VLP)–based, enzyme-linked immuno-
sorbent assays (20). VLPs were produced from recombinant
baculoviruses expressing the major capsid protein of the respective
250 HPV type. Briefly, wells of PolySorp (for detection of HPV types
16 and 31) or MaxiSorp (for detection of HPV types 18 and 35)
microtiter plates (Nunc, Naperville, IL) were incubated overnight
at 4°C with a solution that contained either 66 ng of HPV-16 VLP,
50 ng of HPV-18 VLP, 80 ng of HPV-31 VLP, or 50 ng of
255 HPV-35 VLP in phosphate-buffered saline (PBS; pH 7.2). The
VLP-containing solution was removed and 0.5% (wt/vol) polyvinyl
alcohol (PVA; MW 30 000–70 000; Sigma, St Louis, MO) in PBS
was added to each well, and the plates were incubated at room tem-
perature for 3 hours to block nonspecific protein binding. The
260 blocking solution was replaced with PBS, and the plates were used
immediately as follows. Before the first incubation step and after
each incubation step, the plates were washed four times with PBS
containing 0.05% (vol/vol) Tween 20 (Sigma) in an automatic plate
washer (Skanwasher 300, Skatron, Lier, Norway). Serum samples
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(diluted 1:100 in 0.5% PVA) were added to the wells, and the plates were incubated for 1 hour at 37°C. A horseradish peroxidase (HRP)-conjugated goat antibody against human IgG (Southern Biotech, Birmingham AL) diluted 1:4000 in PBS that contained 0.5% PVA, 0.0025% Tween 20, and 0.8% (wt/vol) polyvinylpyrrolidone (MW 360000; Sigma) was added to each well to detect antigen-bound immunoglobulin, and the plates were incubated for 30 minutes at 37°C. Color development, indicating bound HRP-conjugated antibody, was initiated by adding 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) hydrogen peroxide solution to the wells (Kirkegaard and Perry, Gaithersburg, MD). The reaction was stopped after 20 minutes by the addition of 1% sodium dodecyl sulfate, and the absorbance of each well was measured at 405 nm in an automated microtiter plate reader (Molecular Devices, Menlo Park, CA). Serum samples were tested in duplicate with negative and positive reference sera, and samples that exceeded a preset coefficient of variation (25%) were retested. Samples were considered to be seropositive for HPV if the absorbance was three SDs above the mean optical density value obtained for negative control sera (20).

Statistical Analysis

A cigarette smoker was defined as an individual who had ever smoked one or more cigarettes per day for 1 year or longer, and a heavy smoker was defined as an individual with 20 or more pack-years (1 pack-year = 1 pack per day for a year) of use. Cumulative tobacco dose was calculated using age-specific information about the frequency, dose, and duration of tobacco use that was collected with the use of ACASI. Four cigars or five pipes were considered to be equivalent to one pack of cigarettes for calculation of pack-years (21).

An alcohol drinker was defined as an individual who had ever consumed one or more drinks of alcohol per month for more than 1 year. Cumulative alcohol exposure was calculated by first defining a drink-equivalent (subsequently deferred to as a "drink") as one 12-ounce beer, one glass of wine, one mixed drink, or a 1.5-ounce shot of liquor (16). The average number of drinks per week was calculated from data obtained from ACASI for each 5-year interval (or fraction thereof) of the subject's life. Alcohol consumption within each age interval was then measured as a function of a drink-year, which was defined as the equivalent of drinking 14 drinks per week for 1 year. Total lifetime alcohol consumption was then determined by summing the number of drink-years for each age interval. A heavy drinker was defined as someone with 15 or more drink-years of exposure.

A marijuana user was defined as an individual who reported ever using marijuana at least once per month for 1 year or longer. Cumulative marijuana dose was calculated from age-specific information obtained with the use of ACASI on the method, amount, frequency, and duration of use. Age-specific measures were used to calculate joint-years, a previously defined lifetime measure of marijuana use, which is equivalent to smoking one joint per day for 1 year (22). An individual with 5 or more joint-years was considered to be a heavy user. Cut points for heavy drinking and heavy marijuana use were chosen after exploratory analyses indicated that the choice of cut point did not alter the conclusions.

All analyses were performed after stratification by tumor HPV-16 status. For the case-control analysis, McNemar test was used to compare categorical variables. Separate conditional logistic regres-

sion models were used to estimate the odds ratio (OR) and 95% confidence interval (CI) for a diagnosis of HPV-16-positive or HPV-16-negative cancer. Multivariable models for HPV-16-positive or HPV-16-negative cancers were constructed by a stepwise inclusion of the statistically significant factors in a univariate analysis for either cancer. Therefore, measures were adjusted for race (white, black, other), tobacco use (nonsmoker, 1-20, 21-50, ≥51 pack-years), alcohol use (nondrinker, 0-20, 21-40, ≥41 drink-years), marijuana use (0, 1-4, 5-14, ≥15 joint-years), tooth loss (none, some, all), frequency of tooth brushing (daily, less than daily), and number of oral sex partners (0, 1-5, 6-15, ≥16 lifetime partners) based on cut points that appeared important during exploratory data analysis. Multiplicative interactions were investigated by including an interaction term in the model and using the likelihood ratio test. Trends in odds ratios were tested by modeling each ordinal variable as a single continuous independent variable. For the case-case analysis, Fisher exact test was used to compare categorical variables, and unconditional logistic regression models were used to estimate the odds ratios and 95% confidence intervals for a diagnosis of an HPV-16-positive cancer. Odds ratios in this case-case comparison represent the odds of a diagnosis of HPV-16-positive vs HPV-16-negative cancer among case subjects with head and neck cancer and should not be interpreted as an estimate of the relative risk for HPV-16-positive HNSCC in the general population.

Sensitivity analyses were performed to evaluate the possible effects of race and misclassification of tumor HPV status on observed differences. For race, we repeated the case-control comparisons by restricting the analysis to the case subjects (100% of the 92 HPV-16-positive case subjects and 92% of the 148 HPV-16-negative case subjects) who could be matched to control subjects on age, sex, and race. A second sensitivity analysis was performed to evaluate the potential for inflation in observed associations due to misclassification, that is, to identify potentially spurious associations, as opposed to adjusting the observed results. A 5% nondifferential misclassification rate was assumed as a worst-case scenario. The tumor HPV status for 5% of individuals in each category of tumor HPV status (ie, positive and negative) was reversed, and the analysis was repeated. As a conservative measure, subjects were chosen to maximize the attenuation in the estimated effect size. Therefore, the subjects who were selected to be false-positives were non-tobacco-smoking, HPV-positive case subjects who had a high number of oral sex partners and the subjects who were selected to be false-negatives were heavy tobacco-smoking HPV-negative case subjects with no oral sex partners. This analysis provided a lower bound for the level of association in the presence of misclassification.

Associations were considered to be statistically significant for a two-sided $P \leq .05$. Stata software (version 8.0; Stata Corp., College Station, TX) was used for all statistical analyses.

Results

Two hundred fifty-six case subjects with newly diagnosed HNSCC and 322 control subjects were enrolled in this case-control study. Our analysis was restricted to the 240 case subjects (94%) for whom paraffin-embedded tumor samples were available. Case subjects

with and without tumor samples were similar with respect to age at diagnosis, race, tumor site, and tobacco and alcohol exposure histories; however, case subjects without available tumor samples were more likely to be female ($P = .04$). A subset of the case subjects (100 of 240) and the matched control subjects (196 of 322) in this study participated in a previously reported case-control study that was limited to oropharynx cancers (16). Both this and the previous study were nested within a longitudinal cohort study of patients with newly diagnosed head and neck cancer.

HPV-16 DNA was detected in the tumors of 92 (38%) of the 240 case subjects by in situ hybridization (Table 1). Among the 127 case subjects for whom fresh-frozen tumor samples were available, two (4%) of the 54 HPV-16-positive subjects were also positive for either HPV-33 or HPV-35 and five (7%) of the 73 HPV-16-negative subjects were positive for high-risk HPV-31 ($n = 1$), HPV-33 ($n = 2$), HPV-35 ($n = 1$), or HPV-59 ($n = 1$) by consensus PCR. However, only the two HPV-16-negative case subjects who were positive for HPV-31 or HPV-35 had one or more copies of HPV per tumor cell as measured by quantitative real-time PCR, which is consistent with a possible misclassification rate of less than 5% for HPV-16-negative tumors.

Compared with patients with HPV-16-negative tumors, patients with HPV-16-positive tumors were younger (median age: 54 vs 58 years, $P = .002$) and, in a univariate analysis, were more likely to be white ($P = .006$), married ($P < .001$), college educated ($P = .003$), have an annual income of \$50 000 or higher ($P < .001$) (Table 1), and have been diagnosed with a tumor in the oropharynx, lingual or palatine tonsils, or at an unknown primary site ($P < .001$) (data not shown).

HPV-16 was detected by in situ hybridization in 82 (72%) of 114 oropharynx cancers, four (67%) of six primary cancers of unknown location, one (14%) of seven paranasal sinus cancers, three (6%) of 49 larynx cancers, two (4%) of 54 oral cavity, and none of the seven hypopharynx or the three nasopharynx cancers. The nonoropharyngeal tumors that were HPV-16-positive arose specifically from the oral tongue ($n = 1$), the retromolar trigone ($n = 1$), the true vocal fold ($n = 1$), the supraglottic larynx ($n = 2$), the nasal cavity ($n = 1$), and unknown primary sites ($n = 4$).

Of the 322 control subjects who were enrolled in this study, 157 were age- and sex- matched to an HPV-16-positive case subject and an HPV-16-negative case subject, 27 were matched to a HPV-16-positive case subject only, and 139 were matched to an HPV-16-negative case subject only. Participation rates among eligible case and control subjects were 77% and 70%, respectively. HPV-16-positive case subjects were similar to control subjects with regard to age, sex, education level, and income but were more likely than control subjects to be married and white. HPV-16-negative case subjects were less likely than control subjects to be college educated and in the highest tertile of income (Table 1).

We evaluated whether the well-established risk factors for HNSCC, tobacco and alcohol use, were associated with HPV-16-positive or HPV-16-negative cancers (Table 2). Surprisingly, neither increasing intensity of tobacco smoking (cigarettes smoked per day, $P_{\text{trend}} = .27$) or alcohol consumption (drinks per week, $P_{\text{trend}} = .32$) nor increasing duration (in years) of tobacco ($P_{\text{trend}} = .55$) or alcohol ($P_{\text{trend}} = .19$) use increased the odds of HPV-16-positive

HNSCC. However, very heavy daily users of tobacco (>2 packs or >40 cigarettes per day) had non-statistically significantly increased odds of HPV-16-positive HNSCC (OR = 3.2, 95% CI = 0.73 to 13.9) (Table 2).

By contrast, both tobacco and alcohol use were independently associated with HPV-16-negative HNSCC (Table 2). Odds ratios for HPV-16-negative HNSCC increased with increasing number of cigarettes smoked per day ($P_{\text{trend}} < .001$) and increasing number of years smoked ($P_{\text{trend}} < .001$) and decreased with increasing numbers of years since smoking stopped ($P_{\text{trend}} < .001$). Odds ratios of HPV-16-negative HNSCC also increased with increasing intensity of alcohol drinking ($P_{\text{trend}} = .031$).

Compared with subjects who neither smoked tobacco nor drank alcohol, those with heavy use of tobacco and alcohol had an increased risk of HPV-16-negative HNSCC (OR = 4.8, 95% CI = 1.8 to 12) but not of HPV-16-positive HNSCC (OR = 0.67, 95% CI = 0.29 to 1.9).

Marijuana smoking was strongly associated with HPV-16-positive HNSCC (Table 3). Odds ratios for HPV-16-positive HNSCC increased with increasing intensity (joints per month, $P_{\text{trend}} = .007$) and duration (in years, $P_{\text{trend}} = .011$) of marijuana use. The odds ratios for HPV-16-positive HNSCC declined with increasing number of years since marijuana smoking stopped ($P_{\text{trend}} = .017$). By contrast, observed associations between marijuana use and HPV-16-negative HNSCC were considerably weaker than those for HPV-16-positive HNSCC, although non-statistically significant increases in odds ratios were observed for the highest categories of use (Table 3).

Observed associations with marijuana use could not be explained by confounding by tobacco use or exposure to HPV. For example, measures of intensity or duration of marijuana use did not differ between case subjects who did and did not smoke tobacco (data not shown). In fact, in a multivariable analysis among subjects who smoked marijuana but not tobacco, we observed a strong association between marijuana use and HPV-16-positive HNSCC (OR = 4.5, 95% CI = 1.6 to 13), and the odds ratios for HPV-16-positive HNSCC increased with increasing intensity ($P_{\text{trend}} = .003$) and duration ($P_{\text{trend}} = .005$) of use. Among nonsmokers of tobacco, those with 5 or more joint-years of marijuana use had an 11-fold increase in the odds of HPV-16-positive HNSCC compared with sporadic users or nonusers of marijuana (OR = 11.0, 95% CI = 1.6 to 74). In addition, the observed increase in the odds of HPV-16-positive HNSCC with increasing joint-years of marijuana exposure persisted in multivariable analysis after adjustment for HPV-16 serostatus ($P_{\text{trend}} = .005$). The odds of HPV-16-positive HNSCC associated with 5 or more years of marijuana use were statistically significantly elevated in univariate analysis when restricted to HPV-seropositive case and control subjects (OR = 6.3, 95% CI = 1.3 to 29.6).

We found no evidence of multiplicative interactions between tobacco and alcohol, marijuana and tobacco, or marijuana and alcohol for HPV-16-positive or HPV-16-negative HNSCC (data not shown).

No associations were observed between several measures of oral hygiene (ie, frequency of dental visits, frequency of tooth brushing, number of teeth lost) and HPV-16-positive HNSCC (data not shown). By contrast, several measures of chronic poor

Table 1. Demographic characteristics, oral hygiene, family cancer history, and tobacco and alcohol consumption among HPV-16–positive and HPV-16–negative head and neck squamous cell carcinoma case patients and age- and sex-matched control subjects without cancer*

Explanatory variable	HPV-16–positive case subjects, % (n = 92)	Control subjects, % (n = 184)	Univariate OR (95% CI)	HPV-16–negative case subjects, % (n = 148)	Control subjects, % (n = 296)	Univariate OR (95% CI)
Sex						
Female	15	15	1.0 (referent)	25	25	1.0 (referent)
Male	85	85	Matched design	75	75	Matched design
Age, y						
<50	30	30	1.0 (referent)	25	25	1.0 (referent)
50–64	58	58	Matched design	45	45	Matched design
≥65	12	12	Matched design	30	30	Matched design
Race and ethnicity						
Non-Hispanic white	93	85	1.0 (referent)	82	83	1.0 (referent)
Non-Hispanic black	3	9	0.3 (0.1 to 1.2)	16	11	1.6 (0.9 to 3.0)
Other†	3	3	0.6 (0.2 to 2.2)	2	6	0.3 (0.1 to 1.1)
Education level, y						
<12	8	7	1.0 (referent)	20	7	1.0 (referent)
12–15	41	34	1.1 (0.4 to 3.0)	46	39	0.4 (0.2 to 0.8)
>15	51	59	0.8 (0.3 to 2.1)	34	54	0.2 (0.1 to 0.4)
Annual income, \$						
<30 000	10	12	1.0 (referent)	31	16	1.0 (referent)
30 000–49 999	21	12	2.2 (0.8 to 6.1)	21	17	0.6 (0.3 to 1.2)
≥50 000	70	76	1.1 (0.5 to 2.7)	48	67	0.3 (0.2 to 0.6)
Currently married						
No	14	31	1.0 (referent)	38	31	1.0 (referent)
Yes	86	69	3.0 (1.5 to 6.0)	62	69	0.7 (0.5 to 1.1)
Family history of HNSCC						
No	91	93	1.0 (referent)	91	92	1.0 (referent)
Yes	9	7	1.4 (0.5 to 3.4)	9	8	1.0 (0.5 to 2.1)
Tooth loss						
None	74	78	1.0 (referent)	47	74	1.0 (referent)
Some	12	11	1.2 (0.5 to 2.8)	20	15	2.4 (1.4 to 4.4)
All	14	10	1.5 (0.7 to 3.5)	34	11	5.5 (3.1 to 9.7)
Tobacco and alcohol use						
Nonsmoker, nondrinker	16	16	1.0 (referent)	7	16	1.0 (referent)
<20 pack-years, <15 drink-years	29	40	0.7 (0.35 to 1.6)	24	41	1.3 (0.60 to 2.7)
≥20 pack-years, <15 drink-years	8	6	1.3 (0.41 to 3.9)	15	9	3.6 (1.5 to 8.6)
<20 pack-years, ≥15 drink-years	29	24	1.2 (0.56 to 2.7)	17	21	1.7 (0.77 to 8.7)
≥20 pack-years, ≥15 drink-years	17	14	1.2 (0.51 to 3.0)	36	14	5.7 (2.6 to 12.7)
Smoked marijuana						
Never	67	84	1.0 (referent)	80	86	1.0 (referent)
Former	22	14	2.3 (1.1 to 5.0)	13	11	1.4 (0.7 to 2.7)
Current	11	3	5.2 (1.7 to 16.0)	7	4	2.3 (0.9 to 5.7)
No. of lifetime oral sex partners						
0	8	20	1.0 (referent)	30	26	1.0 (referent)
1–5	47	54	2.7 (1.0 to 7.5)	51	52	0.8 (0.5 to 1.4)
6–15	24	13	5.7 (1.9 to 1.37)	12	13	0.8 (0.4 to 1.6)
>15	22	13	5.6 (1.8 to 17.7)	8	9	0.8 (0.3 to 1.7)
History of heart disease						
No	93	86	1.0 (referent)	91	87	1.0 (referent)
Yes	7	14	0.42 (0.17 to 1.1)	9	13	0.63 (0.33 to 1.2)
History of lung disease						
No	96	89	1.0 (referent)	86	89	1.0 (referent)
Yes	4	11	0.37 (0.12 to 1.1)	14	11	1.3 (0.73 to 2.4)
History of kidney disease						
No	9	98	1.0 (referent)	97	99	1.0 (referent)
Yes	2	2	1.3 (0.22 to 8.2)	3	1	2.7 (0.60 to 12)

(Table continues)

Table 1 (continued).

Explanatory variable	HPV-16-positive case subjects, % (n = 92)	Control subjects, % (n = 184)	Univariate OR (95% CI)	HPV-16-negative case subjects, % (n = 148)	Control subjects, % (n = 296)	Univariate OR (95% CI)
History of diabetes						
No	96	89	1.0 (referent)	92	89	1.0 (referent)
Yes	4	11	0.35 (0.12 to 1.1)	8	11	0.74 (0.38 to 1.5)
History of hypertension						
No	78	66	1.0 (referent)	92	89	1.0 (referent)
Yes	22	34	0.51 (0.28 to 0.94)	8	11	0.67 (0.43 to 1.0)
History of stroke						
No	98	99	1.0 (referent)	95	98	1.0 (referent)
Yes	2	1	2.0 (0.28 to 14)	5	2	3.2 (1.05 to 9.8)
Reason for clinical visit‡						
Hearing loss	NA	22	NA	NA	24	NA
Sinus problem	NA	22	NA	NA	18	NA
Vertigo or dizziness	NA	14	NA	NA	13	NA
Ear blockage, infection, pain	NA	16	NA	NA	14	NA
Ringing in ears	NA	2	NA	NA	3	NA
Benign growth	NA	3	NA	NA	2	NA
Voice change	NA	8	NA	NA	8	NA
Sleep apnea	NA	3	NA	NA	3	NA
Other	NA	10	NA	NA	12	NA
Unknown	NA	2	NA	NA	3	NA

* HPV-16 = human papillomavirus type 16; HNSCC = head and neck squamous cell carcinoma; OR = odds ratio; CI = confidence interval; NA = not applicable.

† Hispanic, Asian, Indian, or Middle Eastern.

‡ Control subjects only.

oral hygiene were independently associated with HPV-16-negative HNSCC. Less than daily tooth brushing increased the odds for HPV-16-negative HNSCC (OR = 4.1, 95% CI = 1.6 to 10.9), and the odds also increased with increasing number of teeth lost ($P_{\text{trend}} = .001$) (Supplementary Table 1, available online).

Several measures of self-reported sexual behavior were strongly associated with HPV-16-positive HNSCC (Table 4), including increasing numbers of lifetime vaginal or oral sex partners, having ever participated in casual sex, infrequent use of barriers during vaginal or oral sex, and ever having had a sexually transmitted disease. By contrast, no measure of self-reported sexual behavior was associated with HPV-16-negative HNSCC (Table 4).

We also evaluated whether oral HPV-16 infection or serologic evidence of exposure to high-risk HPV types 16, 18, 31, or 35 were associated with either HPV-16-positive or HPV-16-negative HNSCC (Table 5). The presence of an oral HPV-16 infection was strongly associated with HPV-16-positive HNSCC (OR = 53, 95% CI = 8.5 to 333) but not with HPV-16-negative HNSCC (OR = 1.1, 95% CI = 0.23 to 4.8). HPV-16 seropositivity was strongly associated with HPV-16-positive HNSCC but not with HPV-16-negative HNSCC; the same was true for seropositivity to HPV-31 and HPV-35. Seropositivity to HPV-18 was not associated with HPV-16-positive or HPV-16-negative HNSCC. Only seropositivity to HPV-35 was associated with HPV-16-negative HNSCC (Table 5).

We also examined lifetime (ie, cumulative) measures of exposures of interest for evidence of dose-response relationships (Figure 1). The odds of HPV-16-positive HNSCC statistically

significantly increased with increasing number of lifetime oral sex partners ($P_{\text{trend}} = .011$) and joint-years of marijuana use ($P_{\text{trend}} = .003$) but not with increasing numbers of pack-years of tobacco smoking, drink-years of alcohol consumption, or teeth lost. By contrast, the odds of HPV-16-negative HNSCC were statistically significantly increased with increasing numbers of pack-years of tobacco smoking ($P_{\text{trend}} < .001$), drink-years of alcohol consumption ($P_{\text{trend}} = .033$), and teeth lost ($P_{\text{trend}} = .001$) but not with increasing number of lifetime oral sex partners or joint-years of marijuana use.

We also conducted an analysis among the case subjects to investigate the odds of being diagnosed with an HPV-16-positive vs an HPV-16-negative HNSCC. In a multivariable analysis, the odds of an HPV-16-positive cancer diagnosis among case subjects increased statistically significantly with increasing number of oral sex partners ($P_{\text{trend}} < .001$) and decreased statistically significantly with increasing number of pack-years of tobacco use ($P_{\text{trend}} < .001$) (Figure 2). The odds of a diagnosis of HPV-16-positive HNSCC increased non-statistically significantly with increasing number of joint-years of marijuana use and decreased non-statistically significantly with increasing number of drink-years of alcohol use or teeth lost (Figure 2; see Supplementary Table 1, available online, for exact odds ratios for Figures 1 and 2).

Finally, because the observed differences in race among HPV-16-positive case subjects and matched control subjects (Table 1) could lead to potential residual confounding, we repeated the analyses with restriction to those case subjects who could be matched to control subjects on age, sex, and race (95% of the total number of case subjects). None of the observed associations reported above were meaningfully changed in these analyses

Table 2. Adjusted ORs for HPV-16–positive and HPV-16–negative head and neck squamous cell carcinoma associated with exposure to tobacco and alcohol*

Risk factor, measure	HPV-16–positive case subjects, % (n = 92)	Control subjects, % (n = 184)	Adjusted OR (95% CI)	<i>P</i> _{trend}	HPV-16–negative case subjects, % (n = 148)	Control subjects, % (n = 296)	Adjusted OR (95% CI)	<i>P</i> _{trend}
Tobacco use								
Never	52	56	1.0 (referent)	.40	27	53	1.0 (referent)	.001
Former smoker	29	29	0.81 (0.39 to 1.7)		33	32	1.7 (0.95 to 3.1)	
Current smoker†	18	15	1.5 (0.63 to 3.8)		40	15	3.2 (1.6 to 6.2)	
No. of cigarettes usually smoked per day								
Nonsmoker	52	56	1.0 (referent)	.27	27	53	1.0 (referent)	<.001
1–20	28	32	0.82 (0.40 to 1.7)		35	32	1.4 (0.75 to 2.6)	
21–40	13	10	1.3 (0.48 to 3.4)		28	10	3.7 (1.7 to 8.1)	
≥41	7	3	3.2 (0.73 to 13.9)		9	4	5.4 (1.9 to 15.3)	
No. of years smoked								
Never	52	56	1.0 (referent)	.55	27	53	1.0 (referent)	<.001
1–20	22	21	1.1 (0.55 to 2.4)		10	20	1.0 (0.47 to 2.3)	
21–30	18	11	1.5 (0.57 to 3.7)		16	11	1.8 (0.84 to 3.9)	
≥31	8	12	0.53 (0.18 to 1.5)		47	16	3.6 (1.9 to 6.8)	
Years since quit smoking								
Current smoker	18	15	1.0 (referent)	.57	40	15	1.0 (referent)	<.001
1–10	7	7	0.35 (0.08 to 1.4)		16	5	1.2 (0.48 to 2.8)	
11–20	12	8	0.92 (0.22 to 3.3)		9	8	0.57 (0.22 to 1.5)	
>20	11	15	0.49 (0.14 to 1.6)		8	19	0.24 (0.10 to 0.59)	
Never smoker	52	56	0.66 (0.27 to 1.6)		27	53	0.34 (0.17 to 0.66)	
Pack-years smoked‡								
Nonsmoker	59	59	1.0 (referent)	.63	29	55	1.0 (referent)	<.001
1–19	18	19	1.0 (0.45 to 2.2)		9	19	0.90 (0.40 to 2.0)	
20–39	10	13	0.82 (0.28 to 2.4)		22	14	2.6 (1.3 to 5.1)	
40–49	4	5	0.56 (0.13 to 2.4)		12	5	3.2 (1.1 to 9.0)	
≥50	9	5	2.0 (0.60 to 6.4)		28	7	4.9 (2.1 to 11.3)	
Alcohol use								
Never	21	21	1.0 (referent)	.40	14	22	1.0 (referent)	.91
Former	18	16	0.74 (0.3 to 2.0)		26	13	2.7 (1.1 to 6.9)	
Current†	61	63	0.70 (0.3 to 1.5)		60	65	1.5 (0.7 to 3.0)	
No. of drinks per week†								
Never drinker	21	21	1.0 (referent)	.32	14	22	1.0 (referent)	.51
1–7	43	44	0.74 (0.31 to 1.6)		48	42	1.7 (0.82 to 3.6)	
8–14	13	10	0.93 (0.49 to 4.0)		8	11	1.7 (0.60 to 4.7)	
15–35	16	19	0.62 (0.24 to 1.6)		14	20	1.3 (0.51 to 3.2)	
>35	8	7	0.49 (0.12 to 2.0)		16	6	2.2 (0.74 to 6.7)	
No. of years drank								
Never drinker	21	21	1.0 (referent)	.19	13	23	1.0 (referent)	.15
1–19	14	13	0.90 (0.33 to 2.5)		18	15	1.9 (0.78 to 4.6)	
≥20	65	66	0.60 (0.27 to 1.4)		69	63	1.9 (0.87 to 4.1)	
No. of drink-years§								
Never drinker	21	21	1.0 (referent)	.87	14	22	1.0 (referent)	.031
0–9	28	32	0.68 (0.30 to 1.6)		29	35	1.5 (0.70 to 3.2)	
10–29	20	24	0.58 (0.23 to 1.5)		16	21	1.5 (0.62 to 3.7)	
30–49	14	10	1.1 (0.37 to 3.0)		10	10	1.4 (0.50 to 4.2)	
≥50	17	13	0.88 (0.26 to 2.9)		31	12	2.8 (1.1 to 7.2)	

* OR = odds ratio; HPV-16 = human papillomavirus type 16; CI = confidence interval. Odds ratios adjusted for race, tobacco use, alcohol use, marijuana use, tooth loss, frequency of tooth brushing, and number of oral sex partners.

† Year before diagnosis.

‡ Cigarettes, cigars, or pipes.

§ Cumulative alcohol measure. One drink-year is the equivalent of 14 drinks per week for 1 year (ie, 28 drinks per week for 0.5 years and 14 drinks per week for 1 year both equal 1 drink-year).

Table 3. Adjusted ORs and 95% CIs for HPV-16–positive and HPV-16–negative head and neck squamous cell carcinoma associated with exposure to marijuana*

Exposure variable	HPV-16–positive case subjects, % (n = 92)	Control subjects, % (n = 184)	Adjusted OR (95% CI)	<i>P</i> _{trend}	HPV-16–negative case subjects, % (n = 148)	Control subjects, % (n = 296)	Adjusted OR (95% CI)	<i>P</i> _{trend}
Smoked marijuana								
monthly for ≥1 y								
Never	67	83	1.0 (referent)	.007	80	86	1.0 (referent)	.26
Former	22	14	2.3 (0.98 to 5.4)		13	11	1.2 (0.52 to 2.8)	
Current	11	3	4.7 (1.3 to 17)		7	4	2.0 (0.62 to 6.5)	
No. of joints usually smoked per mo								
≤1	70	86	1.0 (referent)	.007	85	89	1.0 (referent)	.57
2–13	14	7	2.5 (0.89 to 6.8)		6	6	0.88 (0.29 to 2.7)	
14–29	11	6	3.2 (1.0 to 10)		5	4	1.2 (0.36 to 4.3)	
≥30	5	2	5.4 (1.0 to 28)		4	2	1.7 (0.41 to 6.9)	
No. of years smoked marijuana								
Never	71	85	1.0 (referent)	.011	83	88	1.0 (referent)	.30
1–10	10	9	1.7 (0.58 to 5.1)		8	7	1.3 (0.50 to 3.4)	
11–20	14	3	6.7 (1.8 to 25)		5	2	1.9 (0.39 to 9.2)	
≥21	5	2	3.0 (0.58 to 16)		5	2	1.7 (0.36 to 8.0)	
Years since quit smoking marijuana								
Current marijuana smoker								
1–10	2	2	0.49 (0.05 to 4.7)	.017	7	4	1.0 (referent)	.35
11–20	11	4	0.87 (0.20 to 3.9)		6	3	0.33 (0.04 to 2.9)	
>20	9	8	0.56 (0.13 to 2.5)		5	5	0.79 (0.17 to 3.6)	
Never user	67	83	0.27 (0.08 to 0.93)		80	86	0.55 (0.17 to 1.8)	
No. of joint-years†								
0	67	84	1.0 (referent)	.003	82	86	1.0 (referent)	.29
1–4	13	11	2.0 (0.76 to 5.2)		9	9	1.0 (0.41 to 2.5)	
5–14	10	3	6.0 (1.2 to 29)		6	2	1.7 (0.41 to 7.4)	
≥15	10	2	6.4 (1.6 to 26)		5	2	2.0 (0.50 to 7.8)	

* OR = odds ratio; CI = confidence interval; HPV-16 = human papillomavirus type 16. Odds ratios adjusted for race; tobacco, alcohol, and marijuana use; number of teeth lost; frequency of tooth brushing; and number of oral sex partners. The percentages for some categories do not total 100% due to rounding.

† Cumulative marijuana measure. One joint-year is the equivalent of 1 joint per day for 1 year, or 365 joints (ie, 2 joints per day for 0.5 years and 1 joint per day for 1 year both equal 1 joint-year).

(data not shown). In addition, statistically significant trends in odds ratios for increasing pack-years of tobacco use and HPV-16–negative HNSCC and for increasing number of oral sex partners and HPV-16–positive HNSCC were robust to sensitivity analyses that were designed to evaluate the effect of a worst-case scenario of 5% false-negative and false-positive misclassification for tumor HPV status on the results. Therefore, the effect of misclassification on inferences, if it occurred, was minimal (data not shown).

Discussion

Our data indicate that the risk factors for HPV-16–positive and HPV-16–negative HNSCCs are markedly different. In our previously reported case–control study (16), tobacco smoking, alcohol consumption, poor oral hygiene, a family history of HNSCC, several measures of sexual behavior, HPV-16 exposure, and oral HPV-16 infection were all associated with oropharyngeal cancer. In this study, these risk factors stratified with tumor HPV-16 status. Several measures of sexual behavior, HPV-16 exposure, and oral

HPV-16 infection were associated with HPV-16–positive tumors, whereas tobacco and alcohol use and poor oral hygiene were associated with HPV-16–negative tumors. The difference between the results in this and our previous study (16) can be explained by the etiologic heterogeneity of oropharyngeal cancers, which comprise both HPV-16–positive and HPV-16–negative cancers. Together with previously reported clinical and molecular-genetic distinctions between HPV-positive and HPV-negative HNSCC (1), our data indicate that HPV-positive and HPV-negative HNSCCs should be considered to be two distinct cancers.

Several studies (23–25) have investigated interactions among HPV, tobacco, and alcohol, but none of those analyses stratified the case patients by tumor HPV status. The results of these studies were inconsistent: some (23,24) reported greater than additive risks for combined exposure to HPV and tobacco, whereas one (25) reported greater than additive risks for combined exposure to HPV and alcohol. No interactions were found in our previous case–control study of HPV and oropharyngeal cancers (16). In designing this study, we chose stratification by tumor HPV status to investigate whether tobacco and alcohol use were associated

Table 4. Adjusted ORs and 95% CIs for HPV-16–positive and HPV-16–negative head and neck squamous cell carcinoma associated with self-reported sexual behavior variables*

Variable	HPV-16–positive case subjects, % (n = 92)	Control subjects, % (n = 184)	Adjusted OR (95% CI)	<i>P</i> _{trend}	HPV-16–negative case subjects, % (n = 148)	Control subjects, % (n = 296)	Adjusted OR (95% CI)	<i>P</i> _{trend}
Total lifetime number of vaginal sex partners								
0–1	8	19	1.0 (referent)	<.001	20	24	1.0 (referent)	.58
2–10	38	53	2.5 (0.75 to 8.2)		46	50	0.61 (0.31 to 1.2)	
≥11	54	28	6.4 (1.9 to 22)		34	26	0.74 (0.32 to 1.7)	
Total lifetime number of oral sex partners								
0	8	20	1.0 (referent)	.004	30	26	1.0 (referent)	.47
1–5	47	54	2.1 (0.66 to 6.7)		51	52	0.93 (0.48 to 1.8)	
≥6	46	26	4.3 (1.4 to 14)		20	22	0.70 (0.28 to 1.8)	
History of casual sex								
No	40	61	1.0 (referent)		55	63	1.0 (referent)	
Yes	60	39	2.9 (1.5 to 5.6)		45	37	0.71 (0.39 to 1.3)	
Age at first sexual intercourse, y								
≥19	30	45	1.0 (referent)		44	50	1.0 (referent)	
<19	70	55	1.8 (0.96 to 3.3)		56	50	0.70 (0.39 to 1.2)	
Age at first oral sex, y								
≥21	48	64	1.0 (referent)		77	71	1.0 (referent)	
<21	52	36	1.7 (0.90 to 3.1)		23	29	0.63 (0.35 to 1.1)	
Condom use during vaginal sex								
Usually or always	28	47	1.0 (referent)		41	47	1.0 (referent)	
Never or rarely	72	53	2.5 (1.3 to 4.9)		59	53	1.1 (0.67 to 1.9)	
Barrier use during oral sex								
No oral sex	8	20	1.0 (referent)	.031	30	26	1.0 (referent)	.86
Usually or always	1	5	0.99 (0.10 to 10)		4	5	0.80 (0.22 to 2.9)	
Never or rarely	91	75	3.1 (1.0 to 9.4)		66	69	0.89 (0.45 to 1.7)	
History of sexually transmitted disease								
No	66	83	1.0 (referent)		82	82	1.0 (referent)	
Yes	34	17	2.6 (1.3 to 5.2)		18	18	0.93 (0.48 to 1.8)	

* OR = odds ratio; CI = confidence interval; HPV-16 = human papillomavirus type 16. Odds ratios adjusted for race; tobacco, alcohol, and marijuana use; number of teeth lost and frequency of tooth brushing; and number of oral sex partners, as appropriate. The percentages for some categories do not total 100% due to rounding.

585 with HPV-16–positive HNSCC as an alternative approach to an analysis of interactions among HPV, tobacco, and alcohol among grouped HPV-positive and HPV-negative cases. We found no statistically significant associations between tobacco, alcohol, and HPV-16–positive HNSCC. The distinct associations we observed 590 for behavioral characteristics and HPV-16–positive HNSCCs vs HPV-16–negative HNSCCs are unlikely to be explained by bias introduced by our hospital-based study design. Although hospital-based control subjects may bias results toward the null, such an effect would cause the odds ratios for both HPV-16–positive and 595 HPV-16–negative HNSCC to be similarly underestimated. Furthermore, prevalence estimates for current smoking (15%, Table 2) and HPV-16 exposure (9%, Table 4) among our control population were similar to the 19% prevalence of current tobacco smoking in the general population of Maryland (26) and 600 the 10% seroprevalence for HPV-16 in US men older than 50 years from 1991 to 1994 according to National Health and Nutrition Examination Surveys (27), respectively. The absence of an association between tobacco or alcohol and HPV-16–positive HNSCC suggests that these risk factors and HPV may act at the

same step of multistage carcinogenesis. Thus, in addition to being etiologically heterogeneous, HNSCC may be molecularly homogeneous in the sense that inactivation of the p53 and pRb pathways is important, whether as a consequence of tobacco- and alcohol-induced mutation or by high-risk HPV oncoproteins.

There is insufficient evidence to either implicate or exonerate marijuana as a carcinogen in humans (28). In this study, marijuana use was strongly associated with HPV-16–positive HNSCC. By contrast, no association between marijuana use and HNSCC was observed in a California cohort study (29) or in three previous case–control studies (22,30,31). A dose–response relationship between marijuana use and HNSCC that was reported in a single hospital-based case–control study (32) has been questioned (30) because of the low prevalence of ever marijuana use (7%) among the blood-donor control subjects. In our study, the prevalence of a year or more of marijuana use among control subjects (16%, Table 4) was similar to that observed in a previously published population-based study (approximately 18%; 30) that reported no association between marijuana use and HNSCC. In this study, ACASI technology allowed us to measure lifetime cumulative marijuana

Table 5. Adjusted ORs and 95% CIs for HPV-16–positive and HPV-16–negative head and neck squamous cell carcinoma associated with measures of HPV exposure and infection*

HPV exposure and infection measure	HPV-16–positive case subjects, % (n = 92)	Control subjects, % (n = 184)	Adjusted OR (95% CI)	HPV-16–negative case subjects, % (n = 148)	Control subjects, % (n = 296)	Adjusted OR (95% CI)
Serology						
HPV-16 L1						
Negative	41	91	1.0 (referent)	92	92	1.0 (referent)
Positive	59	9	18.3 (6.8 to 49)	8	8	0.89 (0.36 to 2.2)
HPV-18 L1						
Negative	75	85	1.0 (referent)	80	86	1.0 (referent)
Positive	25	15	2.0 (0.94 to 4.4)	20	14	1.5 (0.71 to 3.1)
HPV-31 L1						
Negative	68	85	1.0 (referent)	86	88	1.0 (referent)
Positive	32	15	2.8 (1.4 to 5.8)	14	12	1.0 (0.50 to 2.2)
HPV-35 L1						
Negative	61	85	1.0 (referent)	76	87	1.0 (referent)
Positive	37	15	3.8 (1.8 to 7.9)	22	13	3.1 (1.5 to 6.3)
Unknown	2	0	NA	3	0	NA
Serology summary						
Seronegative for HPV types 16,18, 31, and 35						
	28	68	1.0 (referent)	67	69	1.0 (referent)
Seropositive for HPV types 16,18, 31, and/or 35						
	72	32	6.9 (3.2 to 14.6)	33	31	0.97 (0.54 to 1.7)
RT-PCR detection of HPV-16 DNA in oral rinse samples						
Negative (<1 copy)	62	97	1.0 (referent)	93	96	1.0 (referent)
Positive (≥1 copy)	33	3	53 (8.5 to 333)	4	3	1.1 (0.23 to 4.8)
Missing	5	0	NA	3	1	NA

* The percentages for some categories do not total 100% because of rounding. OR = odds ratio; CI = confidence interval; HPV = human papillomavirus; NA = not applicable; RT-PCR = real-time polymerase chain reaction. Odds ratios adjusted for race; tobacco, alcohol, and marijuana use; number of teeth lost and frequency of tooth brushing; and number of oral sex partners, as appropriate.

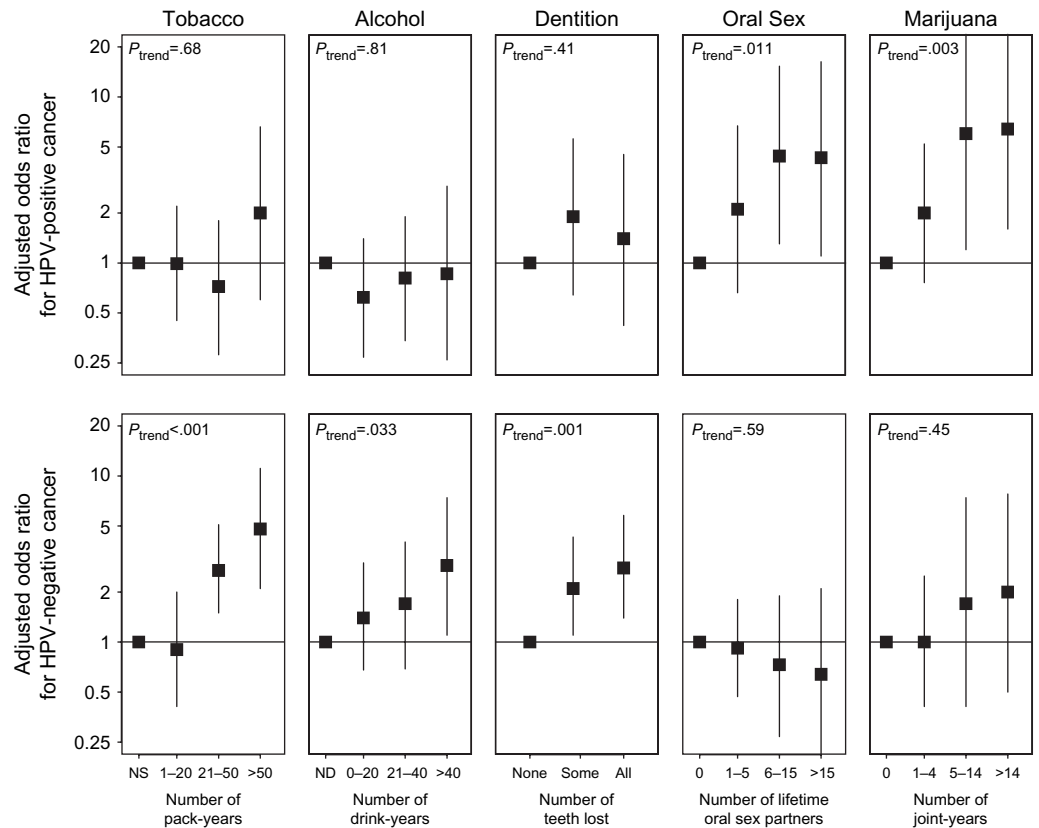
625 exposure and may have minimized underreporting of use (33) relative to interviewer-administered (30) or self-administered surveys used in other studies (31).

630 It is biologically plausible that marijuana could act to promote the development of HPV-positive HNSCC. Carcinogens in marijuana smoke, like those in tobacco smoke (34), can induce molecular alterations of the airway epithelium (35,36). However, although a pathogenic role for DNA-damaging carcinogens in marijuana among HPV-16–positive cases is possible, the known immunomodulatory effects of cannabinoids may be more relevant for a virally mediated cancer. Cannabinoids bind to receptors (CB2) 635 expressed on B cells, natural killer cells, macrophages, dendritic cells, and T cells, including those in human tonsillar tissue (37). Endogenous cannabinoids are increasingly being recognized as important immunomodulatory compounds that act in a paracrine fashion to alter responses in the local microenvironment. In animal models, cannabinoids suppress T helper (T_H-1) proinflammatory cytokines (eg, interleukin 12 and interferon gamma) while augmenting production of T_H-2 anti-inflammatory cytokines (eg, interleukins 4 and 10) (38,39). As a result, cannabinoids suppress humoral and cell-mediated immune responses, reduce host cell 640 resistance to intracellular (eg, *Listeria*, legionella) and viral (eg, herpes simplex virus) pathogens (40), and may also suppress anti-tumor immunity (41,42). Preliminary studies indicate that canna-

binoids may alter immune responses in humans (43). For instance, in marijuana smokers, the function of alveolar macrophages (44) is impaired, and CD4 and natural killer cell numbers and lymphocyte 650 proliferative responses are suppressed (45). Cannabinoid derivatives are in development for treatment of chronic inflammatory disease in human subjects (38,46). It is therefore biologically possible that cannabinoids promote progression of an HPV-positive HNSCC at multiple steps, including by increasing the risk of 655 infection upon exposure, promoting persistence of an infection, and inhibiting antitumor immunity.

We found that several measures of sexual behavior, oral HPV-16 infection, and seropositivity for any of four high-risk HPV types were associated with increased odds of HPV-16–positive 660 HNSCC but not of HPV-16–negative HNSCC. Only HPV-35 seropositivity was associated with HPV-16–negative HNSCC, possibly resulting from misclassification of HPV-35–positive tumors as HPV-16–negative tumors (24,47). We believe that the absence of an observed association between all other measures of 665 HPV exposure and HPV-16–negative HNSCC argues against a “hit and run” mechanism of carcinogenesis in subjects with HPV-16–negative HNSCC (ie, one in which HPV is present during tumor initiation or promotion but is not subsequently 670 found in the tumor). Continued viral oncogene expression has been previously demonstrated to be necessary for the malignant

Figure 1. Dose–response relationships for tobacco, alcohol, tooth loss, oral sex partners, and marijuana use and odds of human papillomavirus type 16 (HPV-16)–positive (top) and HPV-16–negative (bottom) head and neck squamous cell carcinomas (HNSCCs). **Black squares** represent adjusted odds ratios (ORs) of HNSCC for case subjects vs control subjects; **vertical lines** represent 95% confidence intervals; **horizontal lines** represent null associations (ORs = 1). Odds ratios and 95% confidence intervals were derived from conditional logistic regression analysis for case–control comparisons, adjusted for all variables in the figure plus frequency of tooth brushing and race.

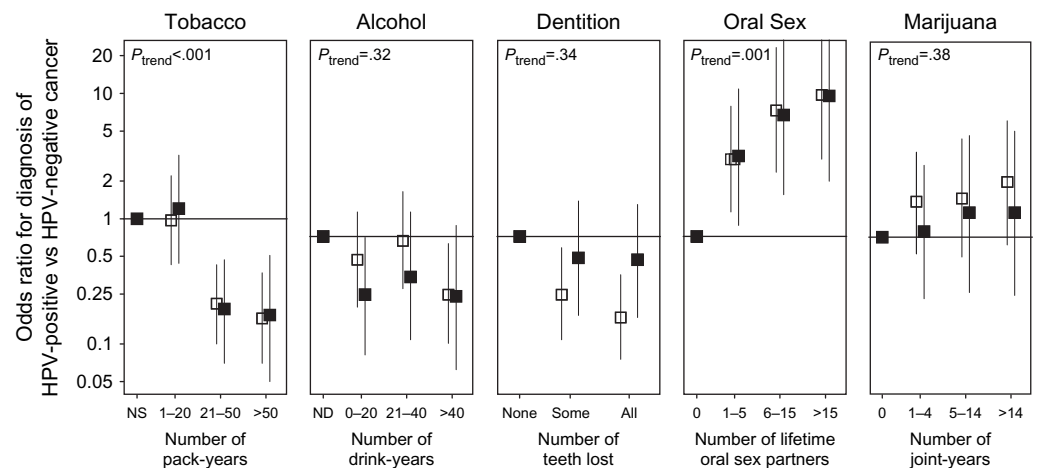


behavior of HPV-associated cancers (48). HPV-16, HPV-31, and HPV-35 seropositivity, but not HPV-18 seropositivity, were each associated with HPV-16–positive tumors (Table 5). Possible explanations for the associations with types other than HPV-16 include cross-reactivity of serum anti-HPV-16 IgG with VLPs of phylogenetically related HPV types, concomitant presence of more than one high-risk HPV type in tumors, or, more likely, given the prevalence of high-risk sexual behaviors, exposure to multiple high-risk HPV infections.

Both molecular and epidemiologic data indicate a strong and consistent association between HPV and oropharyngeal cancers (1). However, the pathophysiologic significance of the low levels of HPV DNA that have been detected by PCR in approximately 24%

of oral cavity and laryngeal cancers in the literature (47) remains unknown. In this study, approximately 11% (10 of 92) of the HPV-16–positive case subjects were classified by the surgeon as having nonoropharyngeal cancers. Four of these 10 patients were diagnosed with unknown primary HNSCCs that could possibly be explained by early regional metastasis of subclinical tumors of the tonsillar crypt epithelium. Misclassification of anatomic site may account for an additional two HPV-16–positive oral cavity carcinomas because they had clinical involvement of the base of tongue or tonsil. However, two HPV-16–positive case subjects with supraglottic laryngeal carcinomas had no apparent base-of-tongue involvement, one case of true vocal fold carcinoma arose in a young subject who did not smoke or drink alcohol but had a family history

Figure 2. Dose–response relationships for tobacco, alcohol, tooth loss, oral sex partners, and marijuana use and the odds of being diagnosed with human papillomavirus type 16 (HPV-16)–positive vs HPV-16–negative head and neck squamous cell carcinoma. **White squares** represent univariate odds ratios (ORs), and **black squares** represent adjusted odds ratios. **Vertical lines** represent 95% confidence intervals, and **horizontal lines** represent null associations (ORs = 1). Odds ratios and 95% confidence intervals were derived from unconditional logistic regression analysis for case–case comparisons that adjusted for all variables in the figure plus age, sex, frequency of tooth brushing, and race.



of laryngeal papillomatosis, and a case subject had a nasal primary tumor that arose from a preexisting inverted papilloma. Therefore, although misclassification of tumor origin and subclinical tonsillar primaries could account for the majority of HPV-16-positive, non-oropharyngeal cancers in this study, our data indicate that it is possible that HPV is involved in the pathogenesis of a small proportion of sinonasal and laryngeal squamous cell carcinomas as well.

In this study, an increasing number of teeth lost was independently associated with HPV-16-negative HNSCC but not with HPV-16-positive HNSCC. The leading cause of tooth loss in the United States is periodontal disease, a chronic inflammatory condition that is caused by chronic bacterial infection. The specific association between tooth loss and HPV-16-negative HNSCC may be explained by chronic inflammation that was localized to the oral cavity (most oral cavity carcinomas were HPV-16-negative). However, arguing against this possibility is the strong association we previously observed between tooth loss and oropharyngeal cancers (16). Socioeconomic differences between HPV-16-negative case subjects and control subjects could also have contributed to this discrepancy if they had resulted in differential access to dental care. Because heavy tobacco (49) and alcohol (50) use are mediating factors for periodontal disease, the higher prevalence of heavy smoking and drinking among HPV-16-negative case subjects than among HPV-16-positive case subjects might also explain the difference in magnitude of observed associations with oral hygiene.

Our study has some important limitations. The use of HPV-16 in situ hybridization alone to stratify cases as HPV-positive or HPV-negative may have misclassified tumor HPV status because high-risk HPV types other than HPV-16 account for approximately 5% of cases in the literature (24,47). However, such misclassification would likely bias our results toward, rather than away from, the null, and our data indicate that misclassification was likely minimal: only two (approximately 3%) of 73 fresh-frozen tumor specimens from case subjects with HPV-16-negative tumors had a viral copy number that was consistent with them being false-negatives. Our control population may not adequately represent the true prevalence of exposures of interest in the general population because eligible control subjects had no history of cancer (indicating that they were possibly at lower risk of HNSCC) but, on the other hand, had appointments at a major referral center (indicating that they were possibly at higher risk of HNSCC). However, although our control population could affect our estimates of risk in unpredictable ways, it would be expected to affect our estimates for HPV-16-positive and HPV-16-negative cancers similarly: eligibility criteria for controls were identical for both groups. Because we observed non-statistically significantly increased odds of HPV-16-positive HNSCC in association with the highest categories of daily tobacco use, we cannot exclude a biologically significant association between HPV-16-positive HNSCCs and very heavy tobacco use. We also cannot exclude the possibility that the associations we observed between marijuana use and HPV-16-positive HNSCC were due, in part, to differential recall bias among case subjects, residual confounding by sexual behavior, or possible confounding by use of other substances (eg, methamphetamines, cocaine, heroin) that were not measured in our study. The data presented in this manuscript are the first, to

our knowledge, to suggest that marijuana use may be a cofactor for HPV-16-mediated carcinogenesis in the head and neck; thus, these results will need to be confirmed in other populations.

In conclusion, our data indicate that HNSCCs, which were once considered to be clinically and pathologically homogeneous, are, in fact, a heterogeneous group of malignancies. Our data support the existence of at least two etiologically distinct pathways for HNSCC pathogenesis, one mediated by tobacco and alcohol and the other by HPV. We cannot exclude the possibility of further etiologic heterogeneity among the cancers we grouped as HPV-negative HNSCCs.

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Funding

Damon Runyon Cancer Research Foundation Clinical Investigator Award (to M.G.); the State of Maryland Cigarette Restitution Fund (to M.G.); the National Institute of Dental and Craniofacial Research (DE016631-01 to M.G.).

Notes

The sources of funding had no role in study design, data collection, analysis, interpretation, or in the writing of this report.

Manuscript received June 26, 2007; revised January 7, 2008; accepted January 17, 2008.