

ORIGINAL ARTICLE

Case–Control Study of Human Papillomavirus and Oropharyngeal Cancer

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ABSTRACT

BACKGROUND

Substantial molecular evidence suggests a role for human papillomavirus (HPV) in the pathogenesis of oropharyngeal squamous-cell carcinoma, but epidemiologic data have been inconsistent.

METHODS

We performed a hospital-based, case–control study of 100 patients with newly diagnosed oropharyngeal cancer and 200 control patients without cancer to evaluate associations between HPV infection and oropharyngeal cancer. Multivariate logistic-regression models were used for case–control comparisons.

RESULTS

A high lifetime number of vaginal-sex partners (26 or more) was associated with oropharyngeal cancer (odds ratio, 3.1; 95% confidence interval [CI], 1.5 to 6.5), as was a high lifetime number of oral-sex partners (6 or more) (odds ratio, 3.4; 95% CI, 1.3 to 8.8). The degree of association increased with the number of vaginal-sex and oral-sex partners (P values for trend, 0.002 and 0.009, respectively). Oropharyngeal cancer was significantly associated with oral HPV type 16 (HPV-16) infection (odds ratio, 14.6; 95% CI, 6.3 to 36.6), oral infection with any of 37 types of HPV (odds ratio, 12.3; 95% CI, 5.4 to 26.4), and seropositivity for the HPV-16 L1 capsid protein (odds ratio, 32.2; 95% CI, 14.6 to 71.3). HPV-16 DNA was detected in 72% (95% CI, 62 to 81) of 100 paraffin-embedded tumor specimens, and 64% of patients with cancer were seropositive for the HPV-16 oncoprotein E6, E7, or both. HPV-16 L1 seropositivity was highly associated with oropharyngeal cancer among subjects with a history of heavy tobacco and alcohol use (odds ratio, 19.4; 95% CI, 3.3 to 113.9) and among those without such a history (odds ratio, 33.6; 95% CI, 13.3 to 84.8). The association was similarly increased among subjects with oral HPV-16 infection, regardless of their tobacco and alcohol use. By contrast, tobacco and alcohol use increased the association with oropharyngeal cancer primarily among subjects without exposure to HPV-16.

CONCLUSIONS

Oral HPV infection is strongly associated with oropharyngeal cancer among subjects with or without the established risk factors of tobacco and alcohol use.

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INFECTION WITH SEXUALLY TRANSMITTED human papillomavirus (HPV) is a cause of virtually all cervical cancers.¹ Molecular evidence also provides support for a role for HPV, particularly HPV-16, in the pathogenesis of a subgroup of squamous-cell carcinomas of the head and neck.² Genomic DNA of oncogenic HPV is detected in approximately 26% of all squamous-cell carcinomas of the head and neck worldwide,³ but the molecular evidence is most rigorous and consistent for oropharyngeal squamous-cell carcinoma, in which viral integration and the expression of viral oncogenes (E6 and E7) have been shown.⁴

The epidemiologic evidence of a causal role for HPV in a subgroup of squamous-cell carcinomas of the head and neck is less rigorous than the molecular evidence. The example of the relationship between HPV and cervical cancer⁵ indicates that high-risk sexual behavior and exposure to and infection with HPV will increase the risk of other cancers caused by HPV.⁶ Although each of these three factors has been found to increase the risk of squamous-cell carcinomas of the head and neck,⁷⁻¹⁴ no single study has shown an association of all three with the development of oropharyngeal cancer.

In this study, we focused exclusively on oropharyngeal cancer, for which the molecular evidence of a causal role for HPV is compelling. Strong epidemiologic data would provide additional support for a causal association between HPV and oropharyngeal cancers and might guide future cancer-prevention programs involving vaccination to prevent oral HPV infection or screening to detect it.

METHODS

PATIENTS

Our case-control study was nested within a longitudinal cohort study of patients with newly diagnosed squamous-cell carcinomas of the head and neck in the outpatient otolaryngology clinic of the Johns Hopkins Hospital in Baltimore from 2000 through 2005. Eligible case patients included those with a confirmed diagnosis of oropharyngeal squamous-cell carcinoma.

The control group consisted of patients without a history of cancer who were seen for benign conditions between 2000 and 2005 in the same clinic from which the case patients were enrolled

(Table 1). Subsequent to enrollment of a case, eligible control patients within the same sex and 5-year age categories were approached until two control patients were individually matched to each case patient. The study protocol was approved by the institutional review board of the Johns Hopkins Hospital. Written, informed consent was obtained from all patients.

DATA COLLECTION

Specimens were collected from case patients before therapy and from control patients at enrollment. Oral-mucosal specimens were collected with the use of a saline oral rinse and 5 to 10 strokes of a cytology brush (Oral CDx, CDx Laboratories) on the posterior oropharyngeal wall. Serum samples were collected and stored at -80°C . For case patients, formalin-fixed, paraffin-embedded tumor specimens and, if possible, snap-frozen fresh tumor specimens were obtained for the detection of HPV.

All patients completed an audio, computer-assisted self-administered interview that obtained information about demographic characteristics, oral hygiene, medical history, family history of cancer, lifetime sexual behaviors, and lifetime history of marijuana, tobacco, and alcohol use (see the Supplementary Appendix, available with the full text of this article at www.nejm.org).

LABORATORY STUDIES

In Situ Hybridization for HPV-16 Detection

We looked for HPV-16 in formalin-fixed and paraffin-embedded tumors from all case subjects, using in situ hybridization-catalyzed signal amplification for biotinylated probes (Dako GenPoint).¹⁵ The HPV-16-positive status of a tumor was defined as specific staining of tumor-cell nuclei for HPV-16.

DNA Purification and Analysis

DNA from oral specimens¹⁶ and fresh-frozen tumors¹⁷ from a subgroup of case subjects was purified as previously described. The tumor specimens were microdissected to ensure that more than 70% of the sample was DNA from the tumor.

We analyzed purified DNA for 37 types of HPV by means of a multiplex polymerase-chain-reaction (PCR) assay targeted to the L1 region of the viral genome, using PGMY09/11 L1 primer pools and primers for β -globin, followed by hybridization to a linear probe array (Roche Molec-

Table 1. Explanatory Variables for Patients with Oropharyngeal Cancer and Control Patients.*

Explanatory Variable	Patients with Oropharyngeal Cancer (N = 100)	Control Patients (N = 200)	Unadjusted Odds Ratio (95% CI)†
<i>number (percent)</i>			
Demographic characteristics			
Sex			
Female	14 (14)	28 (14)	1.0
Male	86 (86)	172 (86)	1.0 (0.5–2.0)
Age			
<50 yr	34 (34)	68 (34)	1.0
50–64 yr	51 (51)	102 (51)	1.0 (0.6–1.7)
≥65 yr	15 (15)	30 (15)	1.0 (0.5–2.0)
Highest educational level			
Some high school	11 (11)	15 (8)	1.0
High-school graduate or some college	41 (41)	71 (36)	0.8 (0.3–1.9)
College graduate	48 (48)	114 (57)	0.6 (0.3–1.4)‡
Race or ethnic group§			
White, non-Hispanic	87 (87)	171 (86)	1.0
Black, non-Hispanic	9 (9)	17 (8)	1.0 (0.5–2.4)
Other	4 (4)	12 (6)	0.7 (0.2–2.1)
Home state			
Maryland	50 (50)	138 (69)	1.0
Other	50 (50)	62 (31)	2.2 (1.3–3.6)
Oral hygiene			
Tooth loss			
None	62 (62)	163 (82)	1.0
Some	16 (16)	20 (10)	2.1 (1.0–4.4)
Complete	22 (22)	17 (8)	3.4 (1.7–6.8)¶
Mouthwash use during past yr			
<1 time/day	55 (55)	126 (63)	1.0
1–2 times/day	40 (40)	71 (36)	1.3 (0.8–2.1)
3–4 times/day	5 (5)	3 (2)	3.8 (0.9–16.5)‖
Daily toothbrushing			
Yes	90 (90)	196 (98)	1.0
No	10 (10)	4 (2)	5.4 (1.7–17.8)
Health history and family history of cancer			
Sexually transmitted disease			
No	71 (71)	161 (80)	1.0
Yes	29 (29)	39 (20)	1.7 (1.0–2.9)
Heartburn or gastric reflux			
No	76 (76)	150 (75)	1.0
Yes	24 (24)	50 (25)	1.0 (0.5–1.7)
Frequency of Pap smear**			
Annually	7 (50)	22 (79)	1.0
Less than annually	7 (50)	6 (21)	3.7 (0.9–14.6)

Table 1. (Continued.)

Explanatory Variable	Patients with Oropharyngeal Cancer (N = 100)	Control Patients (N = 200)	Unadjusted Odds Ratio (95% CI) [†]
<i>number (percent)</i>			
Oral warts or papillomas			
No	93 (93)	196 (98)	1.0
Yes	7 (7)	4 (2)	3.7 (1.1–12.9)
Genital warts			
No	86 (86)	180 (90)	1.0
Yes	14 (14)	20 (10)	1.8 (0.9–3.5)
Sexual partner with genital warts			
No	94 (94)	180 (90)	1.0
Yes	3 (3)	17 (8)	0.4 (0.1–1.2)
Unsure	3 (3)	3 (2)	1.8 (0.4–8.9)
Previous nonoral HPV-associated cancer ^{††}			
No	99 (99)	—	—
Yes	1 (1)	—	—
First-degree relative with squamous-cell carcinomas of the head and neck			
No	92 (92)	196 (98)	1.0
Yes	8 (8)	4 (2)	4.2 (1.2–14.5)
Sibling with cancer at any site			
No	74 (74)	173 (86)	1.0
Yes	26 (26)	27 (14)	2.2 (1.2–4.1)
Tobacco exposure ^{‡‡}			
Current or former use			
No	44 (44)	119 (60)	1.0
Yes	56 (56)	81 (41)	1.6 (1.0–2.6)
No. of years			
<20	62 (62)	143 (72)	1.0
≥20	38 (38)	55 (28)	1.6 (1.0–2.7)
No. of pack-years			
None	44 (44)	119 (60)	1.0
1–19	22 (22)	42 (21)	1.4 (0.8–2.6)
≥20	34 (34)	39 (20)	2.4 (1.3–4.2) ^{§§}
Marijuana smoked monthly for ≥1 yr			
No	69 (69)	165 (82)	1.0
Yes	31 (31)	35 (18)	2.1 (1.2–3.7)
Exposure to second-hand smoke			
During childhood			
No	22 (22)	64 (32)	1.0
Yes	78 (78)	136 (68)	1.7 (1.0–2.9)
Current			
No	81 (81)	177 (88)	1.0
Yes	19 (19)	23 (12)	1.8 (0.9–3.5)

Table 1. (Continued.)

Explanatory Variable	Patients with Oropharyngeal Cancer (N=100) <i>number (percent)</i>	Control Patients (N=200)	Unadjusted Odds Ratio (95% CI) [†]
Alcohol use			
Current or former use			
No ^{¶¶}	15 (15)	41 (20)	1.0
Yes	85 (85)	159 (80)	1.5 (0.8–2.8)
No. of drinks/wk during past 12 mo			
<28	87 (87)	192 (96)	1.0
≥28	13 (13)	7 (4)	4.1 (1.6–10.7)
Consumption of ≥15 drinks/wk			
0 yr	15 (15)	42 (21)	1.0
1–14 yr	51 (51)	122 (61)	1.2 (0.6–2.3)
≥15 yr	34 (34)	36 (18)	2.7 (1.3–5.6)
Medical condition			
Tumor site			
Tonsil	54 (54)	—	—
Base of tongue or lingual tonsil	36 (36)	—	—
Other ^{***}	10 (10)	—	—
Hearing loss	—	50 (25)	—
Sinusitis	—	42 (21)	—
Otitis or cerumen impaction	—	30 (15)	—
Dizziness or vertigo	—	31 (16)	—
Other ^{†††}	—	47 (24)	—

* Two control patients were individually matched to each case patient according to 5-year age intervals and sex. CI denotes confidence interval.

† To evaluate trends in odds, ordinal variables were modeled as single, continuous, independent variables.

‡ P for trend=0.12.

§ Race and ethnic group were self-reported. "Other" races and ethnic groups included Hispanic (7 patients), Asian (6 patients), Indian or South Asian (2 patients), and Middle Eastern (1 patient).

¶ P for trend <0.001.

|| P for trend=0.09.

** The data for the Papanicolaou (Pap) smear are for women only.

†† Nonoral cancers associated with the human papillomavirus (HPV) included cervical, vulvar, vaginal, penile, and anal cancers. The one case reported was vulvar cancer. This category is applicable only to case patients because eligibility required that control patients have no history of cancer.

‡‡ No tobacco exposure was defined as never having smoked cigarettes daily for more than 1 year. Pack-years were reported for cigarettes, cigars, and pipes. Four cigars or five pipes per day for 1 year were deemed equivalent to one cigarette pack-year. Childhood exposure to smoke was defined as living during childhood with an adult who smoked. Current exposure to smoke was defined as exposure for 2 hours or more per day at home or at work during the previous 10 years. P for trend=0.004.

¶¶ No alcohol exposure was defined as never having consumed 1 drink or more per month for 1 year.

|| P for trend=0.006.

*** Other tumor sites included the soft palate (3%), posterior pharyngeal wall (2%), overlapping lesion of the oral cavity and oropharynx (1%), and an unspecified location of the oropharynx (4%).

††† Other medical conditions included voice change (8%), neuroma (6%), sore throat or swollen glands (4%), sleep apnea (4%), laryngeal stenosis (1%), and facial-nerve spasm (1%).

ular Systems).¹⁸ The HPV-16 viral load in purified DNA from oral-mucosal specimens and fresh-frozen tumor specimens was determined with the use of a sensitive real-time PCR assay targeted to the E6 coding region.^{16,19} The viral load was reported for positive samples (those with ≥1 copy of the virus) and was adjusted to the total number of human cells tested with the use of a

real-time PCR assay targeted to a single copy of a human gene (for endogenous retrovirus 3, ERV3).¹⁶

Serologic Analysis

Serum antibodies to the HPV-16 L1 protein were detected with the use of an enzyme-linked immunosorbent assay (ELISA) based on virus-like particles.²⁰ Antibodies against HPV-16 E6 and E7 oncoproteins were detected with the use of ELISA and bacterially expressed full-length E6 or E7 as the antigen.²¹

STATISTICAL ANALYSIS

Cumulative alcohol use was calculated as follows. We defined a drink-equivalent as one 12-oz beer, one 6-oz glass of wine, one 3-oz mixed drink, or one 1.5-oz shot of liquor. The number of drink-equivalents per week was determined for each patient within each 5-year age interval and combined into a measure of lifetime alcohol use, defined as the number of years during which 15 or more drink-equivalents (hereafter called “drinks”) per week were consumed.

We calculated cumulative tobacco use in pack-years using information about the frequency of use (number of cigarettes, pipes, or cigars smoked per day) and duration of use (during 5-year age intervals) and accounting for gaps in use. Four cigars or five pipes per day were deemed equivalent to one pack of cigarettes in the calculation of pack-years.²²

Unconditional and conditional multivariate logistic-regression models were used to estimate odds ratios and the associated 95% confidence intervals (CIs). Results from the unconditional and conditional models were similar, and the results from the unconditional models are presented. Final multivariate models were created through stepwise elimination of variables of interest from univariate analysis while biologically relevant variables were retained. Owing to the collinearity of sexual behaviors, the effect of each behavior on the risk of cancer was evaluated in separate multivariate models adjusted for alcohol use, tobacco use, presence or absence of a family history of head and neck cancer, oral hygiene, age, and sex. To evaluate trends in odds, ordinal variables were modeled as single, continuous, independent variables. Multiplicative interactions among exposure to HPV, tobacco use, and alcohol use were evaluated by including an interaction term in the regression model, and statistical significance was

determined with the use of the likelihood-ratio test. For comparison of our results with those in previous reports,^{9,10} additive interactions were evaluated with the use of a synergy index, calculated as $(\text{odds ratio for tobacco or alcohol use} + \text{odds ratio for HPV}) - 2$.²³ The odds ratio for HPV was for either seropositivity or infection. Attributable risk was calculated as previously described.²⁴ P values of less than 0.05 for associations were considered to indicate statistical significance. Stata 8.0 software (Stata) was used for all analyses.

RESULTS

We enrolled 130 consecutive patients with newly diagnosed oropharyngeal cancer in the longitudinal cohort study from 2000 through 2005, and 100 patients (77%) agreed to participate in our nested case-control study. Case patients who declined enrollment were similar to those who were enrolled with regard to age, race or ethnic group, and anatomical site of the tumor but were more likely to be female ($P=0.001$). Approximately 70% of eligible control patients (200) agreed to participate.

In the univariate analysis, case and control patients were similar with regard to age, sex, race or ethnic group, and education, but case patients were more likely than control patients to live outside of Maryland (Table 1). A history of squamous-cell carcinoma of the head and neck in a first-degree relative, a history of cancer in a sibling, a history of oral papillomas, and poor long-term oral hygiene (some or complete tooth loss or infrequent toothbrushing) were all associated with oropharyngeal cancer (Table 1). A history of heavy tobacco use (20 pack-years or more), a history of heavy alcohol use (15 drinks or more per week for 15 years or more), and a history of regular marijuana use were also associated with oropharyngeal cancer (Table 1). Similar percentages of case and control patients had no history of tobacco or alcohol use (13% and 14%, respectively; odds ratio, 1.0; 95% CI, 0.5 to 1.9).

Certain kinds of sexual behavior were significantly associated with oropharyngeal cancer after adjustment for confounding variables (Table 2). The association with oropharyngeal cancer increased significantly with the number of vaginal-sex partners or oral-sex partners (P for trend = 0.002 and 0.009, respectively) and was markedly elevated among patients with a high lifetime number of such partners (Table 2).

Table 2. Associations of Oropharyngeal Cancer with Sexual Behaviors.*

Sexual Behavior	Patients with Oropharyngeal Cancer (N=100)	Control Patients (N=200)	Adjusted Odds Ratio (95% CI) [†]	
			All Patients	HPV-16+ Patients [‡]
	<i>number (percent)</i>			
Lifetime no. of vaginal-sex partners				
0–5	31 (31)	108 (54)	1.0	1.0
6–25	41 (41)	63 (32)	2.2 (1.2–4.0)	2.7 (1.4–5.5)
≥26	28 (28)	29 (14)	3.1 (1.5–6.5) [§]	4.2 (1.8–9.4) [¶]
Lifetime no. of oral-sex partners				
0	12 (12)	38 (19)	1.0	1.0
1–5	46 (46)	110 (55)	1.9 (0.8–4.5)	3.8 (1.0–14.0)
≥6	42 (42)	52 (26)	3.4 (1.3–8.8)	8.6 (2.2–34.0) ^{**}
Anal sex				
No	55 (55)	129 (64)	1.0	1.0
Yes	45 (45)	71 (36)	1.3 (0.8–2.2)	1.6 (0.9–2.8)
Casual-sex partner ^{††}				
No	42 (42)	120 (60)	1.0	1.0
Yes	58 (58)	80 (40)	1.7 (1.0–3.0)	2.4 (1.2–4.7)
Age at first intercourse				
18 yr or older	30 (30)	87 (44)	1.0	1.0
17 yr or younger	70 (70)	113 (56)	1.3 (0.7–2.3)	2.1 (1.1–3.6)
Condom use				
Usually or always	28 (28)	90 (45)	1.0	1.0
Never or rarely	72 (72)	110 (55)	2.2 (1.2–3.8)	2.1 (1.1–4.0)
Sex with same-sex partner				
No	92 (92)	186 (93)	1.0	1.0
Yes	8 (8)	14 (7)	1.0 (0.4–2.6)	1.1 (0.3–3.3)
Sexual partner with history of HPV-associated cancer ^{‡‡}				
No	86 (86)	190 (95)	1.0	1.0
Yes	3 (3)	2 (1)	3.0 (0.5–20.5)	3.9 (0.6–25.8)
Unsure	11 (11)	8 (4)	2.3 (0.8–6.5)	2.8 (0.9–8.5)

* The study was designed to have statistical power of 80% to detect an odds ratio of 2 or more for associations between sexual behavior and oropharyngeal cancer on the basis of the prevalence of sexual behaviors in case and control patients reported by Schwartz et al.,⁹ a two-tailed α level of 0.05, and a 2:1 ratio of control patients to case patients. CI denotes confidence interval.

[†] Odds ratios were adjusted for age, sex, tobacco use, alcohol use, dentition and toothbrushing, and presence or absence of a family history of head and neck cancer. To evaluate trends in odds, ordinal variables were modeled as single, continuous, independent variables.

[‡] This analysis was restricted to the 72 case patients with HPV-16-positive tumors (determined with the use of in situ hybridization; see Table 3), plus all 200 control patients.

[§] P for trend=0.002.

[¶] P for trend=0.001.

^{||} P for trend=0.009.

^{**} P for trend <0.001.

^{††} A casual-sex partner was defined as a partner in a “one-night stand” or a partner who was a stranger.

^{‡‡} Cancers considered to be associated with HPV included cervical, vulvar, vaginal, anal, penile, and head and neck cancers.

Oropharyngeal cancer was also strongly associated with serologic measures of exposure to HPV-16 and with the presence of oral HPV infection (Table 3). Oropharyngeal cancer was significantly associated with seropositivity for the HPV-16 L1 capsid protein, a validated measure of lifetime HPV-16 exposure (odds ratio, 32.2; 95% CI, 14.6 to 71.3).²⁵ The presence of an oral HPV-16 infection was strongly associated with oropharyngeal cancer (odds ratio, 14.6; 95% CI, 6.3 to 36.6), as was oral infection with any of 37 HPV types (odds ratio, 12.3; 95% CI, 5.4 to 26.4) (Table 3).

To explore whether the association between sexual behaviors and oropharyngeal cancer could be explained by HPV-16 exposure, we reevaluated the associations using multivariate models after adjusting for HPV-16 L1 serologic status. In this analysis, sexual behaviors were no longer signifi-

cantly associated with oropharyngeal cancer (data not shown). However, associations of sexual behaviors with oropharyngeal cancer became stronger when the analysis was restricted to patients with an HPV-16–positive tumor (Table 2). A high lifetime number of oral-sex or vaginal-sex partners, engagement in casual sex, early age at first intercourse, and infrequent use of condoms each were associated with HPV-16–positive oropharyngeal cancer (Table 2).

The association between HPV-16 exposure and oropharyngeal cancer was investigated among patients with varied use of tobacco and alcohol. The association was greatly increased among patients without a history of smoking or drinking who were seropositive for HPV-16 L1 (odds ratio, 44.8; 95% CI, 5.9 to 338.5) or had an oral HPV-16 infection (odds ratio, 43.7; 95% CI, 4.2 to 452.7).

Table 3. Association of Oropharyngeal Cancer with Exposure to HPV and with Biomarkers of Cancer Associated with HPV-16.

Measure of HPV Exposure or Disease	Prevalence		Odds Ratio (95% CI)	
	Case Patients (N=100) number (percent)	Control Patients (N=200) number (percent)	Unadjusted	Adjusted*
HPV-16 L1 serologic status				
Seronegative	43 (43)	186 (93)	1.00	1.00
Seropositive	57 (57)	14 (7)	17.6 (8.8–34.5)	32.2 (14.6–71.3)
Oral HPV-16 infection†				
Negative	68 (68)	192 (96)	1.00	1.00
Positive	32 (32)	8 (4)	11.3 (5.0–25.7)	14.6 (6.3–36.6)
Any oral HPV infection‡				
Negative	63 (63)	189 (94)	1.00	1.00
Positive	37 (37)	11 (6)	10.0 (4.8–20.7)	12.3 (5.4–26.4)
HPV-16 E6 or E7 serologic status				
Seronegative for E6 and E7	36 (36)	192 (96)	1.00	1.00
Seropositive for E6 or E7	64 (64)	8 (4)	33.3 (16.2–68.6)	58.4 (24.2–138.3)
HPV-16 DNA in tumor				
Absent	28 (28)	—	—	—
Present	72 (72)	—	—	—

* Odds ratios were adjusted for age, sex, tobacco use, alcohol use, dentition and toothbrushing, and presence or absence of a family history of head and neck cancer.

† Oral HPV-16 infection was detected with the use of a real-time PCR assay. The median number of cells analyzed for HPV DNA in case patients and control patients was similar (16,282 vs. 11,053 cells per 10-μl sample; P=0.11). The median HPV-16 viral load was 13.0 and 3.5 copies per 1000 cells among case patients and control patients who were positive for HPV-16, respectively.

‡ Infection of the oral cavity with any of 37 types of HPV was detected with the use of consensus-primer PCR. The HPV types detected, in order of prevalence, were 16 (23 patients), 72 (4 patients), 62 (3 patients), 58 (2 patients), 6 (2 patients), and 18, 31, 51, 55, 61, 66, 68, and 73 (1 patient each) among case patients and 58 (2 patients), 62 (2 patients), and 6, 42, 51, 56, 61, 66, 68, 73, and CP6108 (1 patient each) among control patients. Seven case patients and two control patients were infected with multiple types of HPV.

HPV-16 L1 seropositivity and oral HPV-16 infection were also highly associated with oropharyngeal cancer among patients with a history of heavy tobacco and alcohol use and those without such a history (Table 4). Thus, measures of both lifetime and prevalent oral HPV-16 infection were associated with an increased risk of oropharyngeal cancer, whether or not there was a history of use of tobacco, alcohol, or both.

We evaluated whether combined exposure to HPV and tobacco or alcohol further increased the odds that oropharyngeal cancer would develop. No evidence of synergy was found (Table 4, top): combined exposure to HPV and heavy tobacco and alcohol use was not additive (synergy index <1). Moreover, when the analysis was restricted to patients who were seropositive for the HPV-16 L1 protein, the odds of oropharyngeal cancer were not increased among heavy users of tobacco or alcohol (Table 4, bottom). By contrast, among patients who were seronegative for the HPV-16 L1 protein, the odds of oropharyngeal cancer were increased among heavy users of tobacco or alcohol, and the odds of oropharyngeal cancer were further increased among heavy users of both tobacco and alcohol (synergy index >1) (Table 4, bottom). Similar relationships were observed in patients with and those without the presence of an oral HPV-16 infection (Table 4). Therefore, tobacco and alcohol were important risk factors for oropharyngeal cancer, but they may not have acted as cofactors in HPV-mediated carcinogenesis in the oropharynx.

In the multivariate analysis, oropharyngeal cancer was independently associated with HPV-16 L1 seropositivity (odds ratio, 32.2; 95% CI, 14.6 to 71.3), poor dentition (odds ratio, 4.1; 95% CI, 1.6 to 10.6), infrequent toothbrushing (odds ratio, 6.9; 95% CI, 1.6 to 30.3), history of squamous-cell carcinomas of the head and neck in a first-degree family member (odds ratio, 5.4; 95% CI, 1.0 to 30.8), and heavy tobacco use (odds ratio, 2.5; 95% CI, 1.1 to 6.0) after adjustment for age, sex, and alcohol use. These factors were collectively estimated to be responsible for 90% of cases of oropharyngeal cancers (the attributable risk; 95% CI, 72 to 96), with 55% of cases (95% CI, 45 to 63) attributable to HPV-16 exposure alone.

The percentage of oropharyngeal cancers in which HPV-16 genomic DNA was detected by in situ hybridization was 72% (95% CI, 62 to 81) (Table 3 and Fig. 1). Of the 60 specimens of

available fresh-frozen tumor, 35 (58%; 95% CI, 45 to 71) were positive for HPV-16, with a median of 1.2 viral copies per cell (interquartile range, 0.02 to 11) analyzed. Five fresh-frozen specimens were positive for a high-risk type of HPV other than HPV-16 (two for HPV-33, one for HPV-35, and two for both HPV-33 and HPV-16).

To corroborate the in situ data, we tested for serum antibodies against HPV-16 oncoprotein E6, E7, or both, which have high specificity but moderate sensitivity for the detection of invasive cancer associated with HPV-16.²⁶ Such antibodies were found in 64% of the case patients and in 4% of the control patients (odds ratio, 58.4; 95% CI, 24.2 to 138.3; $P < 0.001$) (Table 2).

DISCUSSION

This epidemiologic study provides support for the association between HPV and a subgroup of oropharyngeal cancers. The strength of the evidence is underscored by the associations of high-risk sexual behaviors, oral HPV infection, and HPV-16 exposure (as determined from the results of serologic tests) with oropharyngeal cancer. Furthermore, we found that HPV-16 DNA was specifically localized to tumor-cell nuclei in 72% of 100 paraffin-embedded specimens of oropharyngeal cancers, a finding corroborated by the high prevalence of antibodies for HPV-16 oncoprotein E6, E7, or both (64%) in the patients with oropharyngeal cancer. Although a cause-and-effect relationship cannot be inferred from a single study, our findings confirm and extend those of other case-control studies.⁷⁻¹⁴ Our results are also consistent with a previous report of an increase in the subsequent risk of oropharyngeal cancer by a factor of 14 among HPV-16 L1 seropositive subjects,²⁶ which provides strong evidence that exposure to HPV can precede the appearance of oropharyngeal cancer by 10 years or more.

The degree to which oral HPV infection may interact with tobacco use, alcohol use, or both to increase the risk of squamous-cell carcinomas of the head and neck has been unclear. A greater-than-additive risk has been reported, albeit inconsistently,⁸⁻¹⁰ for patients exposed to both HPV and tobacco⁹ and those exposed to both HPV and alcohol.¹⁰ We found that exposure to HPV increased the association with oropharyngeal cancer regardless of tobacco and alcohol use, but we uncovered no evidence of synergy between expo-

Table 4. Odds Ratios for Associations of Oropharyngeal Cancer with Tobacco Use, Alcohol Use, Seropositivity for HPV-16, and Oral Infection with HPV-16.*

Variable	Odds Ratio (95% CI)		Synergy Index (95% CI)	Odds Ratio (95% CI)		Synergy Index (95% CI)	
	HPV-16 L1 Seronegative	HPV-16 L1 Seropositive		Negative for Oral HPV-16 Infection	Positive for Oral HPV-16 Infection		
Unstratified risk of oropharyngeal cancer							
Tobacco use							
<20 pack-yr	1.0	37.1 (15.6–88.4)		1.0	17.2 (6.4–46.3)		
≥20 pack-yr	2.8 (1.2–6.4)	27.8 (6.7–114.6) [†]	0.7 (0.5–1.1)	2.4 (1.2–4.7)	13.2 (2.4–65.8) [‡]	0.7 (0.2–2.2)	
Alcohol use							
<15 drink-yr	1.0	36.2 (15.1–86.5)		1.0	16.0 (5.8–43.6)		
≥15 drink-yr	2.5 (1.1–5.5)	29.1 (7.4–115.3) [§]	0.8 (0.5–1.2)	2.2 (1.1–4.3)	16.6 (3.6–81.9) [¶]	1.0 (0.7–1.4)	
Tobacco and alcohol use							
<20 pack-yr and <15 drink-yr	1.0	33.6 (13.3–84.8)		1.0	16.0 (5.4–47.7)		
≥20 pack-yr and ≥15 drink-yr	7.7 (2.7–22)	19.4 (3.3–113.9)	0.5 (0.4–0.6)	4.9 (2.0–12)	11.0 (1.0–120.6)	0.5 (0.4–0.7)	
Risk of oropharyngeal cancer stratified by measures of HPV-16 exposure							
Tobacco use							
<20 pack-yr	1.0	1.0		1.0	1.0		
≥20 pack-yr	2.8 (1.2–6.7)	0.8 (0.2–4.0)		2.1 (1.1–4.2)	0.7 (0.1–6.4)		
Alcohol use							
<15 drink-yr	1.0	1.0		1.0	1.0		
≥15 drink-yr	2.6 (1.1–5.9)	0.9 (0.2–4.3)		1.8 (0.9–3.6)	1.2 (0.2–8.6)		
Tobacco and alcohol use							
<20 pack-yr and <15 drink-yr	1.0	1.0		1.0	1.0		
≥20 pack-yr and ≥15 drink-yr	8.9 (3.0–27)**	0.46 (0.07–3.0) ^{††}		5.0 (2.0–12) ^{‡‡}	0.5 (0.0–11.5) ^{§§}		

* As appropriate, odds ratios were adjusted for age, sex, tobacco use, alcohol use, dentition and toothbrushing, and presence or absence of a family history of head and neck cancer. Multiplicative interaction was evaluated by means of inclusion of an interaction term in the logistic-regression model. The synergy index is a test of additive interaction that provides evidence that combined exposures are either superadditive (synergy index >1), compatible with additive (synergy index = 1), or less than additive (synergy index <1). The synergy index was calculated as (odds ratio for tobacco or alcohol use and HPV-1) ÷ [(odds ratio for tobacco or alcohol use + odds ratio for HPV) – 2]. For patients seronegative for HPV-16 L1 and negative for oral HPV-16 infection, the delta method to approximate the 95% confidence interval (CI) for the synergy index could not be performed because of negative values. As an alternative, a Markov chain Monte Carlo approach was used to estimate the logistic-regression model, the synergy index, and its 95% credible interval. A standard logistic model was assumed with noninformative prior distribution for the coefficients and was fit with the use of WinBugs software. The posterior distribution of the synergy index (estimated as a function of the model coefficients) was used to determine the 95% credible interval (i.e., the 2.5th to the 97.5th percentiles of the posterior distribution) as opposed to a CI. Tobacco use was defined as lifetime cigarette, cigar, or pipe use. Four cigars or five pipes per day for a year were deemed equivalent to 1 cigarette pack-year. Alcohol use was defined as the lifetime number of years of consumption of ≥15 drink-equivalents per week (drink-years).

[†] P=0.12 for the interaction between HPV and tobacco.

[‡] P=0.29 for the interaction between HPV and tobacco.

[§] P=0.18 for the interaction between HPV and alcohol.

[¶] P=0.54 for the interaction between HPV and alcohol.

^{||} Results for the full model are not shown.

^{**} P=0.10 for the interaction between tobacco and alcohol.

^{††} P=0.29 for the interaction between tobacco and alcohol.

^{‡‡} P=0.08 for the interaction between tobacco and alcohol.

^{§§} P=0.59 for the interaction between tobacco and alcohol.

The synergy index for tobacco and alcohol among patients who were seronegative for HPV-16 L1 was 1.0 (95% CI, 3.9 to 26). The synergy index for HPV-16 L1 was –0.06 (95% CI, –3.7 to 4.9). The synergy index for tobacco and alcohol among patients who were negative for oral HPV-16 infection was 16 (95% CI, 0.81 to 298). The synergy index for tobacco and alcohol among patients who were positive for oral HPV-16 infection was –0.02 (95% CI, –8.3 to 7.9).

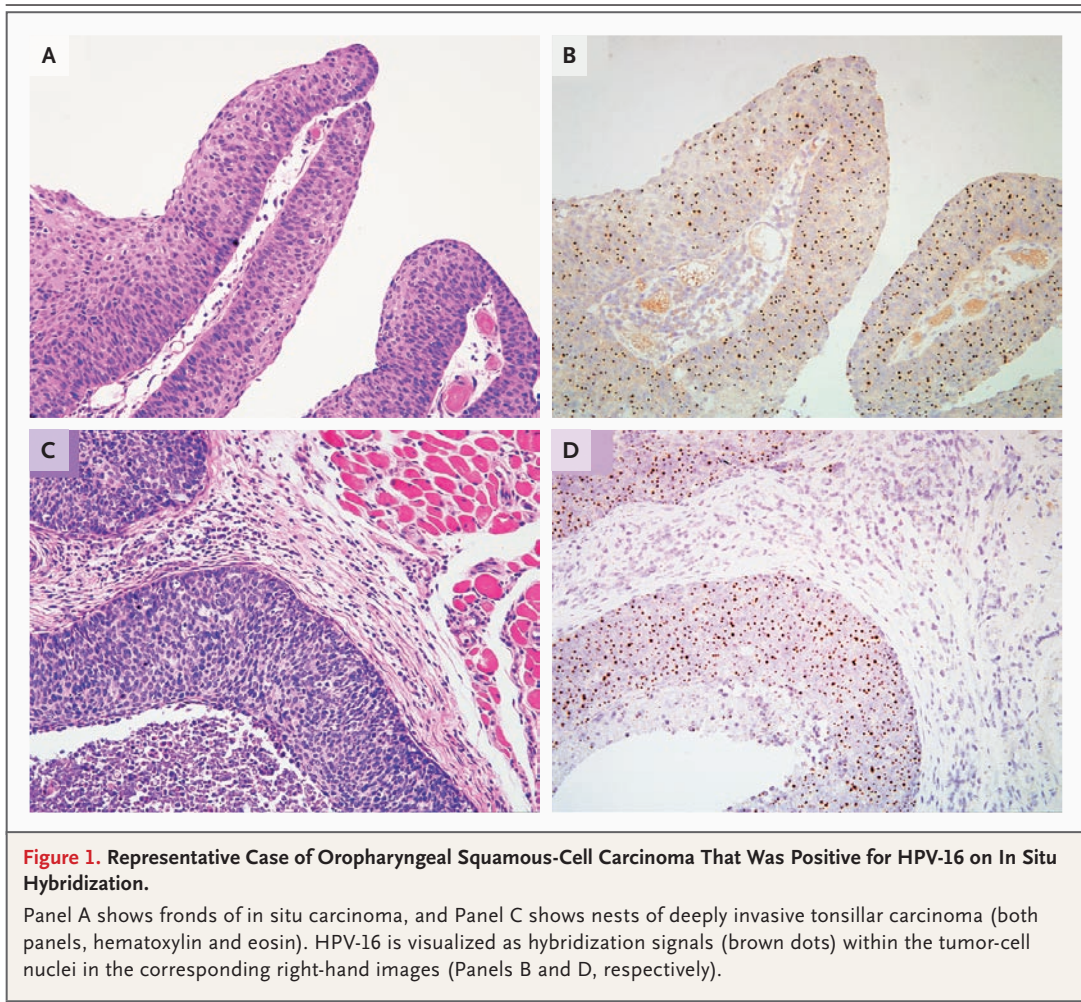


Figure 1. Representative Case of Oropharyngeal Squamous-Cell Carcinoma That Was Positive for HPV-16 on In Situ Hybridization.

Panel A shows fronds of in situ carcinoma, and Panel C shows nests of deeply invasive tonsillar carcinoma (both panels, hematoxylin and eosin). HPV-16 is visualized as hybridization signals (brown dots) within the tumor-cell nuclei in the corresponding right-hand images (Panels B and D, respectively).

sure to HPV and tobacco or alcohol use. For these reasons, our data suggest two distinct pathways for the development of oropharyngeal cancer: one driven predominantly by the carcinogenic effects of tobacco or alcohol (or both) and another by HPV-induced genomic instability.

Our data suggest that oral HPV infection is sexually acquired. Oral–genital contact was strongly associated with oropharyngeal cancer, but we cannot rule out transmission through direct mouth-to-mouth contact or other means. Certain sexual behaviors^{13,14} and a history of oral HPV infection^{7,10} were associated with an increased risk of squamous-cell carcinomas of the head and neck in previous studies in which 25% or more of the tumors from patients were positive for HPV DNA but not those in which less than 25% of the tumors from patients were positive for HPV DNA.^{8,9} Discrepant findings may be explained by

the heterogeneity of the case populations, with variable percentages of cancer cases attributable primarily to tobacco and alcohol use, as compared with HPV infection. In our study, the heterogeneity of case patients was minimized by restricting enrollment to patients with oropharyngeal cancer, 90% of whom had tumors on the tonsil or base of the tongue.

Although HPV-16 alone accounts for more than 90% of cases of HPV-positive squamous-cell carcinomas of the head and neck,⁸ a more accurate and probably higher proportion might be found by testing for other types of HPV (e.g., types 18, 31, 33, and 35), which are infrequently detected in oropharyngeal cancers.

In our study, oropharyngeal cancer was independently associated with a family history of squamous-cell carcinoma of the head and neck and poor oral hygiene, findings that are consis-

tent with other reports.²⁷ The risk of cervical cancer is also increased in women with a family history of that cancer.^{28,29} Until specific genetic markers for the risk of an HPV-associated cancer are identified, familial aggregation due to shared environmental exposures cannot be ruled out as an explanation for these findings. Poor dentition,^{30,31} infrequent toothbrushing,^{31,32} and infrequent dental visits^{30,33} have been associated with an increased risk of squamous-cell carcinomas of the head and neck. Because tooth loss is commonly caused by chronic bacterial infections (e.g., periodontitis), it may serve as a surrogate for chronic infection and inflammation, which may be important in the pathogenesis of cancer. Particular coinfections in the cervix (e.g., infection with *Chlamydia trachomatis*) increase the risk of cancer,³⁴ and our results suggest that bacterial coinfections could play a similar role in the oral region. The absence of data on diet, which is associated with the risk of squamous-cell carcinomas of the head and neck,³⁵ is a limitation of our study but is unlikely to explain the observed associations with HPV infection.

The public health implications of our findings

are underscored by the annual increases in the incidence of tonsillar and base-of-tongue cancers in the United States since 1973.^{36,37} The widespread oral sexual practices among adolescents may be a contributing factor in this increase.³⁸ Our results and those of other studies provide a rationale for HPV vaccination in both boys and girls — since oropharyngeal cancers occur in men and women. If vaccination is as effective in preventing oral HPV-16 infection as it is in preventing cervical infection,³⁹ a substantial reduction in the incidence of oropharyngeal cancer in vaccinated populations would provide the ultimate evidence of causality.

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REFERENCES

- Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* 1999;189:12-9.
- Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst* 2000;92:709-20.
- Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidemiol Biomarkers Prev* 2005;14:467-75.
- Gillison ML. Human papillomavirus-associated head and neck cancer is a distinct epidemiologic, clinical, and molecular entity. *Semin Oncol* 2004;31:744-54.
- Bosch FX, De Sanjose S. Human papillomavirus and cervical cancer — burden and assessment of causality. *J Natl Cancer Inst Monogr* 2003;31:3-13.
- Gillison ML, Shah KV. Role of mucosal human papillomavirus in nongenital cancers. *J Natl Cancer Inst Monogr* 2003;31:57-65.
- Hansson BG, Rosenquist K, Antonsson A, et al. Strong association between infection with human papillomavirus and oral and oropharyngeal squamous cell carcinoma: a population-based case-control study in southern Sweden. *Acta Otolaryngol* 2005;125:1337-44.
- Herrero R, Castellsague X, Pawlita M, et al. Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. *J Natl Cancer Inst* 2003;95:1772-83.
- Schwartz SM, Daling JR, Doody DR, et al. Oral cancer risk in relation to sexual history and evidence of human papillomavirus infection. *J Natl Cancer Inst* 1998;90:1626-36.
- Smith EM, Ritchie JM, Summersgill KF, et al. Human papillomavirus in oral exfoliated cells and risk of head and neck cancer. *J Natl Cancer Inst* 2004;96:449-55.
- Dahlstrom KR, Adler-Storthz K, Etzel CJ, et al. Human papillomavirus type 16 infection and squamous cell carcinoma of the head and neck in never-smokers: a matched pair analysis. *Clin Cancer Res* 2003;9:2620-6.
- Maden C, Beckmann AM, Thomas DB, et al. Human papillomaviruses, herpes simplex viruses, and the risk of oral cancer in men. *Am J Epidemiol* 1992;135:1093-102.
- Smith EM, Ritchie JM, Summersgill KF, et al. Age, sexual behavior and human papillomavirus infection in oral cavity and oropharyngeal cancers. *Int J Cancer* 2004;108:766-72.
- Rosenquist K, Wennerberg J, Schildt EB, Bladstrom A, Goran Hansson B, Andersson G. Oral status, oral infections and some lifestyle factors as risk factors for oral and oropharyngeal squamous cell carcinoma: a population-based case-control study in southern Sweden. *Acta Otolaryngol* 2005;125:1327-36.
- Huang CC, Qiu JT, Kashima ML, Kurman RJ, Wu TC. Generation of type-specific probes for the detection of single-copy human papillomavirus by a novel in situ hybridization method. *Mod Pathol* 1998;11:971-7.
- D'Souza G, Sugar E, Ruby W, Gravitt P, Gillison M. Analysis of the effect of DNA purification on detection of human papillomavirus in oral rinse samples by PCR. *J Clin Microbiol* 2005;43:5526-35.
- Fearon ER, Feinberg AP, Hamilton SH, Vogelstein B. Loss of genes on the short arm of chromosome 11 in bladder cancer. *Nature* 1985;318:377-80.
- Peyton CL, Gravitt PE, Hunt WC, et al. Determinants of genital human papillomavirus detection in a US population. *J Infect Dis* 2001;183:1554-64.
- Gravitt PE, Peyton C, Wheeler C, Apple R, Higuchi R, Shah KV. Reproducibility of HPV 16 and HPV 18 viral load quantitation using TaqMan real-time PCR assays. *J Virol Methods* 2003;112:23-33.
- Viscidi RP, Ahndieh-Grant L, Clayman

- B, et al. Serum immunoglobulin G response to human papillomavirus type 16 virus-like particles in human immunodeficiency virus (HIV)-positive and risk-matched HIV-negative women. *J Infect Dis* 2003;187:194-205.
21. Sehr P, Zumbach K, Pawlita M. A generic capture ELISA for recombinant proteins fused to glutathione S-transferase: validation for HPV serology. *J Immunol Methods* 2001;253:153-62.
22. Benhamou S, Benhamou E, Flamant R. Lung cancer risk associated with cigar and pipe smoking. *Int J Cancer* 1986;37:825-9.
23. Hosmer DW, Lemeshow S. Confidence interval estimation of interaction. *Epidemiology* 1992;3:452-6.
24. Greenland S, Drescher K. Maximum likelihood estimation of the attributable fraction from logistic models. *Biometrics* 1993;49:865-72.
25. Dillner J. The serological response to papillomaviruses. *Semin Cancer Biol* 1999;9:423-30.
26. Mork J, Lie AK, Glatte E, et al. Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. *N Engl J Med* 2001;344:1125-31.
27. Brown LM, Gridley G, Diehl SR, et al. Family cancer history and susceptibility to oral carcinoma in Puerto Rico. *Cancer* 2001;92:2102-8.
28. Hemminki K, Li X, Mutanen P. Familial risks in invasive and in situ cervical cancer by histological type. *Eur J Cancer Prev* 2001;10:83-9.
29. Zelmanowicz Ade M, Schiffman M, Herrero R, et al. Family history as a co-factor for adenocarcinoma and squamous cell carcinoma of the uterine cervix: results from two studies conducted in Costa Rica and the United States. *Int J Cancer* 2005;116:599-605.
30. Bundgaard T, Wildt J, Frydenberg M, Elbrond O, Nielsen JE. Case-control study of squamous cell cancer of the oral cavity in Denmark. *Cancer Causes Control* 1995;6:57-67.
31. Zheng TZ, Boyle P, Hu HF, et al. Dentition, oral hygiene, and risk of oral cancer: a case-control study in Beijing, People's Republic of China. *Cancer Causes Control* 1990;1:235-41.
32. Moreno-Lopez LA, Esparza-Gomez GC, Gonzalez-Navarro A, Cerero-Lapiedra R, Gonzalez-Hernandez MJ, Dominguez-Rojas V. Risk of oral cancer associated with tobacco smoking, alcohol consumption and oral hygiene: a case-control study in Madrid, Spain. *Oral Oncol* 2000;36:170-4.
33. Maier H, Zoller J, Herrmann A, Kreiss M, Heller WD. Dental status and oral hygiene in patients with head and neck cancer. *Otolaryngol Head Neck Surg* 1993;108:655-61.
34. Smith JS, Bosetti C, Munoz N, et al. Chlamydia trachomatis and invasive cervical cancer: a pooled analysis of the IARC multicentric case-control study. *Int J Cancer* 2004;111:431-9.
35. Kreimer AR, Randi G, Herrero R, Castellsague X, La Vecchia C, Franceschi S. Diet and body mass, and oral and oropharyngeal squamous cell carcinomas: analysis from the IARC multinational case-control study. *Int J Cancer* 2006;118:2293-7.
36. Shiboski CH, Schmidt BL, Jordan RC. Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20-44 years. *Cancer* 2005;103:1843-9.
37. Frisch M, Hjalgrim H, Jaeger AB, Biggar RJ. Changing patterns of tonsillar squamous cell carcinoma in the United States. *Cancer Causes Control* 2000;11:489-95.
38. Mosher WD, Chandra A, Jones J. Sexual behavior and selected health measures: men and women 15-44 years of age, United States, 2002. *Adv Data* 2005;362:1-55.
39. Harper DM, Franco EL, Wheeler CM, et al. Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised controlled trial. *Lancet* 2006;367:1247-55.

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