

Human Papillomavirus Type 16 and Squamous Cell Carcinoma of the Head and Neck¹

Elin Ringström, Edward Peters,
Masayuki Hasegawa, Marshall Posner, Mei Liu,
and Karl T. Kelsey²

Department of Cancer Cell Biology, Harvard School of Public Health, Boston, Massachusetts 02115 [E. R., E. P., M. H., M. L., K. T. K.]; Department of Oral Epidemiology and Department of Oral Medicine, Harvard School of Dental Medicine, Boston, Massachusetts 02115 [E. P.]; and Department of Head and Neck Oncology, Dana Farber Cancer Institute, Boston, Massachusetts 02115 [M. P.]

ABSTRACT

Purpose: Human papillomavirus (HPV) has previously been reported to be associated with squamous cell carcinoma of the head and neck. Our objective was to investigate the presence and type of HPV infection in head and neck tumors and determine whether infection was associated with individual tumor characteristics, patients' pattern of tobacco and alcohol exposure, or with clinical outcome.

Experimental Design: Using a case series design, fresh tumor samples were obtained from a series of 89 head and neck squamous cell carcinoma patients, including 64 men and 25 women. The majority of tumors were located in the oral cavity, followed by the oropharynx. A PCR-based technique with restriction fragment length polymorphism analysis was used to detect and type HPV.

Results: Of the 89 patients, 18 (20%) had detectable HPV16 in their tumor samples. HPV16 was detected in 64% of tonsil tumors, 52% oropharyngeal tumors, and 5% oral cavity tumors. The mean age of subjects with HPV16-positive tumors was younger than the patients with HPV-negative tumors. Also, this group consumed less alcohol on a weekly basis and had a better clinical outcome compared with the HPV-negative group. Smoking, clinical stage, tumor grade, and tumor-node-metastasis status were not associated with HPV16 presence.

Conclusions: Our study supports the previous reports that suggest HPV16 is associated with squamous cell cancers located in the oropharynx and oral cavity. The fact that HPV-positive tumors were observed in younger, lighter alcohol-consuming individuals with a better overall and

disease-specific survival suggests a distinct disease process in these patients.

INTRODUCTION

Each year, there is an estimated 45,000 new cases of HNSCC³ diagnosed in the United States (1). Worldwide incident rates are much higher, especially in the Indian subcontinent, Hong Kong, and Europe (2). Despite treatment advances over the past decades, the overall 5-year survival rate in the United States remains at ~55% (3). Established etiologic factors include cigarette, pipe and cigar smoking, heavy alcohol abuse, betel nut chewing, and smokeless tobacco use. However, 15–20% of HNSCC (3) patients have no known tobacco or alcohol exposure (4). This group may be growing (5) and include a large proportion of young adults and women (6–8). Increasingly, research has focused on identifying a possible viral etiologic factor; chiefly, this has included oncogenic HPVs.

HPV subtypes 16, 18, and 33 have been detected in >99% of cervical cancers worldwide (9). These viral types produce oncogenic proteins (E6 and E7) that disrupt p53 and pRb function, respectively, and thereby promote cellular immortalization (10). Epidemiological studies have observed an increased incidence and relative risk for HNSCC among subjects initially presenting with cervical cancer (11, 12). Research thus has focused upon common etiologic factors such as smoking and infection with HPVs. Löning *et al.* (13) first suggested a relationship between HPV and HNSCC in 1985, and evidence of a possible association has since been increasing. Indeed, these proteins and subsequent cell cycle disruption have been demonstrated in HNSCC (14–17).

Previous studies have reported the presence of HPV types 16 and 18 in oropharyngeal cancers. Estimates of HPV prevalence in HNSCC tumors vary with the detection method used and the nature of tissue preservation (18, 19). DNA from fresh tumor tissue subjected to PCR amplification of the L1 or E7 viral region has the highest reported detection rate (20, 21). However information on other risk factors such as tobacco use and alcohol consumption have often been lacking in these reports. Anatomical sites and patient characteristics such as age, gender, clinical stage, smoking, and alcohol consumption have been variably reported as associated with HPV detection. In addition, recently Gillison *et al.* (22) and Schwartz *et al.* (23)

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² To whom requests for reprints should be addressed, at Department of Cancer Cell Biology, Harvard School of Public Health, 665 Huntington Avenue, Building 1, Room 207, Boston, MA 02115-6021. Phone: (617) 432-3313; Fax: (617) 432-0107; E-mail: kelsey@hsph.harvard.edu.

³ The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; HPV16, HPV type 16; HPV18, HPV type 18; TNM, tumor size and nodal presence and metastases staging classification; HR, hazard ratio in Cox regression proportional hazards model; OR, odds ratio; CI, confidence interval; E6, early gene-6 of a HPV genome; E7, early gene-7 of a HPV genome; p53, tumor suppressor protein 53; pRb, retinoblastoma protein; L1, late gene-1 of the HPV genome; pack-years, number of packs of cigarettes smoked/day multiplied by the number of years the subject has smoked.

Table 1 Demographic characteristics of HPV-positive and HPV-negative patients

	Total (n = 89)		HPV-positive (n = 18)		HPV-negative (n = 71)		Fisher's exact test Pr ≤ P
	No.	%	No.	%	No.	%	
Gender							
Male	64	71.9	15	83.3	49	69.0	0.4
Female	25	28.1	3	16.7	22	31.0	
Age							
<59 years	44	49.4	14	77.8	30	42.2	0.009
≥59 years	45	50.6	4	22.2	41	57.8	
Tobacco							
Current smoker	48	53.9	9	50.0	39	54.9	0.9
Former smoker	29	32.6	6	33.3	23	32.4	
Never smoker	12	13.5	3	16.7	9	12.7	
Alcohol							
Current regular	54	60.7	13	72.2	41	57.8	0.6
Former regular	14	15.7	3	16.7	11	15.5	
Never used	20	22.5	2	11.1	18	25.4	
Unknown	1	1.1	0	0	1	1.4	
Alcohol intake							
Ever users (we/wk) ^a n = 68							
Light (≤35)	44	65.7	15	93.8	29	7.1	0.007
Heavy (>35)	23	34.3	1	6.2	22	52.9	
Unknown	1		0		1		

^a we/wk, whiskey-equivalents/wk = the number of glasses of wine, bottles of beer, and shots of hard alcohol consumed in 1 week.

found that HPV16-positive patients had a survival advantage compared with the HPV-negative patients.

Our objective was to determine the presence of high-risk HPV subtypes in HNSCC fresh tumor samples by using PCR-restriction fragment length polymorphism-based detection method. Information on patient age, medical history, smoking status, alcohol usage, clinical management, and follow-up was gathered from medical records, database queries, and detailed patient-completed questionnaires. We then examined the relationship of viral presence with these demographic and clinical parameters.

MATERIALS AND METHODS

Tumor specimens were obtained from 89 HNSCC patients seen at the Dana-Farber Cancer Institute, Brigham and Women's Hospital, and Beth Israel Deaconess Hospital (Boston, MA) between 1994 and 1998. Specimens were snap frozen in liquid nitrogen at the time of excision and stored at -80°C until use. Information on patient gender, age, smoking, and alcohol history was collected from direct patient interviews and medical records. Stage, TNM status, and tumor grade were obtained from clinical notes, pathology, and radiology reports. Staging was done according to International Union Against Cancer Fifth Edition guidelines (24). Follow-up information was obtained from the clinical records and the treating physician. The Social Security Death Index Database was used to confirm the date of death.

DNA extraction of the fresh frozen samples was accomplished by using the QIAamp Extraction Kit (Qiagen, Valencia CA). Integrity of the DNA was confirmed by amplification of β -actin from the DNA sample. To minimize cross contamination, PCR procedures were conducted in a separate laboratory from where the DNA extraction took place. Positive and nega-

tive controls were used during all HPV detection and typing procedures.

Specimens were analyzed for the presence of HPV by PCR amplifying a portion of the viral L1 region as reported previously by Ting and Manos (25). A 50- μ l reaction mixture consisted of 1 μ M of each primer, 1 μ l of DNA sample, 2 μ M of each 2'-deoxynucleoside 5'-triphosphate, 2.0 μ M MgCl₂, and 2 units of recombinant Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT). Initial denaturation occurred at 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C over 1 min. A final extension was done at 72°C for 7 min. PCR products were run on a 4% agarose gel stained with ethidium bromide. A fragment length of 449–458 nucleotide bp was considered positive for HPV. Viral typing was achieved by restriction enzyme digestion of the L1 amplicon by restriction endonuclease RSA1, restriction endonuclease PST1, and *Hae* III (26). Unlike many other HPV types, HPV16 and HPV18 L1 amplicon products are essentially uncut by *Hae* III. Restriction endonuclease RSA1 cleavage of the MY09/11 amplicon product of HPV16 shows a digestion pattern of 310-, 72-, and 70-bp fragments. This enzyme cleaves the product of HPV18 into much smaller distinct fragments of 135, 125, 85, 72, and 38 bp.

χ^2 and Fisher's exact test were used to examine the association of HPV detection with gender, primary site location, stage, TNM status, smoking, and alcohol usage history. Smokers were classified as current, former, or never. A similar classification system was used for alcohol consumption. The intensity of alcohol consumption was measured in whiskey-equivalents/week (1 whiskey equivalent = 1 beer, 1 glass of wine, or 1 shot of hard liquor). The Wilcoxon's rank-sum test was used to examine the difference by HPV status for the mean patient age, number of years smoked, packs/day used, and pack-years. To further examine these relationships, logistic re-

Table 2 Clinical and pathologic characteristics of HPV-positive and HPV-negative patients

	Total (n = 89)		HPV-positive (n = 18)		HPV-negative (n = 71)		Fisher's exact test Pr ≤ P
	No.	%	No.	%	No.	%	
Primary							
Oral cavity	41	46.1	2	11.1	39	55.0	<0.01
Oropharynx	29	32.6	15	83.3	14	19.7	
Hypopharynx	4	4.5	0	0	4	5.6	
Larynx	10	11.2	1	5.6	9	12.7	
Other	5 ^a	5.6	0	0	5	7.0	
T status							
T ₀	4	4.5	0	0	4	5.6	0.3
T ₁	7	7.9	2	11.1	5	7.0	
T ₂	28	31.5	8	50.0	19	26.8	
T ₃	25	28.1	4	16.7	22	31.0	
T ₄	25	28.1	3	22.2	21	29.6	
Lymph Nodes							
Negative	30	33.7	5	27.8	25	35.2	0.8
Positive	59	66.3	13	72.2	46	64.8	
Grade							
Well	24	27.0	4	22.2	20	28.2	0.6
Moderate	45	50.6	8	44.4	37	52.1	
Poor	16	18.0	5	27.8	11	15.5	
Unknown	4	4.5	1	5.6	3	4.2	
Stage							
I	4	4.5	0	0	4	5.6	0.6
II	13	14.6	3	16.7	10	14.1	
III	18	20.2	2	11.1	16	22.5	
IV	54	60.7	13	72.2	41	57.8	

^a Includes 1 maxillary sinus squamous cell carcinoma, 1 nasopharyngeal squamous cell carcinoma, and 3 cervical node tumors with unknown primaries.

gression was performed to calculate the *Ps* and 95% CIs while controlling for age, gender, tobacco, and alcohol consumption. Kaplan-Meier survival estimates and a Cox regression proportional hazards model were used to analyze survival data and calculate HRs. Additional HRs were calculated after adjusting for patient age and stage of disease. Deaths occurring within 2 months of surgery were censored in the survival analysis. All statistics were performed on SAS software with a *P* < 0.05 considered significant (SAS Institute, Cary, NC).

RESULTS

Initial experiments to define the sensitivity of our method used SiHa cells (HPV16-positive with 1–2 copies/cell) and HeLa cells (HPV18-positive with 10–50 copies/cell). These cells were serially diluted with Ca-33A (HPV-negative) cervical cancer cell lines (American Type Culture Collection, Manassas VA). We were able to detect 1–50 HPV copies within a single cell among a background of 100 noninfected cells. These cell lines acted as positive and negative controls for subsequent work.

There were 86 squamous cell carcinomas that represented primary disease and 3 that were local recurrences. The group consisted of 64 men (72%) and 25 women (28%). The mean and median age of the study group were 59 years (Table 1). All tumor specimens were of squamous cell origin. HPV16 was detected in 17 of 89 tumor specimens (20%). No HPV18 was found in the specimens; only HPV16 was detected. None of the recurrent tumors had detectable HPV.

Fifteen of 18 HPV16-positive tumors (83%) were found in the oropharynx; the remaining 3 positive tumors (11%) were in the oral cavity and larynx (Table 2). Sixty-four percent of the tonsillar tumors (*n* = 7 of 11) were positive for HPV16 (*P* < 0.0001).

The mean age of patients who were HPV16-positive was 8.4 years younger than the HPV-negative group (HPV16-positive: mean age = 52.7 years *versus* HPV-negative: mean age = 61.1 years; *P* < 0.01). When patients were stratified into young (<59 years) and old (≥59 years) based upon the median age of the study group, HPV16 was more likely to be detected in the younger patient group (Table 1; *P* = 0.009). After controlling for gender, smoking, and alcohol history by logistic regression models, subjects older than 59 years were less likely to have HPV-positive tumors (OR = 0.18; 95% CI = 0.04, 0.88; *P* < 0.03).

In the univariate analysis, the presence of HPV16 was strongly associated with both fewer whiskey equivalents consumed (*P* < 0.007) and fewer number of years smoking (HPV16-positive group: mean number of years smoked = 30.1 years *versus* HPV-negative group: mean number of years smoked = 36.4 years; *P* < 0.03). The mean number of whiskey-equivalents consumed/day was 3. Logistic regression analysis suggested that there was some confounding; consumption of >3 drinks/day (*P* = 0.02) but not duration of smoking (*P* > 0.05) predicted HPV16-positivity after controlling for age and gender. Age of smoking initiation, packs/day smoked, and pack-years did not differ by HPV status. Twenty-three percent of the men were HPV16-positive compared with 12%

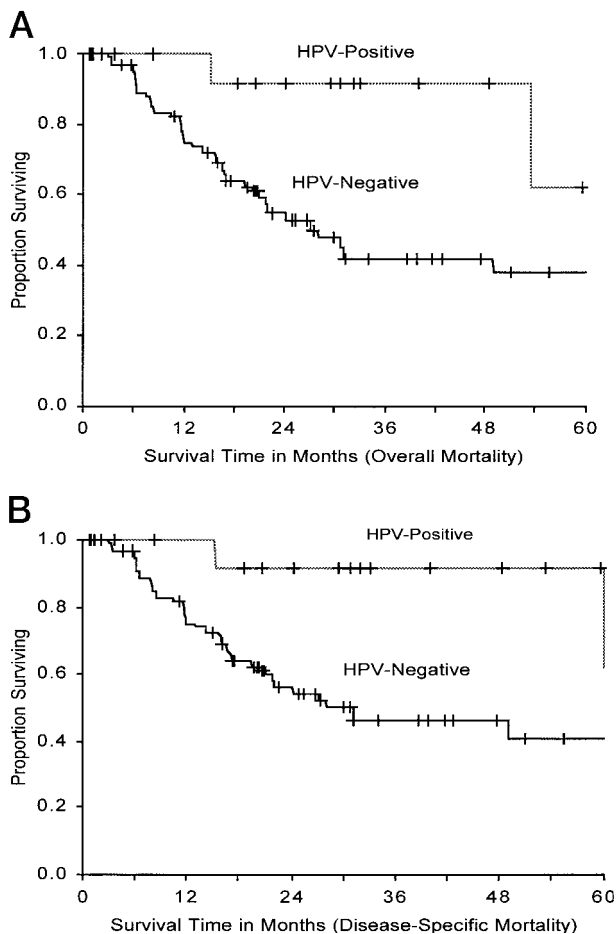


Fig. 1 Graph depicting Kaplan-Meier estimates of overall survival measured in months from diagnosis of HNSCC, by HPV detection (—, HPV negative; ····, HPV positive). Data were analyzed for all-cause mortality (A; Wilcoxon $P = 0.01$) and disease-specific mortality (B; Wilcoxon $P = 0.01$). Censored values noted with +.

of the women. Clinical stage, TNM status, smoking, and alcohol status (current *versus* former *versus* never) were not associated with HPV presence.

Patients with tumors harboring HPV16 had a better clinical outcome than the HPV-negative group. In Kaplan-Meier analysis, HPV16-positive patients had better overall (Fig. 1A; Wilcoxon $P = 0.01$) and disease-specific (Fig. 1B; Wilcoxon $P = 0.01$) survival. Using a Cox proportional hazards model, the unadjusted HR for overall survival was 0.20 ($P = 0.03$), similar to the age and stage adjusted HR = 0.23 ($P = 0.05$). One of 18 HPV16-positive patients died of their disease (5.9%) compared with 33 (46%) of 71 in the HPV-negative group ($P = 0.003$). Three subjects in the study died from causes not specifically related to their head and neck cancer. Overall, 22 (25%) of 89 patients had either a local, regional, or distant recurrence. None of the patients with HPV16-positive tumors developed recurrent disease ($P = 0.002$). Thirteen of the subjects in the HPV-negative group had uncontrollable primary disease. One of the

HPV16-positive patients had uncontrolled disease ($P = 0.002$).

DISCUSSION

Overall, 20% of the HNSCC tumors in our study tested positive for HPV16. No HPV18 was detected in the samples. Previous studies have reported a similar detection rate among HNSCC tumors with 90% of the HPV types identified as HPV16 (22, 27). Our finding of only HPV16 in our subjects, with a PCR-restriction fragment length polymorphism methodology capable over detecting >40 different HPV viral types, could be because of the small sample size or real differences in the population under study. Gillison *et al.* (22) found HPV16 in 22%, HPV18 in 0.4%, HPV33 in 1.2%, and HPV31 in 0.4% of 253 HNSCC patients. Hence, we would expect to detect less than one case of any of these rarer HPV types. Furthermore, HPV16 and HPV18 infection in HNSCC patients could vary by geography. For example, Aggelopouloa *et al.* (27) studying oral cavity cancers and hyperplasias in a Greek population reported a 44% HPV18 and a 22% HPV16 detection rate. In a study of 91 oral cancers in an Indian population, Balaram *et al.* (28) reported a HPV16 and HPV18 detection rate of 42% and 47%, respectively. Both studies also show a different HPV16 and HPV18 type distribution compared with our study and others published in North America (22, 23, 29).

Consistent with other reports, we also found a strong association of HPV presence with oropharyngeal site. Fifty-two percent of the oropharyngeal tumors had detectable HPV16. Furthermore, 64% of tonsillar carcinoma harbored HPV16. Oropharyngeal tumors have been previously associated with 13–57% HPV-positive tumors (22, 30–32). In addition, 21–100% of tonsillar squamous carcinoma specimens have been found to be HPV16, HPV18, or HPV33 positive (17, 22, 32–35). In contrast, the presence of HPV oral cavity tumors is reported from 12–71% (22, 30–32, 36–39) and in the larynx 4–24% (27, 31–33). In our study, 5% of the oral cavity tumors contained HPV16. These results strongly support an anatomical preference for HPV16 infection and are consistent with the literature (15, 22, 30–33, 40).

Our subjects with HPV16 detectable in their tumors were more likely to be <59 years of age compared with those who were negative for the virus. This association was seen in a logistic regression model controlling for gender, pack-years, and alcohol usage. Cruz *et al.* (37) also reported an association of younger patient age with HPV presence. Accounts are variable, but there appears to be an overall trend for HPV to be detected in patients < 60 years of age (19). Even those studies that did not find a significant effect of age have reported point estimates of mean and median age younger in their HPV-positive group. For example, in a study of 14 young and 18 old matched HNSCC patients, Sisk *et al.* (41) report the risk for HPV positivity to be 1.25 greater (95% CI = 0.3, 5.1) among the younger population. Gillison *et al.* (22) studied 253 HNSCC tumors and reported the median age among the HPV-positive group to be 60.5 years compared with 64 years in the HPV-negative group. In light of the increasing incidence of tonsillar and oral cancer in young adults (5), HPV might represent an important etiologic factor in these patients.

In our study, none of the HPV16-positive group developed disease recurrence and only one (5.6%) died of disease. In contrast, 31% of the patients in the HPV-negative group developed a recurrence and 46% died of disease. This finding is consistent with Schwartz *et al.* (23) who reported that HPV16-positive patients had a significantly reduced overall (HR = 0.35) and disease-specific (HR = 0.17) mortality. Gillison *et al.* (22) reported that patients with HPV-positive tumors had a 59% reduction in risk of cancer death compared with the HPV-negative group (HR = 0.41). These observations could have an impact on future clinical management and strongly merit additional investigation.

HPV16 presence was associated with consumption of <3 alcoholic beverages/day. Previous studies also show a trend toward HPV16 association with less alcohol consumed. Gillison *et al.* (22) also found HPV-positive tumors to be less likely to occur among moderate to heavy alcohol drinkers (OR = 0.17; 95% CI = 0.05–0.61) and smokers (OR = 0.16; 95% CI = 0.02–1.4). Portugal *et al.* (42), in their study of 58 oral and 42 tonsillar cancers, found that HPV presence predicted a lower rate of alcohol and tobacco consumption ($P = 0.09$). After selecting out patients with coexpression of mutant p53, they found HPV expression more strongly associated with light drinking ($P = 0.04$).

Oncogenic HPVs, by partially or completely blocking tumor suppressor protein function, likely abrogate the need for extensive carcinogen exposure that may (in uninfected individuals) result in mutation or deletion of the p53 and pRb genes. It is also likely that a local HPV16 infection may not produce a widespread field of cells with altered p53 and pRb functions. HPV16-infected subjects then would be less likely to develop recurrences than the HPV-negative group where carcinogens have produced wide fields of mutant progenitors. Finally, if the E6 and E7 proteins were only partially or temporarily blocking p53 tumor suppression, one might expect a better response to treatment and clinical outcome in the HPV16-positive individuals.

In our study, 82% of the HPV16-positive tumors occurred in men. Previous researchers have found either no association between HPV presence and gender (22, 33) or a higher percentage in males (37, 39). However, males are three times more likely to develop oral and oropharyngeal cancer than females. Premoli-De-Percoco *et al.* (43) studied women with oral squamous cell carcinoma and detected a high percentage of HPV-positive specimens (70%). Of interest, in 23 of the HPV-positive cases, the same HPV type was observed in the patient's cervical mucosa. Small sample size and fewer numbers of female subjects may limit our study and previous reports' ability to detect gender differences in HPV infection.

In our study, we found that HPV16 presence was not significantly associated with clinical stage, tumor size, or nodal status. Previous studies have similarly reported clinical stage and TNM status not to be associated with HPV presence (38, 44). However, most series do not include large proportions of early stage tumors, yielding little power to detect a small association of stage or TNM status with HPV infection. Our series, with >81% of the tumors classified as either stage III or stage IV disease, also includes predominantly large tumors. Hence,

we cannot exclude a small association of clinical stage or TNM status with HPV presence.

In summary, our study demonstrated a HPV16 detection rate of 20% in 89 HNSCC patients. Oropharyngeal site and a younger patient age were strongly associated with HPV16 presence. Also, lighter alcohol consumption was significantly associated with HPV16 infection. Patients who had HPV16-positive tumors had a better overall and disease-specific survival compared with the HPV-negative group. Although no means conclusive, our findings suggest a distinct pattern of head and neck cancer disease in patients with HPV16 infection.

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