Evidence for a Causal Association Between Human Papillomavirus and a Subset of Head and Neck Cancers

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Background: **High-risk human papillomaviruses (HPVs) are etiologic agents for anogenital tract cancers and have been detected in head and neck squamous cell carcinomas (HNSCCs). We investigated, retrospectively, an etiologic role for HPVs in a large series of patients with HNSCC.** ***Methods:*** **Tumor tissues from 253 patients with newly diagnosed or recurrent HNSCC were tested for the presence of HPV genome by use of polymerase chain reaction (PCR)-based assays, Southern blot hybridization, and *in situ* hybridization. The viral E6 coding region was sequenced to confirm the presence of tumor-specific viral isolates. Exons 5–9 of the TP53 gene were sequenced from 166 specimens. The hazard of death from HNSCC in patients with and without HPV-positive tumors was determined by proportional hazards regression analysis.** ***Results:*** **HPV was detected in 62 (25%) of 253 cases (95% confidence interval [CI] = 19%–30%). High-risk, tumorigenic type HPV16 was identified in 90% of the HPV-positive tumors. HPV16 was localized specifically by *in situ* hybridization within the nuclei of cancer cells in preinvasive, invasive, and lymph node disease. Southern blot hybridization patterns were consistent with viral integration. Poor tumor grade (odds ratio [OR] = 2.4; 95% CI = 1.2– 4.9) and oropharyngeal site (OR = 6.2; 95% CI = 3.1–12.1) independently increased the probability of HPV presence. As compared with HPV-negative oropharyngeal cancers, HPV-positive oropharyngeal cancers were less likely to occur among moderate to heavy drinkers (OR = 0.17; 95% CI = 0.05–0.61) and smokers (OR = 0.16; 95% CI = 0.02–1.4), had a characteristic basaloid morphology (OR = 18.7; 95% CI = 2.1–167), were less likely to have TP53 mutations (OR = 0.06; 95% CI = 0.01–0.36), and had improved disease-specific survival (hazard ratio [HR] = 0.26; 95% CI = 0.07–0.98). After adjustment for the presence of lymph node disease (HR = 2.3; 95% CI = 1.4– 3.8), heavy alcohol consumption (HR = 2.6; 95% CI = 1.4–4.7), and age greater than 60 years old (HR = 1.4; 95% CI = 0.8–2.3), all patients with HPV-positive tumors had a 59% reduction in risk of death from cancer when compared with HPV-negative HNSCC patients (HR = 0.41; 95% CI = 0.20–0.88).** ***Conclusions:*** **These data extend recent molecular and epidemiologic studies and strongly suggest that HPV-positive oropharyngeal cancers comprise a distinct molecular, clinical, and pathologic disease entity that is likely causally associated with HPV infection and that has a markedly improved prognosis. [J Natl Cancer Inst 2000; 92:709–20]**

Head and neck squamous cell carcinoma (HNSCC) is a disease largely attributed to environmental exposures. Tobacco use and alcohol consumption are well-established risk factors *(1,2).* However, a small proportion (15%–20%) of HNSCCs occurs in nonsmokers and nondrinkers, suggesting the presence of other risk factors. Recent epidemiologic and molecular data suggest that human papillomavirus (HPV) infection of the upper airway may promote head and neck tumorigenesis *(3,4).*

High-risk HPVs (e.g., HPV16 and HPV18) are known to be tumorigenic in human epithelial tissues. These viruses are a necessary but insufficient cause of cervical squamous cell carcinoma and have been implicated in the development of other anogenital squamous cell cancers. Two viral oncoproteins of high-risk HPVs, E6 and E7, promote tumor progression by inactivating the TP53 gene (also known as p53) and retinoblastoma tumor suppressor gene products, respectively *(5–8).* Accordingly, these viral oncoproteins are capable of transforming primary human keratinocytes from either genital or upper respiratory tract epithelia *(9)* and disrupting cell-cycle regulatory pathways in the genetic progression of HNSCC *(10,11).* The TP53 gene is mutated in approximately 45% of HNSCC *(11)* and, although pRb mutations are rare, expression of upstream regulators of pRb function, such as TP16 *(12)* and cyclin D *(11,13),* is commonly altered in HNSCC. Therefore, HPV infection may represent an alternative, but functionally comparable, molecular pathway for HNSCC tumorigenesis.

Mucosal HPVs are known to infect the upper respiratory tract *(4).* Low-risk HPV6 and HPV11 cause both benign genital condylomata and respiratory papillomas *(14).* HPV genomic sequences have also been identified in HNSCC, but markedly varied estimates of viral prevalence (range, 8%–100%) *(15)* have impeded clarification of the relationship between HPV presence and head and neck cancer development. Despite this variability, studies *(16,17)* have suggested an association of HPVs with cancers in the oropharynx and, especially, with tonsillar carcinomas *(18–20).*

In a recent case–control study *(21),* HPV presence in the oral cavity was associated with increased risk of oral cavity or oropharyngeal cancer (odds ratio [OR] = 3.7; 95% confidence interval [CI] = 1.5–9.3), independent of alcohol and tobacco exposure. Men with cancers at these sites also had sexual risk

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See "Notes" following "References."

factors similar to those for women with cervical cancer and known to be associated with HPV exposure *(22)*. These include young age at first intercourse, a history of multiple sexual partners, and a history of genital warts *(22)*. Seropositivity to HPV16 capsid protein was significantly greater in oral cancer patients than in control subjects, providing further evidence of prior HPV16 exposure in cancer cases *(22)*.

We sought to clarify the role that HPV plays in HNSCC development by performing a detailed virologic analysis of 253 fresh-frozen HNSCC tumor specimens. In addition, patient risk factor profiles, clinical and pathologic data, and tumor p53 status were used to establish epidemiologic, pathologic, and molecular correlates of HPV positivity.

Subjects and Methods

Subjects

Patients with histologically confirmed, newly diagnosed, or recurrent HNSCC were studied. All patients participated in research protocols in the Division of Head and Neck Cancer Research approved by the Institutional Review Board of The Johns Hopkins Hospital, Baltimore, MD, and affiliated institutions and gave written informed consent for banking of tumor tissue. Demographic data, including age at diagnosis, sex, race, and alcohol and tobacco exposures, were obtained from medical records. Tumor site, stage, and grade were determined from review of operative, radiology, and pathology reports. Tumor stage was assigned on the basis of best available staging—clinical or pathologic—by use of the 1997 American Joint Committee on Cancer (AJCC) staging criteria *(23)*. The patient's average weekly consumption of alcohol at diagnosis was recorded in whiskey equivalents consumed per week (one whiskey equivalent = 10 g of alcohol ≈ 1 oz of 86-proof liquor ≈ one 3.6-oz glass of wine containing 12% alcohol ≈ one beer) *(2)*. Patients were classified as light or nondrinkers (<10 whiskey equivalents per week), moderate drinkers (10–20 whiskey equivalents per week), or heavy drinkers (>20 whiskey equivalents per week). Patients were also classified as never, current, or former (former smokers are those who have quit for >12 months before diagnosis) daily tobacco smokers (cigarette, pipe, and/or cigar). All patients with newly diagnosed or recurrent disease were followed prospectively from the date of original diagnosis by the head and neck surgeon. The date and clinical status of patients were recorded at each follow-up appointment (median of every 3 months from diagnosis). Primary treatment modality (radiation therapy, chemoradiation therapy, surgery, etc.) as well as the date and cause of death were documented.

Tumor Specimens

Fresh tumor specimens were frozen within 30 minutes of resection and stored at −80 °C until processing. Microdissection was used to ensure that more than 70% of isolated DNA was from tumor tissue. DNA was purified from samples as described *(24)*. DNA purification and HPV-detection assays were performed in separate laboratories to reduce contaminations.

Review of hematoxylin–eosin-stained slides of available tumor specimens was performed independently by two pathologists (W. H. Westra and M. Viglione) who did not know the results of the HPV testing, and differences were resolved by joint review. Tumor grade was recorded as well, moderate, or poor according to the criteria of the World Health Organization *(25)*. Tumors were further characterized according to the absence or presence of "basaloid" features as defined by small, dark, crowded cells with scant cytoplasm, hyperchromatic nuclei, marked mitotic activity, a predominant lobular pattern of growth, and the absence of prominent keratinization *(26)*.

Cell Lines

SiHa (HPV16-positive), CaSki (HPV16-positive), HeLa (HPV18-positive), C 4II (HPV18-positive), C-33A (HPV-negative), and K562 (HPV-negative) cell lines were used as controls in polymerase chain reaction (PCR), Southern blot, and *in situ* hybridization assays *(27–30)*. High-molecular-weight genomic DNA was isolated from cell lines for Southern blot analysis *(31)*, and cell blocks were prepared from control cell lines for *in situ* hybridization as described previously *(32)*.

Consensus L1 PCR

HPV genomic sequences were detected by PCR amplification by use of consensus degenerate primers (MY09/MY11/HMB01) complementary to the conserved L1 region of HPV as described *(33–35)*. Amplification of a β-globin gene fragment was performed by use of PCO4 and GH20 primers *(34)* to control for target DNA integrity. β-Globin-negative samples were excluded from further analysis. PCR products dot blotted onto Biotrans nylon membranes (ICN Pharmaceuticals, Inc., Costa Mesa, CA) were hybridized with biotin end-labeled oligonucleotide probes for β-globin. A "generic" mixture of HPV probes (HPV16, HPV18, HPV51, and HPV66) and 33 HPV type-specific probes (synthesized at DNA Synthesis Core Facility, The Johns Hopkins School of Hygiene and Public Health, Baltimore, MD) were used for the identification of HPV type. Hybridization signals were detected by use of a chemiluminescence system on Hyperfilm ECL (Amersham Life Science Inc., Arlington Heights, IL).

To ensure the specificity of results, PCR products from all specimens positive for HPV in the dot-blot format were confirmed by Southern blot hybridization. Specimens with amplification products of the expected size were considered to be positive. Specimens positive for HPV by generic probe on Southern blot analysis that could not be further type specified underwent further analysis. PCR products from the L1 region amplification were cloned by use of the TA cloning kit from Invitrogen Corp. (Carlsbad, CA) and sequenced by use of the fluorescent dideoxy terminator method of cycle sequencing following ABD protocols (The Perkin-Elmer Corp., Applied Biosystems Division [ABD], Foster City, CA) *(36)*. A BLAST search was performed to assign L1 sequences to known HPV types.

E7 Type-Specific PCR

Because rare false-negative L1 region amplifications could occur as a result of integration events in that region, all tumor DNAs were also tested for HPV16 and HPV18 by amplification of the viral E7 region by use of type-specific primers. Tumors positive for HPV33 or HPV31 by the L1 primers were confirmed by PCR amplification with type-specific primers for the E7 region as described *(37)* with the following modifications: a 5′ oligonucleotide primer for HPV16 amplification, HPV16 E7.642, 5′-ATTAAATGACAGCTCAGAGGA-3′, was substituted for the HPV16 E7.671 primer described; all reactions contained 3 m*M* $MgCl_2$; and the annealing temperature for the HPV33 type-specific amplifications was reduced to 45 °C. Amplification was followed by dot-blot hybridization *(37)* and confirmed by Southern blot hybridization.

E6 Open Reading Frame Sequencing

Tumor specimens positive for HPV16 were further subclassified into HPV16 variants by amplification and sequencing of the HPV16 E6 coding region (nucleotides 104–559) by use of nested PCR *(38)*. The PCR product was purified for direct sequencing with the use of the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). DNA templates were sequenced by use of the fluorescent dideoxy terminator method of cycle sequencing *(36)*. Coding sequences, including all identified base-pair (bp) substitutions, were confirmed by sequencing both strands of DNA and by repeat amplification and sequencing. Analysis was performed by use of Sequencher Software 3.1 (Gene Codes Corp., Ann Arbor, MI). HPV16 nucleotide variations were compared with reference HPV DNA sequence *(38)*. Placement of HPV16 variants into the major phylogenetic groups was performed by inspection *(39)*.

Southern Blot Hybridization

Tumors positive for HPV16 by PCR, for which at least 2–5 μg of tumor DNA were available, were examined for HPV16 sequences by Southern blot hybridization of unamplified tumor DNAs. Full-length HPV16 genomic DNA (7905 bp) in pGEM II (Promega Corp., Madison, WI) was gel purified and labeled to a high specific activity with [α-^{32}P]deoxycytidine triphosphate by the random primer method (Random Primers DNA Labeling System; Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD). Nonamplified tumor DNA (2–5 μg) was digested with *Pst*I (New England Biolabs, Inc., Beverly, MA), ethanol precipitated, and separated on a 1.25% agarose gel. The DNA was denatured and transferred to a nylon membrane (GeneScreen Plus; Du Pont NEN, Boston, MA). After hybridization to radiolabeled HPV16 probe, the membrane was exposed to film (Kodak, Rochester, NY) for 24–72 hours at −70 °C.

In Situ Hybridization

In situ hybridization was performed by use of the catalyzed reporter deposition system for the detection of HPV16 DNA in formalin-fixed, paraffin-embedded tissues also prepared at the time of resection as described *(32)*.

TP53 Sequencing

A 1.8-kilobase TP53 gene fragment, encompassing exons 5–9, was amplified from purified tumor DNA by PCR *(40)* and sequenced directly *(41)*. This region was chosen because somatic mutations outside this DNA-binding region are rare in human malignancies *(42,43)*. Mutations were confirmed by repeated amplification and sequencing of the tumor DNA.

Statistical Analysis

Factors associated with HPV status were selected on cross-tabulations and logistic regression modeling. Cross-tabulations were analyzed by use of the chi-square test or Fisher's exact test, where appropriate. A logistic regression model was used to determine the effects of multiple factors on HPV status. Results are summarized as ORs and corresponding 95% CIs.

In survival analysis, the primary statistical end points were overall survival (death from all causes) and disease-specific survival (death from HNSCC). Event time distributions for these end points were estimated by use of the method of Kaplan and Meier *(44)* and compared by use of the log-rank statistic *(45)* or the proportional hazards regression model *(46)*. The assumption of proportional hazards for primary and recurrent cancers was not appropriate; therefore, survival models were stratified for this factor. The simultaneous effect of two or more factors was studied by use of stratified multivariate proportional hazards models. Factors tested for prognostic value included sex, age at diagnosis, race, tobacco and alcohol exposures, tumor stage, lymph node status, HPV presence, tumor location, and tumor grade. In the subset of patients for whom TP53 sequence data were available (n = 166), presence of a mutation in the TP53 gene was also evaluated as a prognostic factor for survival. Estimates of relative risk are presented as hazard ratios (HRs) and corresponding 95% CIs. All *P* values reported are two-sided. Computations were performed by use of STATA statistical software (StataCorp, College Station, TX), and Kaplan–Meier curves were created in SAS *(47)*.

RESULTS

Demographics of the Study Population

The study population consisted of 259 patients with a histologically confirmed diagnosis of HNSCC from June 1987 through October 1998. Six of the 259 patients were excluded from further analysis because β-globin DNA could not be amplified from purified tumor DNA. The majority of tumor specimens (from 200 patients) were obtained from the primary tumor at diagnosis. The remaining 53 tumor specimens were obtained by biopsy of recurrent, local disease.

The characteristics of the study population (n = 253) largely reflect the demographics of head and neck cancer patients in the United States (Table 1). Patient ages ranged from 17 to 91 years (median, 63 years; interquartile range, 54–71 years). The primary tumor was located in the nasopharynx (n = 2), oral cavity (n = 84), oropharynx (n = 60), hypopharynx (n = 21), or larynx (n = 86). Seventy percent of the patients (177 of 253) presented with locally advanced, stage III or IV disease. With regard to combined alcohol and tobacco exposures, the study population consisted of 33 (13%) nonsmokers/light or nondrinkers, 100 (39%) current smokers/light or nondrinkers, 64 (25%) current smokers/moderate–heavy drinkers, 40 (16%) former smokers/light or nondrinkers, and nine (4%) former smokers/moderate–heavy drinkers. Exposure information was insufficient to classify seven (3%) patients. No patients in this study were nonsmokers/moderate–heavy drinkers.

Prevalence and Identification of HPV Type in HNSCC

HPV genomic DNA was detected in 55 (22%) of 253 tumor specimens by use of the MY09/MY11/HMBO1 primers to amplify the L1 region of the viral genome. HPV type was identified as HPV16 in 49 tumors, HPV33 in three tumors, and one tumor was positive for HPV16 and HPV31. Two additional tumors positive for HPV by the generic probe could not be type specified. After cloning and sequencing of amplified DNA, one of these two tumors was identified as HPV11. The other tumor was not successfully cloned but was considered to be positive for analysis because the amplified DNA was of the appropriate size on Southern blot when hybridized to the generic probe.

With the use of type-specific primers for the E7 region, seven additional tumors were positive for HPV. Six of seven were positive for HPV16, and one was positive for HPV18 (an oral cavity tumor). All four specimens positive for HPV33 and HPV31 on amplification of the L1 region were confirmed by use of type-specific primers. Therefore, with the use of the combination of the L1 and E7 region primers, 62 (25%) of 253 (95% CI = 19%–30%) tumor specimens were positive for HPV genomic DNA. HPV16 accounted for the majority of the HPVs identified, 90% (56 of 62). Five of six of the remaining viral isolates were high-risk types: HPV31, 2% (one of 62, also HPV16 positive); HPV33, 5% (three of 62); and HPV18, 2% (one of 62). One (2%) of the 62 cases (a laryngeal tumor) remained untyped. HPV11, a low-risk HPV type, was identified by cloning as described above from one oropharyngeal tumor. The HPV positivity at different anatomic sites was as follows: oral cavity, 12% (10 of 84 specimens); oropharynx, 57% (34 of 60); hypopharynx, 10% (two of 21); larynx, 19% (16 of 86); and nasopharynx (none of two).

HPV16 Variant Sequence Analysis

To confirm that the different HPV16 isolates were independent and tumor specific, the E6 open reading frame of HPV16-positive tumors was sequenced. DNA sequencing of the E6 region allows identification of unique viral variants and categorization of these variants within one of six major phylogenetic groups *(38)*. Sequence data were attainable for 52 (93%) of 56 HPV16-positive tumors because DNA from the other four samples had been exhausted. Observed sequence variations were compared with a reference sequence of a European prototype, HPV16 "E-P-350T" (Table 2) *(38)*. Seventeen distinct HPV16 variants were identified in the 52 isolates sequenced; of these, seven were novel variants not previously reported (E-G315T, E-G315G, E-C395G, E-A478T, E-A132T, Af1-C311, and Af1-A389). The majority of isolates could be classified into the same phylogenetic group as the European prototype, 75% (39 of 52) (Table 2). Asian (17% [nine of 52]), North American (4.0% [two of 52]), and African 1 (4.0% [two of 52]) variants were also identified.

Base-pair substitutions occurred overall at 18 (4%) of 455 nucleotide positions in the E6 coding region. The maximum number of base-pair substitutions in a single variant was seven (1.5%) of 455 bp when compared with the reference sequence. Fifteen of the 18-bp substitutions resulted in amino acid substitutions. Base substitutions were confirmed by repeat amplification and sequencing to rule out mutations resulting from PCR artifact.

Table 1. Characteristics of the head and neck cancer study population grouped by HPV status*

Characteristic	Total (n = 253)		HPV-positive group (n = 62)		HPV-negative group (n = 191)		Unadjusted OR
	No.	%	No.	%	No.	%	OR (95% CI)
Univariate analysis							
Sex							
Men†	188	74	49	79	139	73	1.0 (referent)
Women	65	26	13	21	52	27	0.71 (0.36–1.4)
Age at diagnosis, y							
≤60†	108	43	31	50	77	40	1.0 (referent)
>60	145	57	31	50	114	60	0.67 (0.38–1.2)
Race							
Caucasian/Hispanic†	202	80	52	84	150	79	1.0 (referent)
African-American	48	19	10	16	38	20	0.76 (0.35–1.6)
Other	3	1	0	—	3	1	—
Tobacco exposure							
Nonsmoker†	33	13	9	15	24	13	1.0 (referent)
Current smoker	167	66	39	63	128	67	0.81 (0.35–1.9)
Former smoker	51	20	14	23	37	19	1.0 (0.38–2.7)
Unknown	2	1	—	—	2	1	—
Alcohol intake, we/wk‡							
0†	94	37	29	47	65	34	1.0 (referent)
1–10	79	31	16	26	63	33	0.57 (0.28–1.1)
10–20	39	15	10	15	29	15	0.78 (0.33–1.8)
>20	34	13	6	10	28	15	0.48 (0.18–1.2)
Unknown	7	3	1	2	6	3	—
Primary							
Oral cavity†	84	33	10	16	74	39	1.0 (referent)
Oropharynx	60	24	34	55	26	14	9.7 (4.2–22)
Hypopharynx	21	8	2	3	19	10	0.78 (0.16–3.8)
Larynx	86	34	16	26	70	37	1.7 (0.72–4.0)
Nasopharynx	2	1	0	—	2	1	—
Lymph node status							
Negative†	129	51	29	47	100	52	1.0 (referent)
Positive	124	49	33	53	91	48	1.3 (0.70–2.2)
Tumor grade							
Well†	41	16	8	13	33	17	1.0 (referent)
Moderate	141	56	26	42	115	60	0.93 (0.39–2.3)
Poor	59	23	27	44	32	17	3.5 (1.4–8.8)
Unknown	12	5	1	2	11	6	—
Basaloid morphology							
Nonbasaloid†	131	52	25	40	106	55	1.0 (referent)
Basaloid	17	7	14	23	3	2	19.8 (5.3–74)
Not available	105	42	23	37	82	43	—
TP53							
Wild-type†	101	40	26	42	75	39	1.0 (referent)
Mutant	65	26	11	18	54	28	0.59 (0.27–1.3)
Not done	86	34	24	39	62	32	—
Primary therapy							
Surgery + radiation therapy†	123	49	33	53	90	47	1.0 (referent)
Surgery alone	42	17	6	10	36	19	0.45 (0.18–1.1)
Radiation therapy alone	62	25	11	18	51	27	0.59 (0.27–1.3)
Chemoradiation therapy	19	8	8	13	11	6	1.9 (0.74–5.4)
None	2	1	1	1	1	1	—
Unknown	5	2	3	5	2	1	—

	Adjusted OR§	95% CI	*P*
Multivariate analysis			
Oropharyngeal primary	6.2	3.1–12.1	<.001
Poor tumor grade	2.4	1.2–4.9	.01

*HPV = human papillomavirus; OR = odds ratio; CI = confidence interval.

†Reference group for OR calculation. For exposure categories, the unexposed were chosen as the reference group. For ordinal variables (e.g., age and grade), the first category was chosen as the reference group. For other characteristics, the category with the largest number was chosen as the reference group.

‡we/wk = whiskey equivalents per week.

§OR adjusted for other variables in the model (as listed in the table).

Table 2. Variant analysis of 52 human papillomavirus (HPV) type 16-positive head and neck tumors

	Sequence of HPV16 E6 open reading frame (ORF) (base pairs 104–559)																		
Nucleotide position*	109	131	132	143	445	478	267	395	492	111	315	335	350	389	395	406	470	478	
European prototype† (E-P-350T)	T	A	G	C	G	T	G	T	A	T	C	C	T	T	G	C	C	G	
Variant identified																			No.
E-P-350T‡	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	12
E-G131T§	—	G	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3
E-A132T	—	—	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
E-G315T	—	—	—	—	—	—	—	—	—	—	G	—	—	—	—	—	—	—	1
E-A470T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	A	—	—	1
E-A478T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	T	—	3
E-P-350G	—	—	—	—	—	—	—	—	—	—	—	—	G	—	—	—	—	—	6
E-C109G	C	—	—	—	—	—	—	—	—	—	—	—	G	—	—	—	—	—	2
E-G131G	—	G	—	—	—	—	—	—	—	—	—	—	G	—	—	—	—	—	3
E-A267G	—	—	—	—	—	—	A	—	—	—	—	—	G	—	—	—	—	—	2
E-G315G	—	—	—	—	—	—	—	—	—	—	G	—	G	—	—	—	—	—	4
E-C395G	—	—	—	—	—	—	—	—	—	—	—	—	G	—	C	—	—	—	1
As	—	—	—	—	—	G	—	—	—	—	—	—	—	—	—	—	—	—	7
As-A492	—	—	—	—	—	G	—	—	—	—	—	—	—	—	—	—	—	A	2
NA1	—	—	—	—	T	—	—	A	G	—	—	T	G	—	—	—	—	—	2
Af1-C311	—	—	C	G	T	—	—	A	G	C	—	T	—	—	—	—	—	—	1
Af1-A389	—	—	C	G	T	—	—	A	G	—	—	T	—	A	—	—	—	—	1
Total																			52

*Nucleotide positions of the E6 ORF are displayed vertically, e.g., 109, 131, and so forth.

†Nucleotide sequence of the European prototype E-P-350T *(38)* is displayed.

‡Major phylogenetic groups of HPV16 identified include European (E), Asian (As), North American 1 (Na1), and African 1 (Af1).

§Variants within phylogenetic groups are denoted by HPV16 E6 nucleotide position and the substituted nucleotide. For example, E-G131T denotes European variant with guanine at nucleotide position 131. Additional final letter denotes nucleotide at position 350 in European variants. T = thymine, A = adenine, G = guanine, and C = cytosine.

HPV16 Identification by Southern Blot and *In Situ* Hybridization

The presence of HPV16 in tumors found by PCR amplification was further examined by use of two highly specific methods, Southern blot analysis of unamplified tumor DNA with an HPV16 probe and *in situ* hybridization for HPV16 sequences on paraffin-imbedded tumor samples. We verified that a single copy of integrated HPV16 could be detected by Southern blot, with an appropriate band pattern change, using 10 μg of genomic DNA isolated from SiHa cells, known to contain a single copy of integrated virus (data not shown). Fifty-seven percent (12 of 21) of HPV16 PCR-positive (12 of 18 in oropharyngeal and none of three in nonoropharyngeal) tumors tested were positive for HPV16 by Southern blot by the use of 2–5 μg of unamplified tumor DNA (Fig. 1). Banding patterns observed on Southern blot hybridization may help distinguish episomal and integrated forms of the virus. Because HPV16 DNA has six *Pst*I restriction sites, episomal virus has six distinct bands, and viral integration alters this pattern. Banding patterns consistent with episomal (lane 12), both episomal and integrated (lane 7), and integrated (lane 4) forms of the virus were seen (Fig. 1). These results do not exclude the possibility that altered banding patterns were due to unusual multimeric episomal forms with minor rearrangements or deletions *(48)*. Formal confirmation of viral integration in these tumors would require digestion with other informative enzymes (*Hin*dIII and *Bam*HI), two-dimensional gel electrophoresis, and/or cloning of cell genome–viral junctions *(49)*. These procedures could not be performed because of limited quantities of tumor DNA.

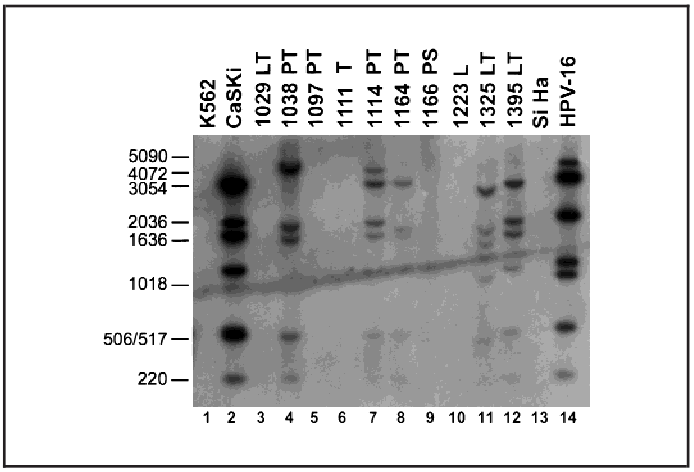

Fig. 1. Southern blot of DNA isolated from human papillomavirus (HPV) type 16 polymerase chain reaction-positive head and neck squamous cell carcinoma (HNSCC) specimens digested with *Pst*I and hybridized to HPV16 probe. **Far left lane** indicates standard 1-kilobase molecular weight markers in base pairs (Life Technologies, Inc.). **Lane 1** = HPV16-negative control (K562 cell line), **lane 2** = HPV16-positive control (CaSki cell line), **lanes 3–12** = contain 2 μg of DNA (**lanes 3 and 10**) or 5 μg (**all others**) of HNSCC specimen DNA, **lane 13** = sensitivity control (10 μg of purified DNA from SiHa cell line), and **lane 14** = specificity control (pGEM HPV16). Tumor samples are labeled by a unique sample identification number followed by location of primary tumor: base of tongue (LT), palatine tonsil (PT), anterior tongue (T), pyriform sinus (PS), and larynx (L). Prototype pattern of episomal HPV16 is seen in lanes 2, 8, 11, and 12. Patterns consistent with integrated (lane 4) and both integrated and episomal (lane 7) HPV16 were also observed. In pGem HPV16 (lane 14), HPV16 is cloned into the *Bam*HI site; therefore, banding pattern is altered from that expected when episomal HPV16 is cut with *Pst*I. Faint bands are detectable in the SiHa lane (lane 13).

In situ hybridization was performed on paraffin sections of available HPV16 PCR-positive tumors to identify the location of the virus. As shown in Fig. 2, an HPV DNA hybridization signal was specifically located within tumor cell nuclei of preinvasive (panel A) and invasive (panel B) squamous cell carcinomas, as well as in the nuclei of tumor implants in regional lymph nodes (panel C). A hybridization signal was never present in the surrounding stroma or in the nondysplastic surface epithelium.

Analysis of Clinical and Pathologic Factors Related to the Presence of HPV in Tumors

There were significant associations between HPV presence and both location and grade of the primary tumor (Table 1). Oropharyngeal tumors were significantly more likely to be HPV positive (34 [57%] of 60; OR = 9.7; 95% CI = 4.2–22) than oral cavity tumors (10 [12%] of 84) or all tumors arising from nonoropharyngeal sites (28 [14%] of 193; OR = 7.7; 95% CI = 4.0–15). HPV-positive oropharyngeal tumors arose predominantly from the palatine or lingual tonsils (32 [94%] of 34): HPV positivity was 62% (32 of 52) for tumors on the tonsil/base of tongue and 25% (two of eight) for tumors at other oropharyngeal sites. The association of HPV presence with tonsillar location was very strong (OR = 9.1; 95% CI = 4.6–18) when compared with all nontonsillar tumors. Poorly differentiated tumors were more likely to be HPV positive (OR = 3.5; 95% CI = 1.4–8.8) than well-differentiated and moderately differentiated tumors (Table 1). For the subset of cases reviewed for basaloid characteristics (n = 148) (*see* "Subjects and Methods" section), HPV-positive tumors were significantly more likely to have a characteristic basaloid morphology (OR = 19.8; 95% CI = 5.3–74) than HPV-negative tumors (Table 1).

The median age of patients with HPV-negative tumors was 64 years and was not significantly different from that of patients with HPV-positive tumors, 60.5 years (Wilcoxon rank sum; $P = .42$). There was a trend for patients with HPV-positive tumors to be nondrinkers or light drinkers, but this difference was not statistically significant (Table 1). Other factors unrelated to HPV status on univariate logistic regression analysis included sex, race, presence of lymph node disease, primary therapy received, and tobacco exposure (Table 1). Neither the tumor specimen tested (primary or recurrent) nor the AJCC stage was associated with the presence of HPV (data not shown).

Direct sequencing of exons 5–9 of the TP53 gene has been completed for an unselected subset (166 [66%] of 253) of tumors in this study. TP53 mutations were identified in 39% (95% CI = 32%–47%) of sequenced tumors. HPV-positive tumors appeared less likely to harbor a TP53 mutation, but this difference was not statistically significant (Table 1). Although specimens taken from the primary tumor were more likely to have undergone TP53 sequencing than recurrent tumors (chi-squared$_{(1\ df)}$; $P = .04$), the subset of patients with TP53 sequence data did not differ significantly from those without with regard to HPV status, location of the primary tumor, sex, race, stage, lymph node status, age, smoking status, or alcohol consumption (chi-squared$_{(1\ df)}$; $P > .10$).

A logistic regression model of factors related to the presence of HPV in HNSCC was constructed. Both poor tumor grade (OR = 2.4; 95% CI = 1.2–4.9) and oropharyngeal tumor site (OR = 6.2; 95% CI = 3.1–12.1) independently increased the probability of HPV presence (Table 1). The magnitude and direction of these associations were not altered appreciably by the addition of other nonsignificant factors to the model, including age, race, sex, tumor site, tumor grade, lymph node status, and alcohol or tobacco exposure.

Because the majority of HPV-positive tumors arose from the oropharynx, we compared risk factors for HPV positivity for HNSCC located in the oropharynx (n = 60) versus other sites (n = 193) (Table 3). Regardless of location, there were no differ-

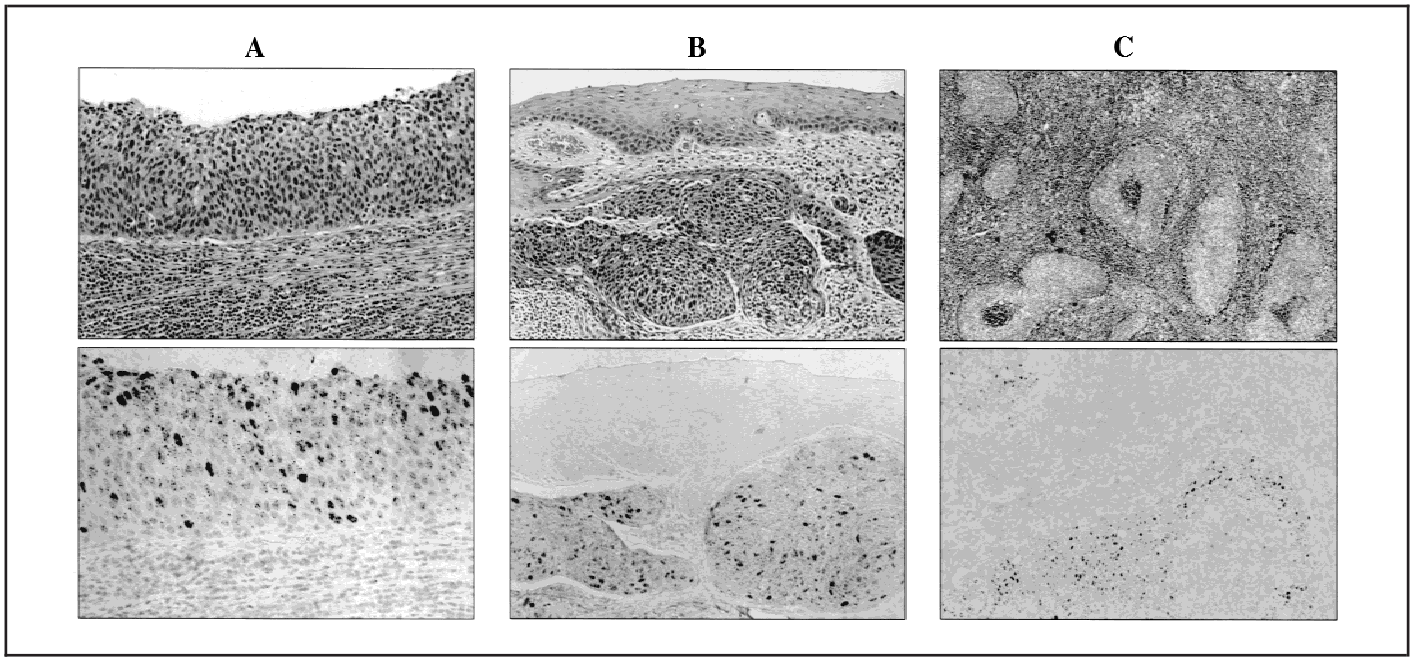

Fig. 2. Human papillomavirus (HPV) type 16 *in situ* hybridization of three head and neck squamous cell carcinoma specimens. **Panel A**—*in situ* and microinvasive disease. **Panel B**—invasive disease. **Panel C**—lymph node disease. **Top**—hematoxylin–eosin-stained slides. In B and C, nests of squamous cell carcinoma with prominent basaloid features infiltrate the submucosa and lymph node tissue, respectively. **Bottom**—HPV16 *in situ* hybridization. The HPV16 hybridization signal is strictly confined to the tumor cells; it is not seen in the surrounding stroma, in lymphoid tissue, or in the non-neoplastic surface epithelium.

Table 3. Univariate analysis of patient/tumor characteristics and HPV presence stratified by oropharyngeal and nonoropharyngeal tumor site*

Characteristic	Oropharynx (n = 60)			Nonoropharynx (n = 193)		
	HPV status			HPV status		
	No. positive (n = 34)	No. negative (n = 26)	OR (95% CI)	No. positive (n = 28)	No. negative (n = 165)	OR (95% CI)
Tobacco exposure						
Nonsmoker†	7	1	1	2	22	1
Smoker (current + former)	27	24	0.16 (0.02–1.4)	26	142	2.0 (0.45–9.1)
Unknown	—	1	—		1	
Alcohol intake, we/wk‡						
<10†	29	11	1	16	117	1
⩾10	5	11	0.17 (0.05–0.61)	11	46	1.7 (0.75–4.0)
Unknown	—	4		1	2	
Lymph node status						
Negative†	9	7	1	20	93	1
Positive	25	19	1.0 (0.32–3.2)	8	72	0.5 (0.22–1.2)
Tumor grade						
Well/moderate†	14	18	1	20	130	1
Poor	20	8	3.2 (1.1–9.4)	7	24	1.9 (0.72–5.0)
Unknown	—	—		1	11	
Basaloid morphology						
Not present†	10	17	1	15	89	1
Present	11	1	18.7 (2.1–167)	3	2	8.9 (1.4–58)
Not available	13	8		10	74	
TP53						
Wild-type†	17	5	1	9	70	1
Mutant	2	10	0.06 (0.01–0.36)	9	44	1.6 (0.59–4.3)
Not done	15	11		10	51	
Southern/*in situ*, positive	17/24	—		1/13	—	
HR cancer death§ (95% CI)	0.26 (0.07–0.98)	1		0.62 (0.26–1.5)	1	

*HPV = human papillomavirus; OR = odds ratio; CI = confidence interval.

†Reference group for OR calculation.

‡we/wk = whiskey equivalents per week; one whiskey equivalent is defined as 10 g of alcohol.

§HR = hazard ratio as estimate of relative risk of death from head and neck cancer in univariate proportional hazards modeling.

ences between HPV-positive and HPV-negative tumors with respect to sex, age at diagnosis, race, and lymph node status. However, in the oropharynx, poor tumor grade, basaloid morphology, and wild-type TP53 increased the probability of HPV positivity, while moderate to heavy alcohol intake decreased the probability of HPV positivity (Table 3). In the oropharyngeal subset, TP53 mutations were inversely associated with HPV; 67% of HPV-negative but only 10% of HPV-positive tumors harbored TP53 mutations (Fisher's exact test; $P = .001$) (Table 3). Nonsmokers were more frequent in the HPV-positive group (seven [21%] of 34) than in the HPV-negative oropharynx group (one [4%] of 25), but this difference was not statistically significant. By contrast, at nonoropharyngeal sites, only basaloid morphology significantly increased the probability of HPV positivity (Table 3).

The virologic characteristics of HPV–PCR-positive tissues were also different in the two locations. Seventy-one percent (17 of 24) of the PCR-positive oropharyngeal tumors tested by more stringent Southern blot and/or *in situ* hybridization assays were positive; by comparison, only one (one [8%] of 13) of the PCR-positive HNSCC at nonoropharyngeal sites (a retromolar trigone tumor positive by *in situ*) was positive by those corroborative assays (Fisher's exact test; $P<.001$).

Survival Analysis

To determine whether the presence of HPV in head and neck tumors had prognostic significance, we analyzed both overall survival and disease-specific survival. Survival data were available for 252 of the 253 patients in this study. One hundred deaths, including 71 from head and neck cancer and 29 from unrelated causes, have occurred in the study population. Patients were followed for a median of 31 months (range, 5 days to 241 months). The status of eight patients (3%) who transferred their care to another institution after being followed at Johns Hopkins Hospital (Baltimore, MD) for 7 weeks to 96 months (median, 16 months) was unknown. Therefore, these patients were considered lost to follow-up. The estimated median overall survival of the 252 patients was 85 months. Seventy percent of all patients survived for 2 years and 58% survived for 5 years. The estimated median survival of the HPV-negative group was 76 months, while that of the HPV-positive group was estimated to be greater than 91 months.

For the entire group of 252 subjects, patients with HPV-positive tumors had significantly improved overall survival when compared with patients with HPV-negative tumors (HR = 0.57; 95% CI = 0.34–1.0). Factors found to be predictive of poor overall survival by univariate proportional hazards regression analysis included presence of lymph node disease (HR = 1.8; 95% CI = 1.2–2.8) or advanced locoregional disease (stage 3 or 4 versus stage 1 or 2) at diagnosis (HR = 1.7; 95% CI = 1.1–2.7), age greater than 60 years (HR = 1.9; 95% CI = 1.2–2.9), and heavy weekly consumption of alcohol (HR = 2.1; 95% CI = 1.2–3.6) (Table 4). Sex, race, and tumor location were not prognostically significant. After adjustment for lymph

Table 4. Cox proportional hazards regression models for overall survival and disease-specific survival in head and neck cancer patients*

Characteristic	Univariate analysis			Multivariate analysis†		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
All patients (n = 252)‡						
Overall survival						
HPV-positive tumor	0.57	0.34–1.0	.04	0.6	0.35–1.0	.07
Lymph node-positive disease	1.8	1.2–2.8	.004	1.9	1.3–2.9	.002
Age >60 y	1.9	1.2–2.9	.005	1.8	1.2–2.8	.008
Heavy drinker	2.1	1.2–3.6	.007	1.9	1.1–3.2	.02
Disease-specific survival						
HPV-positive tumor	0.40	0.19–0.84	.015	0.41	0.20–0.88	.02
Lymph node-positive disease	2.2	1.3–3.7	.002	2.3	1.4–3.8	.001
Age >60 y	1.4	0.85–2.4	.18	1.4	0.82–2.3	.23
Heavy drinker	2.8	1.5–5.1	<.001	2.6	1.4–4.7	.002
Subset of patients with TP53 sequence completed (n = 166)§						
Overall survival						
HPV-positive tumor	0.68	0.36–1.3	.25	—	—	
Lymph node-positive disease	1.8	1.1–3.0	.016	1.8	1.1–3.0	.015
Age >60 y	2.7	1.5–4.8	.001	2.5	1.4–4.6	.002
Heavy drinker	2.3	1.2–4.2	.008	2.1	1.1–3.8	.022
Mutant p53	0.84	0.51–1.4	.49	—	—	
Disease-specific survival						
HPV-positive tumor	0.44	0.17–1.1	.08	0.46	0.18–1.2	.12
Lymph node-positive disease	2.2	1.2–4.0	.009	2.5	1.3–4.6	.004
Age >60 y	2.2	1.1–4.2	.024	1.9	0.98–3.8	.06
Heavy drinker	3.3	1.6–6.5	<.001	3.0	1.5–6.1	.002
Mutant p53	0.85	0.47–1.5	.60	0.70	0.38–1.3	.26

*The assumption of proportional hazards for primary and recurrent cancers was not appropriate; therefore, survival models were stratified for this factor. HPV = human papillomavirus; 95% CI = 95% confidence interval.

†Adjusted for other variables in the model presented in the table and stratified for primary or recurrent disease.

‡100 total deaths, 71 from HNSCC.

§70 total deaths, 50 from head and neck squamous cell carcinomas.

node status, age, and alcohol consumption, patients with HPV-positive tumors were estimated to have approximately a 40% reduction in risk of death from all causes (HR = 0.60; 95% CI = 0.35–1.0) when compared with patients with HPV-negative tumors (Table 4).

When the risk of death from cancer (disease-specific survival) was evaluated, both the presence of lymph node disease (HR = 2.2; 95% CI = 1.3–3.7) and heavy consumption of alcohol (HR = 2.8; 95% CI = 1.5–5.2) were associated with increased risk in univariate analysis (Table 4). Patients with HPV-positive tumors had a statistically significant 60% reduction in risk of death from cancer (HR = 0.40; 95% CI = 0.19–0.84) when compared with HPV-negative patients (Fig. 3). In multivariate analysis, patients with HPV-positive tumors were estimated to have a 59% reduction in risk of cancer death (HR = 0.41; 95% CI = 0.20–0.88) after adjustment for age, lymph node disease, and heavy alcohol consumption (Table 4).

Survival analysis was performed separately for patients with completed tumor TP53 sequencing data (n = 166) (Table 4). In multivariate analysis, lymph node disease (HR = 1.8; 95% CI = 1.1–3.0), age greater than 60 years (HR = 2.5; 95% CI = 1.4–4.6), and heavy alcohol consumption (HR = 2.1; 95% CI = 1.1–3.8) increased all-cause mortality; however, TP53 mutations and HPV presence did not significantly affect survival (Table 4). After adjustment for age, lymph node status, heavy alcohol consumption, and the presence of TP53 mutations, patients with HPV-positive HNSCC were estimated to have approximately half the risk of death from cancer (HR = 0.46; 95% CI = 0.18–1.2) of patients with HPV-negative tumors (Table 4).

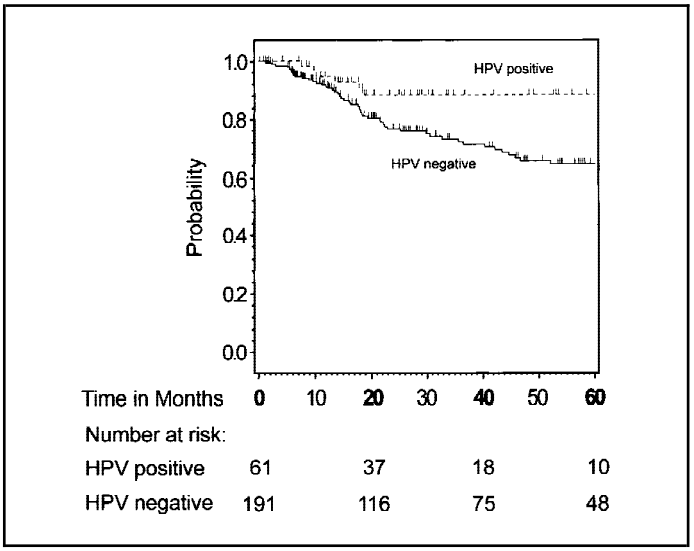

Fig. 3. Kaplan–Meier plot of disease-specific survival for head and neck squamous cell carcinoma patients with human papillomavirus (HPV)-positive and HPV-negative tumors. **Vertical ticks** represent censored events. Patients with HPV-positive tumors had significantly improved disease-specific survival when compared with patients with HPV-negative tumors (log-rank, chi-squared$_{(1\ df)}$ = 5.33; P = .02).

Survival data were also compared separately for oropharyngeal and nonoropharyngeal HNSCC patients. There were three cancer deaths in 34 patients with HPV-positive and 10 cancer deaths in 26 patients with HPV-negative oropharyngeal cancers.

Disease-specific survival was significantly improved in the HPV-positive oropharyngeal group (HR = 0.26; 95% CI = 0.07–0.98; $P = .05$). Among patients with nonoropharyngeal cancers, there were six cancer deaths in 28 patients with HPV-positive and 52 cancer deaths in 165 patients with HPV-negative cancers. Disease-specific survival was similar in the two groups (HR = 0.62; 95% CI = 0.26–1.5; $P = .28$).

DISCUSSION

Although HPV genomic DNA has been detected in head and neck cancers *(16,17,22,50)*, its etiologic role in HNSCC development has remained unclear. In conjunction with recent epidemiologic studies, our data provide strong evidence that HPVs are etiologically linked to a defined subset of head and neck cancers. HPV-positive oropharyngeal cancers in particular may comprise a distinct molecular and pathologic disease entity that is causally associated with HPV infection and has a markedly improved prognosis.

A predisposition of the oropharyngeal mucosa to malignant transformation by HPV was first suggested when HPV16 was detected in tumors of the tongue, tonsil, and pharynx but not in control tissues *(51)*. Another study *(50)* found HPV in oropharyngeal cancers three times as often as other HNSCC primaries (18.6% versus 6.1%; $P = .02$). In two case series, 50%–60% of tonsillar carcinomas (a subset of oropharyngeal tumors) were HPV positive in comparison to 6%–10% of tumors at other sites ($P<.001$) *(16,17)*. Consistently high prevalence of HPV in tonsillar carcinomas has been found by use of various methods of HPV detection *(16–20,52,53)*, and active viral oncogene transcription and genomic integration have been observed in case studies of these cancers *(19,53,54)*.

We have now confirmed and extended previous studies showing a strong association between HPV and oropharyngeal cancer. Our findings suggest that HPV-positive oropharyngeal cancers arising from the lingual and palatine tonsils are a distinct molecular–pathologic entity etiologically linked to infection by high-risk HPVs, especially HPV16. Moreover, a clonal association of HPV with cancer cells is strongly supported by the specific localization of HPV in tumor cells at all cancer stages (preinvasive, invasive, and lymph node) and its probable integration into the genome of some tumors. In contrast to HPV-negative oropharyngeal cancers, these cancers have distinct pathology (more frequently basaloid), tumor biology (fewer p53 mutations), risk factors (less associated with alcohol consumption and perhaps smoking), and clinical course (improved survival) (Table 3).

An etiologic link between HPV and nonoropharyngeal tumors is less firmly established. Nonoropharyngeal HPV-positive tumors were not statistically significantly different from HPV-negative tumors with regard to alcohol consumption and tobacco exposure and survival. Also, nonoropharyngeal cancers that were HPV positive by PCR were rarely HPV positive by Southern blot or *in situ* hybridization. We have no reason to suspect that HPV presence by PCR in these tumors represents false-positive findings. However, because of PCR's exquisite sensitivity, latent infections pathologically unrelated to the tumor could be detected. Nevertheless, single case reports of nonoropharyngeal tumors have provided incontrovertible evidence that the HPV16 genome was integrated into cancer cells and that the viral genome was transcriptionally active *(55)*.

HPV-associated HNSCCs have a morphologic appearance that deviates from conventional keratinizing squamous cell carcinoma *(17,18)*. In our detailed histopathologic evaluation, HPV-positive tumors not only were poorly differentiated and nonkeratinizing but also were strongly associated with a "basaloid" morphology (OR = 18.7; 95% CI = 2.1–167). A basaloid subtype is a well-recognized morphologic variant of HNSCC *(26)* and, like the subset of HPV-positive HNSCC tumors, basaloid tumors predominantly occur in the oropharynx *(19,52,56–58)*. In this study, basaloid features were statistically significantly associated with HPV-positive tumors, both in and outside the oropharynx. The association between HPV and basaloid differentiation is substantiated further by the basaloid phenotype of HPV-associated squamous cell carcinomas of the anus *(59)*, penis *(60,61)*, and vulva *(62,63)*.

The role of p53 mutations in the pathogenesis of certain head and neck cancers *(64,65)* may be substituted by HPV infection because viral E6 protein can inactivate p53 by targeting the protein for ubiquitination and degradation *(7)*. Although p53 mutations were found in about one third of all tumors, there was a marked difference in p53 mutation frequency between HPV-positive and HPV-negative tumors in the oropharynx. The inverse relationship (67% versus 10%) between p53 mutation and HPV strengthens the etiologic role of HPV in oropharyngeal cancers. The overall frequency of p53 inactivation in HNSCC (p53 mutation and/or high-risk HPV infection) was 55% in this entire series and 85% in the oropharynx, emphasizing the importance of p53 abrogation in HNSCC progression.

The coexistence of both HPV and p53 mutations in a single tumor, observed in 11 cases, may be explained by the variable sensitivity of certain p53 mutants to E6 degradation *(66,67)* and/or by tumor promotion via p53-independent viral mechanisms *(68,69)* such as disruption of pRb function by HPV E7 *(70)*. Evidence for activity of high-risk HPV E7 was previously observed in a subset of HNSCCs found to have reduced pRb expression by immunohistochemistry. These tumors tended to arise from the tonsil, were poorly differentiated, were more likely to occur in nonsmokers, and were HPV16 or HPV33 positive *(52)*. Similar findings were reported in another study of tonsillar cancers *(19)*.

The inverse association between p53 mutations and HPV presence in the oropharynx further suggests two parallel or overlapping pathways of HNSCC development: one driven by environmental toxins (e.g., tobacco and alcohol) and another driven by an infectious agent (e.g., high-risk HPVs). Although in previous studies *(7,50)* nonsmokers were more likely to have HPV-positive head and neck tumors than smokers, viral infections may act synergistically with tobacco and alcohol exposures. Exposure of HPV16- or HPV18-immortalized human keratinocyte cell lines to tobacco-related carcinogens resulted in substantially more genetic alterations leading to cellular transformation not seen in keratinocytes transfected with low-risk HPV or without HPV and exposed to the same carcinogens *(71–74)*. This synergy was also supported by a recent case–control study of oral cavity and oropharyngeal cancer patients. HPV16-seropositive nonsmokers had a twofold and HPV16-seronegative current smokers had a 5.8-fold risk of HNSCC compared with seronegative nonsmokers–light drinkers; HPV16-seropositive current smokers had an approximately 15-fold increase in HNSCC risk *(22)*.

The "case–case" or "case-only" format of this study was used specifically to investigate the differences between HPV-positive

and HPV-negative head and neck cancers to enhance the specificity of the association between HPV and head and neck cancer *(75)*. Multivariate analysis of the data shown in Table 1 indicates that the two populations of patients may be more similar than different with respect to the major environmental risk factors of alcohol and tobacco exposure. However, a healthy control group would be required to further investigate possible synergistic interactions between tobacco, alcohol, and HPV exposures. Alcohol and tobacco exposure histories in this study were limited to qualitative categories because of the dependence on medical record data. Indeed, our qualitative data may underestimate differences between HPV-positive and HPV-negative patients that may become more evident with measurements of lifetime cumulative exposures.

We found that HPV-positive HNSCC patients had significantly improved disease-specific survival when compared with patients with HPV-negative tumors, even after adjustment for age, lymph node status, and heavy alcohol consumption (Table 4). Because of retrospective data collection, we were unable to adjust for possible confounding factors, such as nutrition and performance status or the presence of comorbid illness. Prior survival analyses led to contradictory conclusions but were limited by small sample size, short follow-up, and/or lack of disease-specific survival analysis *(17,52,76–78)*.

The improved disease-specific survival in patients with HPV-positive HNSCC is somewhat surprising and remains unexplained. Because HPV-positive tumors may be less associated with alcohol and tobacco exposure and HPV infections tend to be focal, field cancerization (in which the upper respiratory epithelium is repeatedly exposed to carcinogens) *(10)* may be less applicable. Patients with these tumors may be less susceptible to the development of synchronous or metasynchronous tumors in the lungs, esophagus, and elsewhere in the head and neck that could adversely affect long-term survival. Because the majority of HNSCC recurrences occur within approximately 18 months of diagnosis, this hypothesis is supported by the separation of disease-specific survival curves at 18 months (Fig. 3) and the rarity of cancer deaths beyond 18 months in the HPV-positive group. Because of the retrospective nature of the study, we were unable to evaluate the effect of HPV presence on response to primary therapy, including commonly employed radiation therapy. Although there is no evidence that the presence of HPV alters the radiosensitivity of tumors *(79–81)*, radiation therapy has been shown to increase both major histocompatibility complex class I and E6 and E7 expression, and an increased immune surveillance could contribute to improved survival *(82)*.

The predominance of oncogenic, high-risk viral types (HPV16, HPV18, HPV31, and HPV33) in HNSCC *(16,17,19, 52,78,83,84)* previously identified as the major HPV types in cervical carcinomas worldwide *(85,86)* argues for a potentially analogous role for these viruses in the development of malignancy in the upper airway. However, these results need to be interpreted within the context of the study's limitations. As a retrospective case series at a tertiary referral center, it is unclear how these results extend to other populations with different environmental exposures and genetic backgrounds. However, similar HPV prevalence estimates have been reported in a population-based study *(22)* and in case series in the United States *(17)* and in Europe *(50)*.

Discrete, tumor-specific HPV16 E6 variants were identified in 52 of 56 HPV16-positive tumors after repeated amplification and sequencing, arguing against artifacts, such as laboratory contamination or PCR-induced mutations. Infection by particular HPV16 variants may increase both the risk of progression to high-grade cervical *(87)* and anal *(88)* intraepithelial neoplasia and invasive cervical cancer *(89)*. The striking similarity between the frequency distribution of HPV16 variants in HNSCC and that previously reported in cervical cancers in North America *(39)* is consistent with a predisposition of certain HPV16 variants to increase the risk of invasive cancer *(87,90)*. Alternatively, this similarity may merely reflect the overall frequency distribution of HPV infections in the populations studied. Future evaluation of HPV variants in the upper airway of noncancer subjects, estimated to have a prevalence in adults of 5%–11% *(4)*, may help to distinguish between these possibilities.

The means by which HPV is transmitted to the upper airway is unclear. Although oral HPV infection is rare in newborn children of infected mothers *(91)* and in children prior to sexual activity *(92)*, infections increase after onset of sexual activity *(93)*. Epidemiologic studies [reviewed in *(94)*] of cervical cancer have clearly demonstrated that high-risk mucosatropic HPVs are transmitted by sexual contact. Although HPV presence in head and neck cancers has not yet been convincingly linked to specific sexual practices such as oral sex *(21,22,95)*, HPV positivity has been linked to the number of sexual partners in three case–control studies *(21,22,95)*. The presence of HPV in preinvasive and invasive disease as well as lymph node metastases suggests that viral presence precedes disease progression. However, a prospective, seroepidemiologic study of HPV exposure and subsequent development of head and neck cancer is needed to determine that exposure precedes disease *(96)*.

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NOTES

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