Improved Survival of Patients With Human Papillomavirus–Positive Head and Neck Squamous Cell Carcinoma in a Prospective Clinical Trial

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Background The improved prognosis for patients with human papillomavirus (HPV)–positive head and neck squamous cell carcinoma (HNSCC) relative to HPV-negative HNSCC observed in retrospective analyses remains to be confirmed in a prospective clinical trial.

Methods We prospectively evaluated the association of tumor HPV status with therapeutic response and survival among 96 patients with stage III or IV HNSCC of the oropharynx or larynx who participated in an Eastern Cooperative Oncology Group (ECOG) phase II trial and who received two cycles of induction chemotherapy with intravenous paclitaxel and carboplatin followed by concomitant weekly intravenous paclitaxel and standard fractionation radiation therapy. The presence or absence of HPV oncogenic types in tumors was determined by multiplex polymerase chain reaction (PCR) and in situ hybridization. Two-year overall and progression-free survival for HPV-positive and HPV-negative patients were estimated by Kaplan–Meier analysis. The relative hazard of mortality and progression for HPV-positive vs HPV-negative patients after adjustment for age, ECOG performance status, stage, and other covariables was estimated by use of a multivariable Cox proportional hazards model. All statistical tests were two-sided.

Results Genomic DNA of oncogenic HPV types 16, 33, or 35 was located within tumor cell nuclei of 40% (95% confidence interval [CI] = 30% to 50%) of patients with HNSCC of the oropharynx or larynx by in situ hybridization and PCR. Compared with patients with HPV-negative tumors, patients with HPV-positive tumors had higher response rates after induction chemotherapy (82% vs 55%, difference = 27%, 95% CI = 9.3% to 44.7%, $P = .01$) and after chemoradiation treatment (84% vs 57%, difference = 27%, 95% CI = 9.7% to 44.3%, $P = .007$). After a median follow-up of 39.1 months, patients with HPV-positive tumors had improved overall survival (2-year overall survival = 95% [95% CI = 87% to 100%] vs 62% [95% CI = 49% to 74%], difference = 33%, 95% CI = 18.6% to 47.4%, $P = .005$, log-rank test) and, after adjustment for age, tumor stage, and ECOG performance status, lower risks of progression (hazard ratio [HR] = 0.27, 95% CI = 0.10 to 0.75), and death from any cause (HR = 0.36, 95% CI = 0.15 to 0.85) than those with HPV-negative tumors.

Conclusion For patients with HNSCC of the oropharynx, tumor HPV status is strongly associated with therapeutic response and survival.

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Human papillomavirus (HPV) is recognized to play a role in the pathogenesis of a subset of head and neck squamous cell carcinomas (HNSCCs). Detailed analyses of tumors for HPV genomic DNA and viral oncogene expression and case–control studies have indicated that HPV infection is most strongly associated with HNSCC of the oropharynx, where it is observed in 40%–60% of patients (1,2). HPV-positive oropharyngeal tumors are clinically and molecularly distinct from HPV-negative tumors and may be associated with different prognostic outcomes (1).

Analyses of retrospective case series have consistently demonstrated that patients with HPV-positive tumors have a better prognosis than patients whose tumors are HPV negative (3–5). Several hypotheses (3), each based on a factor specific to the HPV-positive patient (these factors include the absence of field cancerization, immune surveillance to viral-specific tumor antigens, and an intact apoptotic response to radiation) have been proposed to explain this difference. However, retrospective survival analyses should be

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CONTEXT AND CAVEATS

Prior knowledge

Analyses of retrospective case series had demonstrated that patients with head and neck squamous cell carcinoma (HNSCC) whose tumors were human papillomavirus (HPV)-positive had a better prognosis than patients whose tumors were HPV-negative, but this remained to be confirmed in a study that adequately controlled for factors of known prognostic value.

Study design

The association of tumor HPV status with therapeutic response and survival was evaluated in patients participating in a trial of chemotherapy and radiation therapy by Kaplan–Meier analysis and a Cox proportional hazards model.

Contribution

The presence of HPV in tumors of patients with HNSCC was positively associated with response to treatment and overall survival after adjustment for a set of other factors known to be associated with clinical outcome.

Implications

The risks and benefits of current therapies may need to be assessed separately according to the HPV status of the patient's tumor.

Limitations

Larger samples may be needed to more thoroughly evaluate the possibility of confounding by smoking and other variables.

interpreted with caution because of the relatively poor quality of retrospectively collected data and the absence of information on factors of known prognostic value (eg, performance status, weight loss, anemia, and comorbidities). Further grounds for caution are that patient populations in retrospective studies are often heterogeneous with regard to primary tumor site and treatment. Thus, observed differences in survival for HPV-positive and -negative patients could be explained by flawed study design rather than representing true differences in treatment response and survival.

To better understand the association between HPV status and prognosis for patients with HNSCC, we prospectively evaluated the effect of tumor HPV status on treatment response and survival outcomes among patients with oropharyngeal or laryngeal squamous cell carcinoma who were uniformly treated with induction chemotherapy and chemoradiation as participants in a phase II trial conducted by the Eastern Cooperative Oncology Group (ECOG).

Patients and Methods

Study Population

All patients enrolled in ECOG protocol 2399, a phase II trial of chemoradiation for organ preservation in resectable stage III or IV squamous cell carcinomas of the larynx or oropharynx, were eligible for this correlative study, which was included in the original protocol. Eligibility criteria for ECOG 2399 (for which the details, design, and results were reported previously) (6) included a diagnosis of histologically confirmed and resectable squamous cell carcinoma of the oropharynx or larynx of clinical stage III or IV (tumor–node–metastasis [TNM] stage T2N1-3 or T3-4N0-3M0) as defined by the American Joint Committee on Cancer (AJCC): absence of prior chemotherapy, radiation therapy, or surgical treatment; age $\geq$ 18 years; and ECOG performance status < 3. The ECOG 2399 protocol was approved by the institutional review board of all participating institutions, and written informed consent was obtained from all patients for this correlative study.

Treatment

Patients in ECOG 2399 were administered two cycles of induction chemotherapy that consisted of paclitaxel 175 mg/m^2 as a 3-hour intravenous infusion and carboplatin (area under the curve 6, Cockcroft–Gault) on day 1 of a 21-day cycle. Response to chemotherapy was formally evaluated within 21 days of administration of the second cycle of induction chemotherapy by use of standard criteria (ie, Response Evaluation Criteria in Solid Tumors) for the overall treatment response at the primary and nodal sites (7). Patients with a partial or complete response to induction chemotherapy at the primary site were eligible to receive chemoradiation. Patients with stable or progressive disease were referred for surgical resection. However, patients who refused surgery were allowed to proceed with concurrent chemotherapy and radiation.

Chemoradiation consisted of seven weekly intravenous doses of paclitaxel (30 mg/m^2) administered concurrently with standard-fractionation external beam radiation therapy at a total dose of 70 Gy in 35 fractions over 7 weeks. Treatment response was clinically evaluated by direct fiberoptic endoscopy and computed axial tomography imaging 6–8 weeks after completion of chemoradiation. Surgical resection was performed on all patients with residual disease. Patients were evaluated for disease progression every 3, 4, and 6 months for the first, second, and third or more years, respectively. Histologically confirmed second primary tumors diagnosed during follow-up were reported to ECOG. Patients were followed until death or for a maximum of 5 years.

Laboratory Analysis

For each patient, formalin-fixed and paraffin-embedded specimens from the surgically obtained diagnostic biopsy of the primary tumor were prepared. The research team was blinded to all clinical data until after the laboratory analysis was complete and results had been reported to ECOG. For each patient, 10 sections (5 μm thick) were cut from the formalin-fixed, paraffin-embedded tumor specimen. All specimens were reviewed by a study pathologist (WHW) to confirm the presence of tumor on hematoxylin-and-eosin–stained slides. Tumor differentiation was also scored on an ordinal scale as well, moderately, or poorly differentiated. The presence and degree of basaloid features were scored on a scale of 0–2 (0 = absent, 1 = present but only partially developed, 2 = present and fully developed), with the term basaloid denoting squamous cell carcinomas that exhibit lobular to solid growth with peripheral cellular palisading with tumor cells demonstrating prominent basophilia due to high nuclear to cytoplasmic ratio not seen in HPV-negative keratinizing squamous cell carcinoma (*see* Figs. 1, A and B) (8).

HPV Detection

All tumors were evaluated for the presence of HPV16 DNA by use of the in situ hybridization–catalyzed signal amplification method

for biotinylated probes (GenPoint; Dako, Carpinteria, CA) (9). Briefly, tissue sections were subjected to deparaffinization, heat-induced target retrieval, and digestion with proteinase K (Roche Diagnostics, Indianapolis, IN), as described previously (10). Tumor specimens were then hybridized to a biotinylated type-specific HPV16 probe (Dako, code X1415) or a negative control probe (Dako, code OQ002). After low- and high-stringency washes, the Dako TSA System kit was used for signal amplification. An HPV16-positive tumor specimen was used as a positive control. Slides were scored as positive for HPV16 if a punctate signal specific to tumor cell nuclei was present. Positive samples were scored as 1+ (one focal hybridization signal in scattered tumor cell nuclei), 2+ (one focal hybridization signal per nucleus), or 3+ (two or more hybridization signals per nucleus).

DNA was purified from paraffin by deparaffinization, proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. No attempt was made to microdissect tumor from associated tissue. Purified DNA was screened for HPV DNA by multiplex polymerase chain reaction (PCR) with PGMY09/11 L1 primer pools and primers for β-globin (11). β-globin was amplified as a positive control for the quality of DNA purified from paraffin. PCR products were denatured and hybridized to a prototype HPV probe array for genotyping of 37 HPV types and β-globin (Roche Molecular Systems, Inc., Alameda, CA). Samples positive for β-globin and/or HPV DNA were considered to be evaluable, and HPV type was reported for all positive samples.

For tumors positive by multiplex PCR for an HPV type other than 16, the specificity of the HPV DNA to tumor cell nuclei was confirmed by in situ hybridization analysis, as described above. A probe for HPV types 31/33 (Dako) or a probe cocktail for 13 high-risk types (Dako) was used as appropriate depending on the HPV type detected.

HPV16 viral load was determined by use of a real-time, quantitative TaqMan PCR method targeted to the E6 region of the viral genome (12) and normalized to human diploid genomic equivalents that were present in the PCR by use of a modified TaqMan real-time PCR targeted to a single-copy gene on chromosome 7, human endogenous retrovirus 3 (13).

p16 Immunohistochemistry

The expression status of p16 is strongly correlated with tumor HPV status (14) and therefore was evaluated by immunohistochemistry, as previously described (15). The protein is a cyclin-dependent kinase inhibitor whose expression increases in response to pRb inactivation by the high-risk HPV E7 protein in cervical cancers (16). It is constitutively expressed in tonsillar crypt basal epithelium (15). Briefly, after 5-μm sections were deparaffinized, antigen retrieval was performed by use of heat-induced epitope retrieval with 10 mM citrate buffer. Sections were incubated with a mouse monoclonal antibody against p16 (MTM Laboratories, Heidelberg, Germany) at 1 : 500 dilution. The p16 antibody was detected using the avidin-biotin-peroxidase technique (Dako LSAB Kit, Dako). On histopathologic review, the pattern of p16 expression was generally dichotomous according to tumor sample, with p16 staining either absent (negative) or present with strong and diffuse nuclear and cytoplasmic staining (positive). Immunohistochemical interpretation was performed blinded to the HPV status and identity of the patient from whom the tumor originated.

Statistical Analysis

ECOG 2399 was powered for a primary outcome of organ preservation rate, as previously described (6). Our correlative study was designed and executed as part of the original protocol.

HPV-positive and -negative patients were compared by use of Fisher exact test for dichotomous (eg, sex, race, ECOG performance status, AJCC stage, and AJCC tumor site and subsite) or categorical (eg, weight loss, smoking status, alcohol and tobacco consumption, tumor differentiation) variables and Wilcoxon rank-sum test for continuous variables (eg, age, hemoglobin). Differences in HPV viral load as expressed by the ordinal score assigned in interpretation of in situ hybridization experiments were analyzed by the Kruskal–Wallis test.

Overall survival was defined as the time from the date of registration to the date of death or to the date of censorship (ie, the last date of follow-up). Progression-free survival was defined as the time from registration to local or distant recurrence. Death without documented progression was censored at the date of death. Survival data were analyzed using the Kaplan–Meier method, and survival curves were compared by use of the log-rank test in univariate analysis. In multivariable analyses, a Cox proportional hazards model was used to adjust for covariates of statistical significance in univariate analysis, including age, ECOG performance status, and stage, and to estimate the relative hazard of mortality or progression over the follow-up period. Proportional hazards were demonstrated by visual inspection of log–log survival curves and of Schoenfeld residuals. All statistical tests were two-sided, and a P value of .05 or less was considered statistically significant.

Results

Of the 111 patients enrolled in ECOG 2399, 105 met the eligibility criteria and 101 (96%) consented to participate in this correlative study. Tumor samples were unavailable for five of 101 patients; therefore, the study population consisted of 96 patients.

Genomic DNA of an oncogenic HPV type was specifically localized to tumor cell nuclei in 38 of 96 patients (40%, 95% confidence interval [CI] = 30% to 50%) by in situ hybridization. HPV16 was detected in 36 tumor samples by in situ hybridization and multiplex and type-specific PCR. Two additional samples were positive for HPV33 or 35 by multiplex PCR and were confirmed as positive by in situ hybridization. The intensity of the nuclear hybridization signal for HPV in tumor cell nuclei was scored as negative for 58 tumors, as 1+ for 11 tumors, as 2+ for seven tumors, and as 3+ for 20 tumors (Fig. 1, C–E). The intensity of nuclear staining by HPV16 in situ hybridization was strongly associated with HPV16 viral copy number as determined by real-time PCR ($P < .001$, Kruskal–Wallis test). The median viral copy number per cell DNA equivalent (DNA was purified from tumor specimens without microdissection) in samples scored as 0, 1+, 2+, and 3+ by in situ hybridization was 0, 0.29, 2.4, and 17.6, respectively.

Thirty-six (95%) of 38 HPV-positive tumors had sufficient material for p16 immunohistochemistry; all had high (strong and

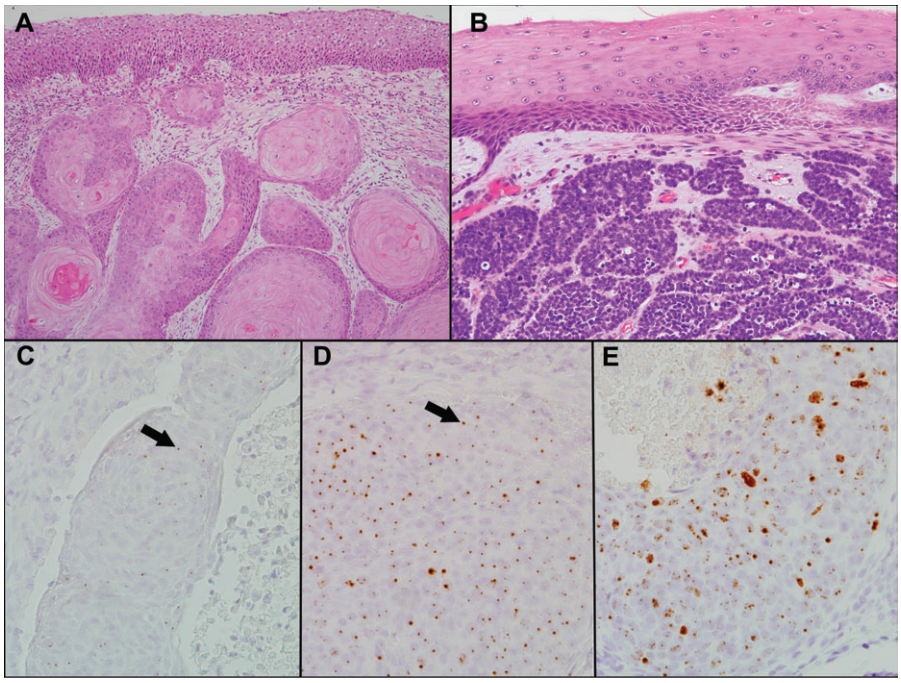

Fig. 1. Histopathologic classification of basaloid features and ordinal scoring for human papillomavirus 16 (HPV16) infections as determined by in situ hybridization. **A**) HPV-negative, conventional keratinizing squamous cell carcinoma. The lamina propria is infiltrated by nests of keratinized squamous cells with abundant glassy eosinophilic cytoplasm. **B**) HPV-positive basaloid squamous cell carcinoma. Here the carcinoma infiltrates as lobules of basaloid cells characterized by enlarged hyperchromatic nuclei and scant cytoplasm. **C**, **D**, and **E**) In situ hybridization signal of HPV16-positive squamous cell carcinomas. Tissue sections were probed with a biotinylated type-16 specific probe. In **C**, **D**, and **E**, the in situ hybridization signal was scored as 1+, 2+, and 3+, respectively.

diffuse nuclear and cytoplasmic staining) expression of p16. p16 expression was strongly associated with HPV positivity (100% in HPV-positive tumors vs 28% in HPV-negative tumors, $P < .001$, Fisher exact test).

We analyzed the association of patient variables (eg, age, sex, race, presence of comorbid conditions, weight loss, performance status, and alcohol and cigarette consumption) and tumor characteristics (eg, AJCC tumor stage and histologic features) according to tumor HPV status (Table 1). A diagnosis of an HPV-positive tumor was associated with white race ($P = .02$, Fisher exact test) and better performance status (ECOG 0 vs 1–2, $P = .01$) and only marginally associated with sex ($P = .07$) and weight loss ($P = .07$). Patients with HPV-positive and -negative tumors were similar with regard to neurologic or gastrointestinal comorbidities at diagnosis (Table 1).

Similar proportions of patients with HPV-positive and -negative tumors reported a history of cigarette smoking (82% vs 95%, respectively). However, patients with HPV-positive tumors were statistically significantly less likely than patients with HPV-negative tumors to have 20 or more pack-years of exposure to cigarettes (45% vs 90%, $P < .001$).

Tumor HPV status was associated with the tumor's primary site, stage, and histopathology (Table 1). Thirty-eight of 60 (63%, 95% CI = 50% to 75%) oropharyngeal vs 0 of 34 (0%, 95% CI = 0% to 10%) laryngeal cancers were HPV positive ($P < .001$). HPV-positive tumors were more likely than HPV-negative tumors to arise from the tonsil or base of the tongue ($P < .001$). Although nodal status and overall AJCC TNM stage did not differ by HPV status, HPV-positive tumors were more likely than HPV-negative tumors to have an AJCC tumor stage of T2 vs T3–T4 ($P = .02$). HPV-positive tumors were also more likely than HPV-negative tumors to be poorly differentiated ($P = .03$) and to have basaloid features ($P < .001$).

In analyses restricted to patients with oropharyngeal tumors, similar differences were observed. Compared with those with HPV-negative tumors, patients with HPV-positive oropharyngeal tumors were more likely to be white (95% and 71%, $P = .02$) and patients whose tumors were HPV positive had higher performance status (ECOG performance status was 0 in 66% of those with HPV-positive tumors and 33% of those with HPV-negative tumors, $P = .01$). Patients with HPV-positive tumors were more likely to report less than 20 pack-years of cigarette use (37% vs 0%, respectively, $P < .001$) and to be diagnosed with lingual or palatine tonsil primary tumors (84% vs 63%, respectively, $P = .07$) than those with HPV-negative tumors. However, no differences between HPV-positive and HPV-negative patients in tumor, nodal, or overall AJCC tumor stage were observed in the subset of patients with oropharnygeal tumors.

Response Rates

Response rates after induction chemotherapy and chemoradiation were evaluated according to HPV status. Response was evaluable for 91 (95%) of 96 patients after induction chemotherapy and for 85 (89%) of 96 patients after chemoradiation. Compared with patients with HPV-negative tumors, patients with HPV-positive tumors had higher response rates after induction chemotherapy (82% vs 55%, difference = 27%, 95% CI = 9.3% to 44.7%, $P = .01$) and after chemoradiation treatment (84% vs 57%, difference = 27%, 95% CI = 9.7% to 44.3%, $P = .007$). Similar differences were observed when the analysis was restricted to oropharyngeal cancers. The difference in response could not be attributed to the site of the primary tumor: among patients whose tumors were HPV negative, the response rate after induction chemotherapy was 58% among those who had oropharyngeal cancers vs 53% among those with laryngeal cancers, $P = .58$. Similarly, among HPV-negative patients treated with radiation, the response rate was 54% among those with

Table 1. Comparison of baseline characteristics of study population, stratified by tumor human papillomavirus status*

Patient or tumor characteristic	HPV positive, n = 38	HPV negative, n = 58	*P*†
Age			
Median (range)	56 (41–79)	60 (24–83)	.19‡
Sex, No. (%)			
Male	34 (90)	43 (74)	.07
Female	4 (10)	15 (26)	
Race, No. (%)			
White	36 (95)	45 (78)	.02
Nonwhite	2 (5)	13 (22)	
ECOG performance status			
0	25 (66)	22 (38)	.01
1–2	13 (34)	36 (62)	
Weight loss in last 6 mo, No. (%)			
<5%	25 (65)	38 (65)	.07
5% to <10%	8 (21)	7 (12)	
≥10%	1 (3)	10 (18)	
Unknown	4 (11)	3 (5)	
Hemoglobin (g/dL), median (range)	13.5 (11.7–14.0)	13.5 (11.0–15.3)	.10‡
Chronic gastrointestinal disease, No. (%)			
Absent	27 (71)	47 (81)	.32
Present	11 (29)	11 (19)	
Chronic neurologic disease, No. (%)			
Absent	35 (92)	53 (91)	.9
Present	3 (8)	5 (9)	
Average alcohol consumption per wk at diagnosis, No. (%)			
<10 ounces	21 (55)	30 (52)	.6
10–32 ounces	9 (24)	10 (17)	
>32 ounces	6 (16)	13 (22)	
Unknown	2 (5)	5 (9)	
Smoking history, No. (%)			
Never	5 (13)	2 (3)	.07
Ever	31 (82)	55 (95)	
Unknown	2 (5)	1 (2)	
Cigarettes smoked (pack-years), No. (%)			
<20	14 (37)	1 (2)	<.001
≥20	17 (45)	55 (95)	
Never/unknown	7 (18)	3 (5)	
Tumor site, No. (%)			
Oropharynx	38 (100)	24 (41)	<.001
Larynx	0 (0)	34 (59)	
Tumor subsite, No (%)			
Base of tongue and tonsil	32 (84)	15 (32)	<.001
Other	6 (16)	43 (88)	
Overall AJCC stage, No. (%)			
Stage III	11 (29)	23 (40)	.62
Stage IV	21 (71)	35 (60)	
AJCC tumor stage, No. (%)			
T2	22 (58)	19 (33)	.02
T3–T4	16 (42)	39 (67)	
AJCC nodal stage, No. (%)			
N0–N1	13 (34)	29 (50)	.13
N2–N3	25 (66)	29 (50)	
Tumor differentiation, No. (%)			
Well or moderate	20 (53)	43 (74)	.03
Poor	13 (34)	11 (19)	
Unevaluable	5 (13)	4 (7)	
Basaloid histologic features, No. (%)			
Present	25 (66)	12 (21)	<.001
Absent	10 (26)	42 (72)	
Unevaluable	3 (8)	4 (7)	

* HPV = human papillomavirus; ECOG = Eastern Cooperative Oncology Group; AJCC = American Joint Commission on Cancer Staging; T = tumor; N = node.

† Fisher exact test unless otherwise indicated.

‡ Wilcoxon rank-sum test.

oropharyngeal cancers vs 59% among those with laryngeal cancers ($P = .76$).

Survival Analysis

We evaluated whether or not improved response rates among patients with HPV-positive tumors were associated with improved survival outcomes. Median follow-up time for the entire study population was 39.1 months (range = 20.1–58.9 months). Disease progression occurred during follow-up in five of the 38 HPV-positive patients with oropharyngeal cancer (two had local/regional progression and three had distant metastases), nine of the 24 patients with HPV-negative oropharyngeal cancer (eight experienced local/regional progression and one experienced distant metastasis), and 11 of the 34 patients with HPV-negative laryngeal cancer (four with local/regional progression, seven with distant metastases). There were seven deaths among patients with HPV-positive oropharyngeal cancer, 12 among patients with HPV-negative oropharyngeal cancer, and 12 among patients with HPV-negative laryngeal cancer.

Based on Kaplan–Meier estimates, overall survival for patients with HPV-positive tumors was improved to a statistically significant extent compared with that of patients with HPV-negative tumors ($P = .005$, log-rank test). The estimated 1- and 2-year overall survival rates were 97% (95% CI = 92% to 100%) and 95% (95% CI = 87% to 100%), respectively, among HPV-positive patients. By contrast, 1- and 2-year survival rates among HPV-negative patients were 90% (95% CI = 92% to 100%) and 62% (95% CI = 49% to 74%). The difference in overall survival between HPV-positive and -negative patients was 7% (95% CI = 2.4% to 16.4%) at 1 year and 33% (95% CI = 18.6% to 47.4%) at 2 years (Fig. 2, A).

HPV-positive patients also had statistically significantly better progression-free survival than HPV-negative patients ($P = .02$, log-rank test). One- and 2-year progression-free survival rates among HPV-positive patients were 91% (95% CI = 81% to 100%) and 86% (95% CI = 74% to 99%), respectively. Corresponding estimates of 1- and 2-year progression-free survival rates among HPV-negative patients were 69% (95% CI = 55% to 82%) and

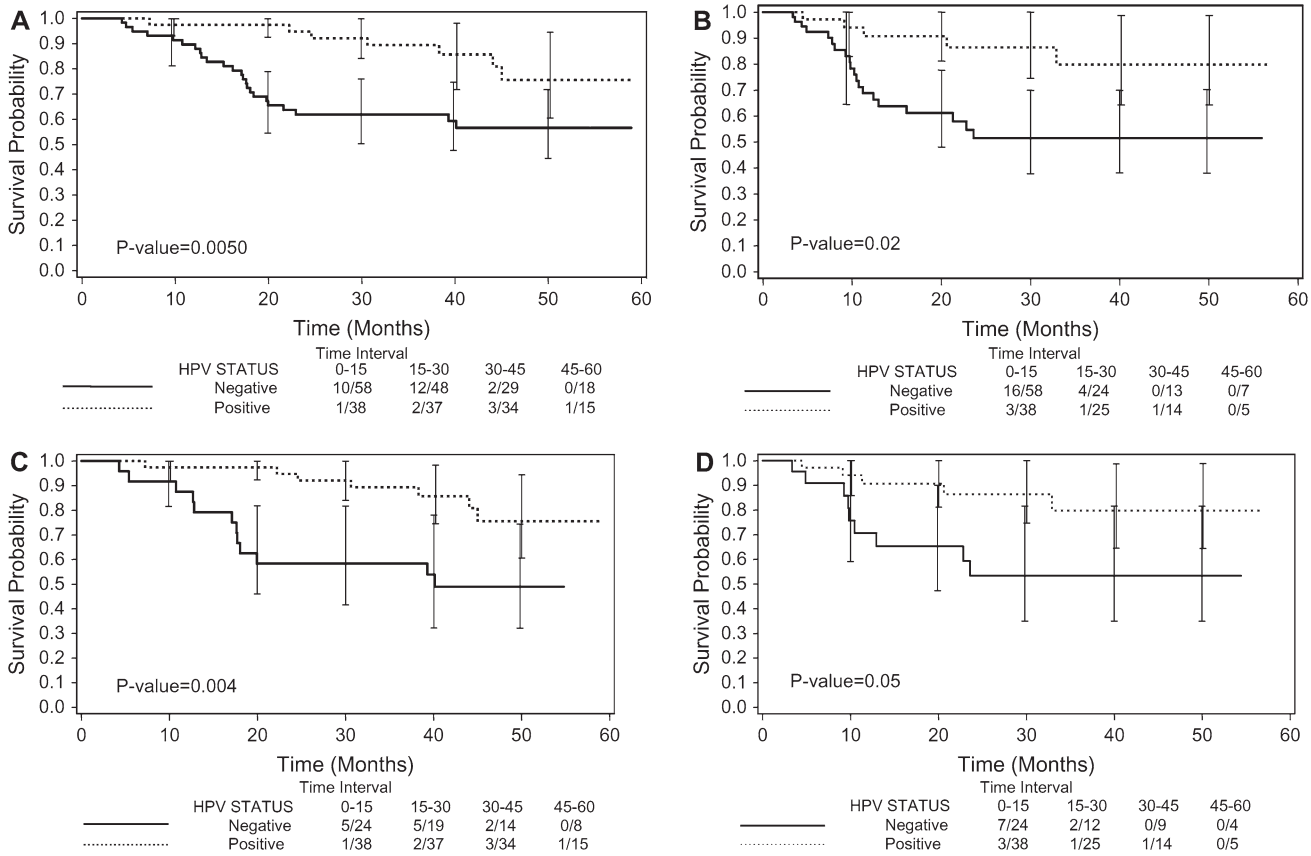

Fig. 2. Kaplan–Meier curves for overall and progression-free survival stratified by tumor human papillomavirus (HPV) status. **A**) Overall survival (OS) for the entire study population. **B**) Progression-free survival (PFS) for the entire study population. **C**) OS for patients with oropharynx cancer only. **D**) PFS for patients with oropharynx cancer only. For all curves, 95% confidence intervals for survival estimates at several time points are shown. Events per patients at risk are indicated for 15-month intervals.

53% (95% CI = 36% to 67%) (Fig. 2, B). The difference in progression-free survival between HPV-positive and -negative patients was 22% (95% CI = 5.0% to 39%) at 1 year and 33% (95% CI = 12.7% to 53.3%) at 2 years.

Among patients with cancers of the oropharynx, HPV-positive patients had better overall (P = .004, log-rank test) and progression-free (P = .05, log-rank test) survival than patients with HPV-negative tumors (Figs. 2, C and D). Survival outcomes for patients with HPV-negative oropharyngeal cancer and patients with HPV-negative laryngeal cancer were similar to one another (data not shown).

Univariate analysis was performed to evaluate factors potentially associated with overall and progression-free survival. Anemia, weight loss, and comorbid conditions (neurologic or gastrointestinal) were not important determinants of overall or progression-free survival (data not shown). However, age, tumor stage, ECOG performance status, and tumor HPV status were associated with overall or progression-free survival outcomes (Table 2). Performance status (ECOG 1–2 vs 0, hazard ratio [HR] = 3.79, 95% CI = 1.69 to 8.49), tumor stage (AJCC stage IV vs III, HR = 3.52, 95% CI = 1.35 to 9.18), and tumor HPV status (positive vs negative, HR = 0.35, 95% CI = 0.15 to 0.80) were associated with overall survival. Age (>60 vs ≤ 60 years, HR = 2.50, 95% CI = 1.10 to 5.65), performance status (ECOG 1–2 vs 0, HR = 2.55, 95% CI = 1.12 to 5.79), and tumor HPV status (positive vs negative, HR = 0.28, 95% CI = 0.11 to 0.75) were associated with progression-free survival. The association of tumor HPV status with survival could not be explained by smoking: patients with HPV-positive tumors with and without a history of smoking had a similar reduction in risk of mortality when compared with their HPV-negative counterparts (HR = 0.36, 95% CI = 0.14 to 0.87 and HR = 0.36, 95% CI = 0.02 to 5.9, respectively).

We then performed multivariable analysis to estimate the association of tumor HPV status with survival outcomes (Table 2). In this analysis, advanced tumor stage (AJCC stage IV vs III, adjusted HR = 5.32, 95% CI = 1.97 to 14.3, P = .001) and poorer performance status (ECOG 1–2 vs 0, adjusted HR = 2.77, 95% CI = 1.20 to 6.38, P = 0.02) were associated with elevated mortality risk after adjustment for age and tumor HPV status. Tumor HPV status was independently associated with mortality risk after adjustment for age, tumor stage, and ECOG performance status: patients with HPV-positive tumors had a 64% lower risk of death than patients with HPV-negative tumors (adjusted HR = 0.36, 95% CI = 0.15 to 0.85, P = .02).

After adjustment for age, tumor stage, and ECOG performance status, tumor HPV status was also statistically significantly associated with progression-free survival. Patients with HPV-positive tumors had a risk of progression that was 73% lower than that of patients with HPV-negative tumors (adjusted HR = 0.27, 95% CI = 0.10 to 0.75, P = .01).

Table 2. Univariate and multivariable models for overall and progression-free survival*

Characteristic	Univariate		Multivariable†	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Entire study population				
Overall survival				
Age (>60 vs ≤60 y)	1.88 (0.92 to 3.84)	.08	1.99 (0.94 to 4.18)	.07
Sex (male vs female)	1.24 (0.48 to 3.23)	.66		
Race (Caucasian vs non-Caucasian)	0.9 (0.35 to 2.35)	.83		
Performance status (1, 2 vs 0)	3.79 (1.69 to 8.49)	.002	2.77 (1.20 to 6.38)	.02
Stage (IV vs III)	3.52 (1.35 to 9.18)	.01	5.32 (1.97 to 14.33)	.001
Tumor HPV status (positive vs negative)	0.35 (0.15 to 0.80)	.01	0.36 (0.15 to 0.85)	.02
Smoking (ever vs never)	1.26 (0.30 to 5.29)	.75		
Progression-free survival				
Age (>60 vs ≤60 y)	2.5 (1.10 to 5.65)	.03	2.81 (1.20 to 6.60)	.02
Sex (male vs female)	0.83 (0.33 to 2.09)	.69		
Race (Caucasian vs non-Caucasian)	0.78 (0.27 to 2.07)	.64		
Performance status (1, 2 vs 0)	2.55 (1.12 to 5.79)	.03	1.88 (0.81 to 4.35)	.14
Stage (IV vs III)	2.27 (0.85 to 6.06)	.09	3.55 (1.28 to 9.80)	.01
Tumor HPV status (positive vs negative)	0.28 (0.11 to 0.75)	.01	0.27 (0.10 to 0.75)	.01
Smoking (ever vs never)	2.71 (0.37 to 20.04)	.33		
Oropharynx cancers only				
Overall survival				
Age (>60 vs ≤60 y)	1.63 (0.66 to 4.0)	.29		
Sex (male vs female)	2.67 (0.36 to 20.01)	.34		
Race (Caucasian vs non-Caucasian)	0.57 (0.19 to 1.71)	.31		
Performance status (1, 2 vs 0)	3.39 (1.28 to 8.98)	.01	2.49 (0.89 to 6.92)	.09
Stage (IV vs III)	1.98 (0.66 to 5.98)	.23		
Tumor HPV status (positive vs negative)	0.29 (0.11 to 0.74)	.01	0.39 (0.15 to 1.05)	.06
Smoking (ever vs never)	1.08 (0.25 to 4.69)	.92		
Progression-free survival				
Age (>60 vs ≤60 y)	2.34 (0.81 to 6.76)	.12		
Sex (male vs female)	2.16 (0.28 to 16.5)	.46		
Race (Caucasian vs non-Caucasian)	0.35 (0.11 to 1.12)	.08		
Performance status (1, 2 vs 0)	2.79 (0.93 to 8.39)	.07	2.26 (0.74 to 6.97)	.15
Stage (IV vs III)	1.73 (0.48 to 6.22)	.4		
Tumor HPV status (positive vs negative)	0.32 (0.11 to 0.94)	.04	0.38 (0.12 to 1.15)	.09
Smoking status (ever vs never)	2.09 (0.27 to 15.97)	.48		

* HR = hazard ratio; CI = confidence interval; HPV = human papillomavirus.

† Adjusted for all other covariates listed in the column by use of Cox proportional hazards model.

When the analysis was restricted to patients with oropharyngeal cancer, patients with HPV-positive tumors had a 61% lower risk of death (HR = 0.39, 95% CI = 0.15 to 1.05, *P* = .06) and a 62% lower risk of progression (HR = 0.38, 95% CI = 0.12 to 1.15, *P* = .09) than patients with HPV-negative tumors after adjustment for ECOG performance status (Table 2).

The number of patients who developed a second malignancy during follow-up did not differ to a statistically significant extent by tumor HPV status: 11% of patients whose tumors were HPV positive and 5% of patients whose tumors were HPV negative developed second malignancies (*P* = .43). Second primary tumors among HPV-positive patients included skin cancer (n = 1), multiple myeloma (n = 1), and prostate cancer (n = 2), and among HPV-negative patients they included skin cancer (n = 1), renal carcinoma (n = 1), cancer of the oral cavity (n = 1), and an unknown primary tumor (n = 1).

Discussion

This is one of the first studies to prospectively evaluate in a multicenter clinical trial the association of tumor HPV status with response to treatment and survival in patients with HNSCC. The data confirm the improved survival outcomes for patients with HPV-positive HNSCC observed in retrospective survival analyses and are consistent with an increased sensitivity of these cancers to chemotherapy and chemoradiation. Our results, however, should not be construed as evidence for a difference in natural history between HPV-positive and HPV-negative cancers in the absence of therapy.

Consistent with the existing literature, the risk factors and demographic and tumor characteristics of HPV-positive patients differed from those of HPV-negative patients (1). HPV-positive tumors were more likely than HPV-negative tumors to arise from the oropharynx, to be poorly differentiated, and to have basaloid features. Additionally, patients with HPV-positive tumors had less cumulative exposure to tobacco. Thus, the data provide further evidence that HPV-positive tumors are a unique clinical entity distinct from HPV-negative tumors.

Several case series have supported an inverse association between tumor HPV status and the presence of p53-inactivating mutations in head and neck cancers (3,17). The better response to chemotherapy and radiation observed for HPV-positive tumors

compared with HPV-negative ones that were observed in this study and in retrospective studies (18,19) may therefore be due to the presence of an intact p53-mediated apoptotic response to chemotherapy-induced stress in the HPV-positive tumors (20). However, in the study by Licitra et al. (21), survival for patients with HPV-positive oropharyngeal cancers was improved relative to that of HPV-negative patients both with and without p53 mutations in their tumors and was observed in patients treated with and without radiation therapy. Therefore, the biologic basis for the improved survival among the HPV-positive patients is unclear and warrants further study.

Both AJCC tumor stage and ECOG performance status were associated with tumor HPV status. Nevertheless, HPV status of the tumor was associated with survival after adjustment for these factors. Additional variables of potential prognostic importance, such as race, smoking status, weight loss, and sex were marginally associated with tumor HPV status. Sample size constraints limited the number of variables that could be included in our models. Hence, factors not included in our models may be important. However, we found no evidence in our stratified analysis that the association of tumor HPV status with survival outcomes could be attributed to confounding by tobacco use. Although this work provides prospective evidence that tumor HPV status is an independent prognostic factor for survival, larger confirmatory studies are needed to provide definitive evidence.

Although lifetime exposure to tobacco was less for patients with HPV-positive tumors, the rate of second primary tumors during the period of observation was not. The majority of these second primary tumors are not established as smoking related. Therefore, in contrast to what was observed in a recent analysis of retrospective data (21), the improved prognosis observed in this study cannot be explained by differences in the development of second primary tumors or by the absence of tobacco-associated field cancerization in HPV-positive patients. It is possible that differences in the rate or type of second primary tumors could be observed with longer follow-up time.

This correlative study was designed in 1999, based on the work of one of its investigators (3). Based on that study (3), 57% of oropharyngeal and 19% of laryngeal cancers were estimated to be HPV associated, whereas in this study a similar proportion of oropharyngeal cancers but none of the larynx cancers were HPV associated. Because subsequent molecular and epidemiologic data [reviewed in (1)] suggest that HPV is associated primarily with oropharyngeal cancers, we analyzed these cancers separately. Our findings were similar to those of the overall analyses: point estimates for hazard ratios remained largely unchanged, but confidence intervals widened as expected because of smaller sample size.

The proportion of oropharynx cancers attributable to HPV infection is unclear. The International Agency on Treatment of Cancer (IARC) Multicenter Study estimated that 18% of oropharynx cancers worldwide are HPV associated (22). In a separate IARC literature summary, this proportion was estimated to be 38%, and that study found that oropharynx cancer patients in North America were more likely than those in Europe to be HPV positive (47% vs 28%) (2). The prevalence estimate for HPV in oropharynx cancers of 63% in the United States from this multicenter trial should be considered to be reliable for a number of reasons. Patients from throughout the United States participated. Tumor HPV status was evaluated and confirmed by several different assays. Cancers were analyzed for all 18 of the known oncogenic HPV types by sensitive PCR-based methods. The detected HPV was specific to tumor cell nuclei by in situ hybridization in patterns consistent with integration and confirmed to be of high copy number by real-time, quantitative PCR. Consistent with other studies (1,2,22), the overwhelming majority (~95%) of HPV-positive tumors were HPV16 positive.

The association of tumor HPV status with response to treatment and survival observed in this prospective study is consistent with prior retrospective analyses and sufficiently strong to warrant consideration in the design and analysis of current and future clinical trials of treatments for head and neck cancer patients. Failure to take tumor HPV status into account could lead to confounding—that is, in the absence of data from this correlative study in ECOG protocol 2399, the clinical trial with which it was associated could be interpreted as demonstrating that larynx cancers were less likely than oropharynx cancers to respond to the treatment regimen. In fact, HPV-negative oropharynx and larynx cancers respond similarly and less favorably than HPV-positive tumors.

Limitations to this study include the small sample size, which restricted the number of variables that could be included in our models, and the inclusion of laryngeal cancer patients. Analysis of a larger study limited to oropharyngeal cancer patients could more thoroughly evaluate the possibility of confounding by smoking via analysis of different levels of tobacco consumption. Although statistically significant differences in response and survival were observed for HPV-positive and -negative tumors with this treatment regimen, the findings from this study may not necessarily extrapolate to other treatment regimens or chemotherapeutic agents.

Nevertheless, our data suggest that the risks and benefits of intensive combined modality therapies should be considered separately for HPV-positive and -negative patients. The current AJCC staging system does not yet reflect different effects of treatment and survival for the HPV-positive and -negative patient with HNSCC. Tumor HPV status or an appropriate clinical surrogate (eg, p16 immunohistochemistry) should now be included as a stratification factor for clinical trials that include oropharyngeal cancer patients.

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