VOLUME 32 · NUMBER 35 · DECEMBER 10 2014

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

p16 Protein Expression and Human Papillomavirus Status As Prognostic Biomarkers of Nonoropharyngeal Head and Neck Squamous Cell Carcinoma

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Published online ahead of print at www.jco.org on September 29, 2014.

Supported by Radiation Therapy Oncology Group Grant No. U10 CA21661 and National Cancer Institute Community Clinical Oncology Program Grant No. U10 CA37422.

Presented orally at the 49th Annual Meeting of the American Society of Clinical Oncology, Chicago, IL, May 31-June 4, 2013.

Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Clinical trial information: NCT00047008, NCT00265941, NCT00084318.

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0732-183X/14/3235w-3930w/$20.00

DOI: 10.1200/JCO.2013.54.5228

ABSTRACT

Purpose
Although p16 protein expression, a surrogate marker of oncogenic human papillomavirus (HPV) infection, is recognized as a prognostic marker in oropharyngeal squamous cell carcinoma (OPSCC), its prevalence and significance have not been well established in cancer of the oral cavity, hypopharynx, or larynx, collectively referred as non-OPSCC, where HPV infection is less common than in the oropharynx.

Patients and Methods
p16 expression and high-risk HPV status in non-OPSCCs from RTOG 0129, 0234, and 0522 studies were determined by immunohistochemistry (IHC) and in situ hybridization (ISH). Hazard ratios from Cox models were expressed as positive or negative, stratified by trial, and adjusted for clinical characteristics.

Results
p16 expression was positive in 14.1% (12 of 85), 24.2% (23 of 95), and 19.0% (27 of 142) and HPV ISH was positive in 6.5% (six of 93), 14.6% (15 of 103), and 6.9% (seven of 101) of non-OPSCCs from RTOG 0129, 0234, and 0522 studies, respectively. Hazard ratios for p16 expression were 0.63 (95% CI, 0.42 to 0.95; $P = .03$) and 0.56 (95% CI, 0.35 to 0.89; $P = .01$) for progression-free (PFS) and overall survival (OS), respectively. Comparing OPSCC and non-OPSCC, patients with p16-positive OPSCC have better PFS and OS than patients with p16-positive non-OPSCC, but patients with p16-negative OPSCC and non-OPSCC have similar outcomes.

Conclusion
Similar to results in patients with OPSCC, patients with p16-negative non-OPSCC have worse outcomes than patients with p16-positive non-OPSCC, and HPV may also have a role in outcome in a subset of non-OPSCC. However, further development of a p16 IHC scoring system in non-OPSCC and improvement of HPV detection methods are warranted before broad application in the clinical setting.

J Clin Oncol 32:3930-3938. © 2014 by American Society of Clinical Oncology

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous disease occurring in various sites within the head and neck region, including the oral cavity, oropharynx, hypopharynx, and larynx. The most common risk factors are tobacco and alcohol use and high-risk human papillomavirus (HPV) infection.[1,2] Although the detection rates vary depending on assay selection and study populations, approximately 57% to 72% of oropharyngeal squamous cell carcinomas (OPSCCs) and 1.3% to 7% of non-OPSCCs, including cancers of the oral cavity, hypopharynx, and larynx, are HPV positive.[3-8]

HPV status in tumors can be determined by several assays, including HPV DNA detection by in situ hybridization (ISH) or polymerase chain reaction (PCR), HPV E6/E7 RNA expression detected by quantitative reverse transcriptase–PCR (qRT-PCR), and/or p16 protein expression by immunohistochemistry (IHC) staining as a surrogate marker of oncogenic HPV infection.[5-7,9-11] Among these assays, detection of HPV E6/E7 RNA expression, which indicates active viral oncogene transcription in tumor cells, is considered to be a gold standard.[9,10]

However, because RNA isolation for qRT-PCR requires additional sample preparation steps and a larger amount of tumor cells compared with other assays, the most widely used assays are HPV ISH and p16 IHC. When the results of various assays are compared, the concordance rate between HPV ISH and p16 IHC is approximately 90% in OPSCC, where HPV infection is frequent.[5] However, in oral cavity squamous cell carcinoma (SCC), where infection is relatively less common than in the oropharynx, sensitivity of p16 IHC compared with high-risk HPV E6/7 RNA expression is 79%, specificity is 93%, positive predictive value (PPV) is 41%, and negative predictive value is 99%, indicating that p16 IHC is a poor surrogate biomarker of HPV infection in non-OPSCC sites.[7]

It is also well established that patients with HPV-positive/p16-positive OPSCC have a more favorable prognosis compared with those with HPV-negative/p16-negative OPSCC.[5,6,11,12] However, the prognostic significance of p16 expression in non-OPSCC with or without evidence of HPV infection has not been clearly delineated. The p16 protein is an important tumor suppressor and cell-cycle regulator.[13] In HPV-positive tumors, the viral protein E7 binds to retinoblastoma susceptibility protein (Rb) through cullin 2 ubiquitin ligase complex and rapidly degrades Rb by ubiquitination.[14,15] Loss of Rb results in upregulation of p16 protein expression by a feedback interaction.[16,17] However, increased p16 protein expression is not specific to Rb loss caused by E7 oncoprotein, because other molecular events associated with loss of Rb function, such as *RB1* inactivating mutation, or deletion or chromosomal loss, can result in the same phenotype.

In this study, we evaluated p16 protein expression by IHC and HPV status by HPV ISH as potential prognostic biomarkers in non-OPSCC tumors, where HPV infection is less common than in the oropharynx, in patients enrolled onto three prospective Radiation Therapy Oncology Group (RTOG) clinical trials.

PATIENTS AND METHODS

Protocol

RTOG 0129 was a phase III trial evaluating standard fractionation radiotherapy with concurrent cisplatin versus accelerated fractionation by concomitant boost radiotherapy with concurrent cisplatin for patients with locally advanced HNSCC (N = 743).[5] RTOG 0234 was a phase II randomized trial testing whether radiation therapy with concurrent cetuximab and either cisplatin or docetaxel improved disease-free survival over a historical cohort of patients treated with radiation therapy and concurrent cisplatin (RTOG 9501) for patients at high risk for recurrence after surgical resection of advanced HNSCC (N = 238).[18] RTOG 0522 was a phase III trial testing the addition of cetuximab to radiation therapy with concurrent cisplatin for patients with locally advanced HNSCC (N = 940).[19]

Laboratory Studies

IHC was performed to determine p16 expression using a p16 mouse monoclonal antibody (predilute, mtm-CINtech, E6H4) as previously described.[5] p16 was considered to be positive when defined as strong and diffuse nuclear and cytoplasmic staining in ≥ 70% of the tumor cells, which is the same scoring criteria used in the study by Ang et al.[5] High-risk HPV status was determined by ISH using a cocktail probe that detects HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66 (GenPoint HPV probe cocktail; Dako, Carpinteria, CA).[5] HPV ISH was interpreted as positive when nuclear-specific staining was detected in the tumor cells.

Statistical Analysis

The end points, progression-free (PFS) and overall survival (OS), were as defined in the RTOG 0522 protocol. Nonachievement of PFS was defined as locoregional recurrence or progression, distant metastasis, or death resulting from any cause. Nonachievement of OS was defined as death resulting from any cause. Rates were estimated by the Kaplan-Meier method[20] and compared with a two-sided stratified (by study) log-rank test.[21] Univariable and multivariable hazard ratios (HRs) were estimated using the Cox proportional hazards model,[22] stratified by study. The ϕ coefficient was used to measure the association between HPV and p16 status.

RESULTS

Expression of p16 and High-Risk HPV ISH Status Determination

A total of 683 eligible patients with non-OPSCC tumors, including primary sites in the oral cavity, hypopharynx, and larynx, were identified among the 1,921 patients enrolled onto RTOG 0129, 0234, and 0522 studies. Tumors from 356 (52.1%) of 683 patients with non-OPSCC were tested for p16 expression, which could be determined in 90.4% (322 of 356) of the tumors. The results are summarized in Table 1. Characteristics and outcomes were similar between patients with known and unknown p16 status in each trial (Appendix Table A1, online only). Overall, 19.3% (62 of 322) were p16 positive. The rates of p16 positivity were 14.1%, 24.2%, and 19.0% for RTOG 0129, 0234, and 0522, respectively. Cancer of the oral cavity had the highest rate of p16 positivity (21 [26.3%] of 80), followed by the larynx (31 [17.1%] of 181) and hypopharynx (10 [16.4%] of 61). Patient characteristics by p16 expression status are summarized in Appendix Table A2 (online only).

A total of 311 (45.5%) of 683 tumors had tumor material available for high-risk HPV testing by ISH (Table 1). Of these, HPV status could be determined in 95.5% (297 of 311). Overall, 9.4% (28 of 297) were HPV ISH positive. The HPV ISH–positive rates were 6.5%, 14.6%, and 6.9% for RTOG 0129, 0234, and 0522, respectively. Cancer of the oral cavity had the highest rate of HPV ISH positivity (13 [14.6%] of 89), followed by the larynx (12 [7.9%] of 151) and hypopharynx (three [5.3%] of 57). Patient characteristics by high-risk HPV ISH status are summarized in Appendix Table A3 (online only). When the two tests were compared, there was moderate correlation between p16 and HPV status (ϕ coefficient, 0.46; 95% CI, 0.34 to 0.58; Table 2). The tests were concordant more often when the primary site was the hypopharynx (ϕ coefficient, 0.54; 95% CI, 0.28 to 0.81) or larynx (ϕ coefficient, 0.52; 95% CI, 0.35 to 0.68) compared with the oral cavity (ϕ coefficient, 0.35; 95% CI, 0.13 to 0.57).

Survival Outcomes Based on p16 and HPV ISH Status

We examined survival outcome differences based on p16 status in patients with non-OPSCC tumors. Univariable analysis showed that patients with p16-positive tumors had better PFS and OS than patients with p16-negative tumors (Figs 1A and 1B). For PFS, the HR estimate was 0.65 (95% CI, 0.44 to 0.98), reflecting a 35% reduction in PFS nonachievement for patients with p16-positive tumors ($P = .04$). For OS, the HR was 0.57 (95% CI, 0.36 to 0.90), reflecting a 43% reduction in the death rate for patients with p16-positive tumors ($P = .01$). Furthermore, the effects of p16 expression in PFS and OS were examined after adjustment for known prognostic factors, including age, sex, T stage, and N stage (Table 3). The adjusted HRs were 0.63

Table 1. Summary of p16 Expression and High-Risk HPV Status

Status	RTOG 0129 (n = 288) No.	RTOG 0129 (n = 288) %	RTOG 0234 (n = 129) No.	RTOG 0234 (n = 129) %	RTOG 0522 (n = 266) No.	RTOG 0522 (n = 266) %	Total (N = 683) No.	Total (N = 683) %
HPV tested								
Tested, value known	93	32.3	103	79.8	101	38.0	297	43.5
Tested, value unknown	10	3.5	1	0.8	3	1.1	14	2.0
Not tested	185	64.2	25	19.4	162	60.9	372	54.5
HPV status	n = 93		n = 103		n = 101		n = 297	
Negative	87	93.5	88	85.4	94	93.1	269	90.6
Positive	6	6.5	15	14.6	7	6.9	28	9.4
HPV status, oral cavity	n = 14		n = 75		n = 0*		n = 89	
Negative	12	85.7	64	85.3			76	85.4
Positive	2	14.3	11	14.7			13	14.6
HPV status, hypopharynx	n = 20		n = 7		n = 30		n = 57	
Negative	20	100.0	6	85.7	28	93.3	54	94.7
Positive	0	0.0	1	14.3	2	6.7	3	5.3
HPV status, larynx	n = 59		n = 21		n = 71		n = 151	
Negative	55	93.2	18	85.7	66	93.0	139	92.1
Positive	4	6.8	3	14.3	5	7.0	12	7.9
p16 tested								
Tested, value known	85	29.5	95	73.6	142	53.4	322	47.1
Tested, value unknown	18	6.3	4	3.1	12	4.5	34	5.0
Not tested	185	64.2	30	23.3	112	42.1	327	47.9
p16 status	n = 85		n = 95		n = 142		n = 322	
Negative	73	85.9	72	75.8	115	81.0	260	80.7
Positive	12	14.1	23	24.2	27	19.0	62	19.3
p16 status, oral cavity	n = 12		n = 68		n = 0*		n = 80	
Negative	8	66.7	51	75.0			59	73.8
Positive	4	33.3	17	25.0			21	26.3
p16 status, hypopharynx	n = 20		n = 7		n = 34		n = 61	
Negative	20	100.0	6	85.7	25	73.5	51	83.6
Positive	0	0.0	1	14.3	9	26.5	10	16.4
p16 status, larynx	n = 53		n = 20		n = 108		n = 181	
Negative	45	84.9	15	75.0	90	83.3	150	82.9
Positive	8	15.1	5	25.0	18	16.7	31	17.1

Abbreviations: HPV, human papillomavirus; RTOG, Radiation Therapy Oncology Group.
*Patients with oral cavity tumors were not eligible for RTOG 0522.

(95% CI, 0.42 to 0.95; $P = .03$) and 0.56 (95% CI, 0.35 to 0.89; $P = .01$) for PFS and OS, respectively.

We also examined survival outcome differences based on HPV ISH in these patients. Univariable analysis showed that patients with HPV ISH–positive tumors did not have significantly better PFS (HR, 0.77; 95% CI, 0.44 to 1.33; $P = .35$) or OS (HR, 0.64; 95% CI, 0.34 to 1.21; $P = .17$) than patients with HPV ISH–negative tumors (Figs 1C and 1D). In addition, PFS and OS in p16-positive/HPV-positive, p16-positive/HPV-negative, p16-negative/HPV-positive, and p16-negative/HPV-negative groups were evaluated (Figs 1E and 1F); no significant differences were found (PFS, $P = .23$; OS, $P = .21$), probably because of the small sample sizes for the first three groups (n = 20, 33, and 7, respectively) compared with the p16-negative/HPV-negative group (n = 213).

Survival Outcomes Based on p16 Status and Primary Site

We also examined survival outcomes based on p16 status and three primary sites (oral cavity, hypopharynx, or larynx) separately. Although only PFS in the hypopharynx reached statistical significance because of the small sample size in each site, more favorable outcomes in p16-positive patients compared with p16-negative patients were suggested (Appendix Figs A1 and A2, online only). For the oral cavity, the HRs for PFS and OS were 0.8 (95% CI, 0.42 to 1.55; $P = .52$) and 0.70 (95% CI, 0.33 to 1.47; $P = .34$), respectively. For the larynx, the HRs for PFS and OS were 0.61 (95% CI, 0.33 to 1.13; $P = .11$) and 0.54 (95% CI, 0.27 to 1.10; $P = .08$), respectively. For the hypopharynx, the HRs for PFS and OS were 0.33 (95% CI, 0.12 to 0.96; $P = .03$) and 0.33 (95% CI, 0.10 to 1.09; $P = .06$), respectively, favoring p16-positive patients.

We further examined survival outcomes based on p16 status and primary sites, comparing non-OPSCC with OPSCC to examine the survival difference in non-OPSCC in the context of OPSCC, where the prognostic power of p16 is well established. p16 expression data in OPSCC from the three RTOG trials have been previously published.[5,18,19] With both PFS and OS, there was a significant interaction between p16 status and primary site (interaction $P = .01$ for both; Table 4; Fig 2). Patients with p16-positive OPSCC had half the risk of death when compared with patients with p16-positive non-OPSCC (OS: HR, 0.48; 95% CI, 0.30 to 0.78). However, patients with p16-negative OPSCC and non-OPSCC had similar survival, even after

Table 2. Association Between p16 Expression and High-Risk HPV Status

HPV Status	p16 Status: Negative No.	Negative %	Positive No.	Positive %	Total No.	Total %
All primary sites						
Negative	213	78.0	33	12.1	246	90.1
Positive	7	2.6	20	7.3	27	9.9
Total	220	80.6	53	19.4	273	100.0
Correlation (ϕ coefficient)			0.46			
95% CI			0.34 to 0.58			
Oral cavity only						
Negative	53	67.1	13	16.5	66	83.5
Positive	5	6.3	8	10.1	13	16.5
Total	58	73.4	21	26.6	79	100.0
Correlation (ϕ coefficient)			0.35			
95% CI			0.13 to 0.57			
Hypopharynx only						
Negative	45	83.3	6	11.1	51	94.4
Positive	0	0.0	3	5.6	3	5.6
Total	45	83.3	9	16.7	54	100.0
Correlation (ϕ coefficient)			0.54			
95% CI			0.28 to 0.81			
Larynx only						
Negative	115	82.1	14	10.0	129	92.1
Positive	2	1.4	9	6.4	11	7.9
Total	117	83.6	23	16.4	140	100.0
Correlation (ϕ coefficient)			0.52			
95% CI			0.35 to 0.68			

Abbreviation: HPV, human papillomavirus.

adjustment for other prognostic variables (OS: HR, 0.97; 95% CI, 0.74 to 1.24). Although these data are intriguing, there is significant heterogeneity within the non-OPSCC patient population that is inherent to the complex anatomy of the head and neck region, with numerous anatomic subsites and varying clinical outcomes (Appendix Table A4, online only).

Survival Outcomes Based on p16 Status and Smoking Status

Seventy-five percent of patients with non-OPSCC with p16-positive tumors and 84% of those with p16-negative tumors had > 10 pack-years of exposure. Median number of pack-years was 33.8 (interquartile range, 10 to 51) and 40 (interquartile range, 20 to 60) for patients with p16-positive and p16-negative non-OPSCC tumors, respectively; the difference was not statistically significant ($P = .12$; Appendix Table A2, online only). Adding pack-years to the multivariable models did not change HRs for p16 status, suggesting that survival outcome differences observed between the p16-positive and p16-negative patients were not a function of smoking history (data not shown).

DISCUSSION

Similar to results in patients with OPSCC, our data suggest that patients with p16-positive non-OPSCC have significantly better outcomes than patients with p16-negative disease. Although the result is intriguing and biologically plausible, p16 being an important tumor suppressor for cellular function, the potential biologic or clinical significance of our results is unclear in this heterogeneous group of patients because of several limitations.

Although the relevance of p16 expression as a prognostic biomarker is well established in the context of p16 as a surrogate for HPV infection, the significance of p16 decoupled from HPV is unclear. Numerous studies have developed and validated the scoring system used for defining p16 as positive (diffuse staining > 70% of tumor) in OPSCC, where HPV infection is common.[5,6,9,10,23] However, use of this scoring system may not accurately reflect the significance of p16 expression independent of HPV infection in non-OPSCC. One commonly used method to develop an independent IHC scoring system is to assess an H factor (ie, intensity X percent positive tumor cells) of the p16 staining and identify an optimal threshold for separating the prognostic groups by using test and validation sets. In a recent study of oral cavity SCC, where 409 tumors were evaluated with multiple HPV detection methods, p16 expression was assessed with the H score, but again, the H score cutoff of 60 used was derived from an OPSCC study rather than independently derived from non-OPSCC.[7,10] Unfortunately, our study is limited by an inability to determine H scores, because a majority of our data were generated using tissue microarrays, where the assessment of percent positive tumor cells in a representative core of tumor may not have accurately reflected the tumor overall. However, close evaluation of tumors with available whole-tumor sections showed that only rare samples exhibited strong p16 staining in < 70% of the tumor cells (one [2%] of 50 tumors), and few

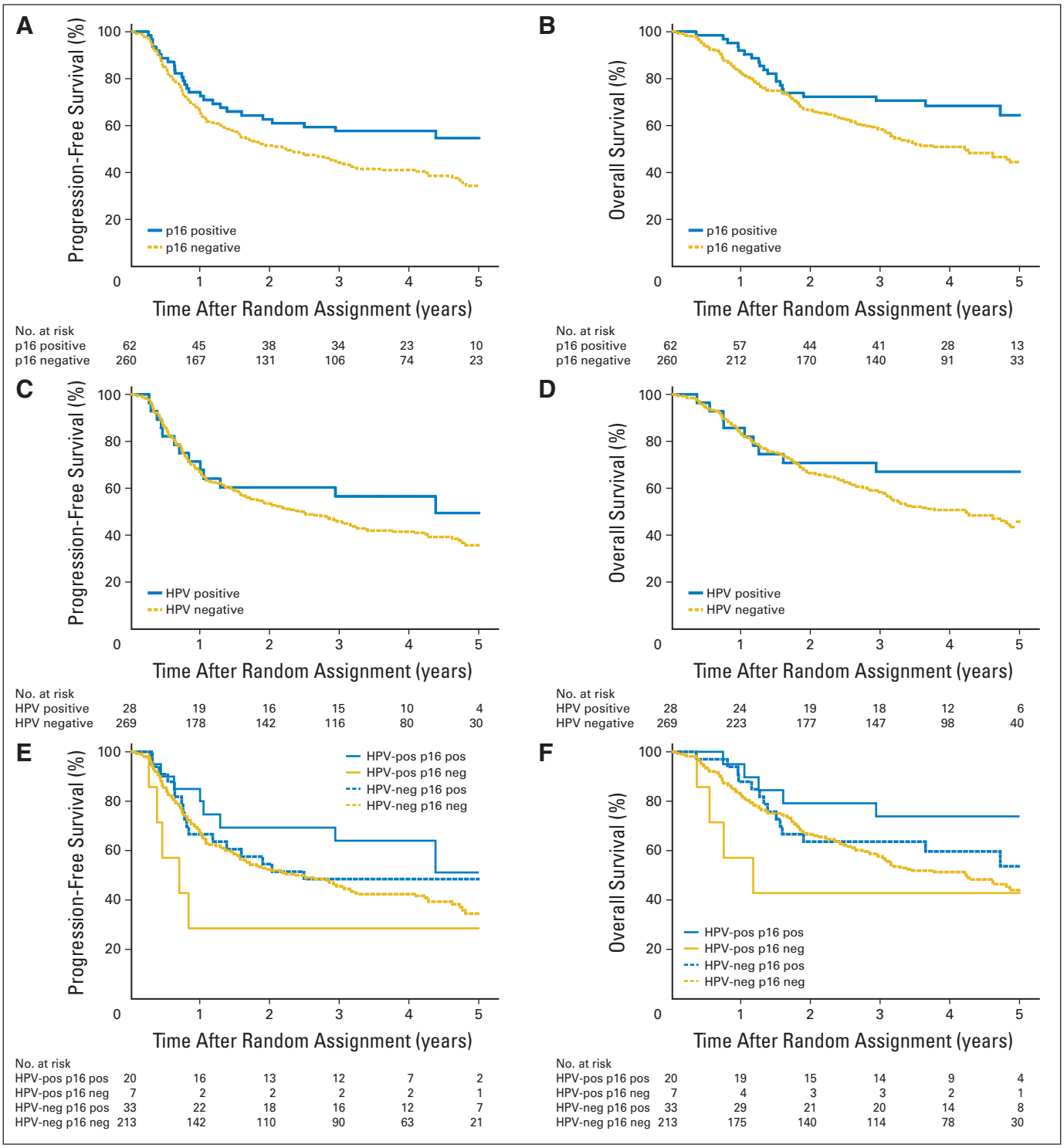

Fig 1. (A) Progression-free (PFS) and (B) overall survival (OS) by p16 expression for patients with nonoropharynx tumors. Patients with p16-positive tumors had significantly longer PFS ($P = .04$) and OS ($P = .01$) than patients with p16-negative tumors. Regarding PFS, 28 of 62 patients with p16-positive tumors and 160 of 260 patients with p16-negative tumors experienced progression; 5-year PFS estimates were 54.7% (95% CI, 41.6 to 67.8) and 34.3% (95% CI, 27.5 to 41.1), respectively. Regarding OS, 21 of 62 patients with p16-positive tumors and 134 of 260 patients with p16-negative tumors died; 5-year OS estimates were 64.4% (95% CI, 50.8 to 77.9) and 44.4% (95% CI, 37.4 to 51.4). (C) PFS and (D) OS by human papillomavirus (HPV) in situ hydridization (ISH) status for patients with nonoropharynx tumors. There was no significant difference in PFS (hazard ratio [HR], 0.77; 95% CI, 0.44 to 1.33; $P = .35$) or OS (HR, 0.64; 95% CI, 0.34 to 1.21; $P = .17$) between HPV ISH–positive and –negative patients. (E) PFS and (F) OS by HPV ISH status and p16 expression. There was no significant difference in PFS or OS (HPV positive/p16 positive *v* HPV positive/p16 negative *v* HPV negative/p16 positive *v* HPV negative/p16 negative: PFS, $P = .23$; OS, $P = .21$; HPV positive/p16 positive *v* HPV negative/p16 positive: PFS, $P = .66$; OS, $P = .46$; HPV positive/p16 positive *v* HPV negative/p16 negative: PFS, $P = .20$; OS, $P = .09$; HPV positive/p16 positive *v* HPV negative/p16 positive or p16 negative: PFS, $P = .23$; OS, $P = .12$).

Table 3. Multivariable Analysis of PFS and OS

End Point Variable	HR*	95% CI	*P*
PFS			
p16 status (positive *v* negative)	0.63	0.42 to 0.95	.03
Age (continuous)	1.02	1.00 to 1.03	.03
Sex (male *v* female)	1.44	1.00 to 2.07	.05
Clinical T stage (T4 *v* T1-3)	1.38	1.01 to 1.88	.04
Clinical N stage (N1-3 *v* N0/NX)	2.63	1.78 to 3.89	< .001
OS			
p16 status (positive *v* negative)	0.56	0.35 to 0.89	.01
Age (continuous)	1.03	1.01 to 1.05	.002
Sex (male *v* female)	1.62	1.07 to 2.45	.02
Clinical T stage (T4 *v* T1-3)	1.55	1.11 to 2.17	.01
Clinical N stage (N1-3 *v* N0/NX)	2.18	1.44 to 3.28	< .001

Abbreviations: HR, hazard ratio; OS, overall survival; PFS, progression-free survival.
*HRs estimated from Cox models stratified by protocol.

samples (three [6%] of 50 tumors) resulted in significant interobserver variability (positive *v* negative) between two pathologists (C.S.K., R.C.J.). This suggests that the OPSCC scoring system may also be applicable in non-OPSCC, but additional evaluation of the scoring system specific to non-OPSCC is required before broad clinical application.

The rare cases of intermediate p16 staining (< 70%) may be explained by data recently presented by the Cancer Genome Atlas (TCGA), which included a comprehensive analysis of 279 HNSCCs (HPV positive, n = 35; HPV negative, n = 244). In this extensive analysis, *CDKN2A* (gene coding p16 protein) was found to be inactivated in up to 90% of the HPV-negative tumors through mutation, deletion, abnormal splicing, or DNA methylation.[24] This is consistent with historical data suggesting that the lack of p16 expression in HPV-negative tumors is not the result of undetectable expression of p16 protein, as in normal tissue,[25,26] but rather the result of a lack of functional *CDKN2A* and subsequent loss of p16 expression. The few tumors with intermediate p16 staining may be the result of incomplete loss of p16 expression or rare molecular alterations that require further investigation. The clinical significance of tumors with intermediate p16 staining is currently unknown, given the limited number of patient cases.

Furthermore, determination of the prognostic value of p16 independent of HPV infection requires identification of true HPV-negative patients, but our study is hampered by limited tumor availability, which precluded HPV E6/7 RNA detection by the gold-standard qRT-PCR assay. This is a major limitation, because HPV ISH, used in our study, is known to be specific but less sensitive than other HPV detection methods.[6,9,10,23] Our data show that 12% of non-OPSCCs were p16 positive/HPV negative, suggesting this group may represent a mixture of true HPV-positive patients with false-negative HPV ISH results and true HPV-negative patients, in whom

Table 4. Interaction of p16 Status and Primary Site

	Model One*		Model Two†	
End Point Comparison	HR	95% CI	HR	95% CI
PFS				
p16 positive *v* negative				
Oropharynx	0.37	0.29 to 0.47	0.39	0.31 to 0.50
Nonoropharynx	0.67	0.45 to 1.00	0.65	0.43 to 0.97
Oropharynx *v* nonoropharynx				
p16 positive	0.54	0.36 to 0.82	0.48	0.32 to 0.74
p16 negative	0.99	0.77 to 1.26	0.80	0.62 to 1.03
P for interaction	.01		.04	
OS				
p16 positive *v* negative				
Oropharynx	0.29	0.22 to 0.38	0.31	0.24 to 0.42
Nonoropharynx	0.58	0.36 to 0.91	0.57	0.36 to 0.90
Oropharynx *v* nonoropharynx				
p16 positive	0.48	0.30 to 0.78	0.44	0.27 to 0.72
p16 negative	0.97	0.74 to 1.27	0.80	0.60 to 1.05
P for interaction	.01		.03	

Abbreviations: HR, hazard ratio; OS, overall survival; PFS, progression-free survival.
*Model one: HRs estimated from Cox models stratified by protocol, with covariates p16 status, primary site, and interaction of p16 status and primary site.
†Model two: HRs estimated from Cox models stratified by protocol, with covariates p16 status, primary site, interaction of p16 status and primary site, age, sex, T stage, and N stage.

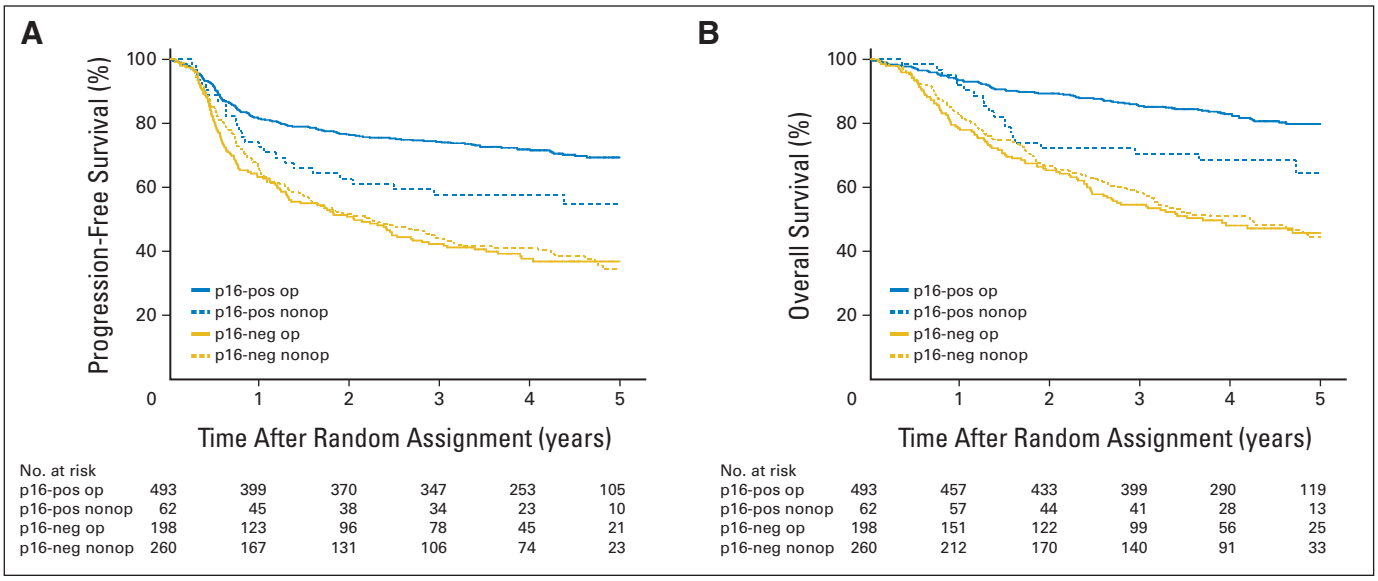

Fig 2. (A) Progression-free (PFS) and (B) overall survival (OS) by p16 expression and primary site (oropharyngeal squamous cell carcinoma [OPSCC] *v* non-OPSCC); 5-year PFS estimates were 69.3% (95% CI, 65.0% to 73.6%), 54.7% (95% CI, 41.6% to 67.8%), 36.8% (95% CI, 29.8% to 43.9%), and 34.3% (95% CI, 27.5% to 41.1%) for patients with p16-positive OPSCC (p16 pos op), p16-positive non-OPSCC (p16 pos nonop), p16-negative OPSCC (p16 neg op), and p16-negative non-OPSCC (p16 neg nonop), respectively; 5-year OS estimates were 79.8% (95% CI, 76.0% to 83.7%), 64.4% (95% CI, 50.8 to 77.9), 45.8% (95% CI, 38.1% to 53.5%), and 44.4% (95% CI, 37.4% to 51.4%) for patients with p16-positive OPSCC, p16-positive non-OPSCC, p16-negative OPSCC, and p16-negative non-OPSCC, respectively. There was significant interaction between p16 status and primary site for both PFS ($P = .01$) and OS ($P = .01$).

other molecular mechanisms led to overexpression of p16 in the absence of HPV infection. Therefore, p16 prognostic significance may be driven by HPV as well as *RB1* loss, which can induce p16 overexpression. TCGA data show *RB1* mutations in 4% of HPV-negative tumors.[16,17,24] In addition, 2.6% of non-OPSCCs in our study were p16 negative/HPV positive, and TCGA data show that no HPV-positive tumors had *CDKN2A* inactivation.[24] Even considering the relatively few HPV-positive tumors in the TCGA data set, our data suggest that the p16-negative/HPV-positive patient cases may be at least in part the result of false-negative p16. Therefore, additional research using multimodality testing in non-OPSCC and development of more accurate HPV testing are indicated.

We have in addition observed differences in the concordance between p16 and HPV ISH among the subsites of the oral cavity, hypopharynx, and larynx, where oral cavity tumors have the worst concordance. This difference could be the result of the biologic and anatomic heterogeneity among these sites. For example, large tumors in the retromolar trigone may be indistinguishable from a tonsillar primary because of the anatomic proximity of these sites, resulting in misclassification. In addition, ectopic tonsillar tissue, albeit rare, has been reported in the floor of the mouth, where HPV infection can lead to the development of a tumor.[27,28] Again, in the published study of oral cavity SCC, poor concordance between p16 and HPV ISH was primarily because of poor sensitivity of p16 IHC in detecting HPV.[7] The PPV of HPV ISH was 95.2%, but the PPV of p16 IHC was only 40.4% when compared with HPV E6/7 RNA expression.[7] Although this study determined that p16 is a poor surrogate marker of HPV infection in the oral cavity, consistent with our data, it did not evaluate whether p16 expression is prognostic independent of HPV status. In recent studies of oral tongue SCC (further delineating subsite even within oral cavity), Lim et al[29] evaluated *CDKN2A* (p16) aberrations and did not observe a significant correlation with clinical characteristics or outcome, whereas Harris et al[30] reported a link between p16 expression and improved outcomes. These data demonstrate that each anatomic subsite needs separate evaluation with a larger sample size to clearly determine the prognostic role of p16 expression, and our findings should be interpreted with caution because of the small sample sizes in each site.

In addition, presence of p16 expression may affect treatment outcomes and may have contributed to the survival differences seen in our study. In a recent study, p16 was shown to sensitize HPV-positive cells to ionizing radiation by inhibiting homologous recombination-mediated DNA damage response and downregulating cyclin D1 expression. The inhibition of the DNA damage response by p16 is thought to be independent of its cell-cycle regulation–inhibiting CDK4/6 activity.[31] A majority of patients with oral cavity tumors in our study were treated with primary surgery followed by adjuvant therapy (68 [85%] of 80 in RTOG 0234), whereas a majority of patients with tumors in the larynx or hypopharynx were treated with primary concurrent chemoradiotherapy (161 [89%] of 181 and 54 [89%] of 61 in RTOG 0129 and 0522, respectively). Although we could not clearly delineate the prognostic impact of p16 expression in each subsite or in HPV-negative/p16-negative and HPV-negative/p16-positive groups because of the limited sample sizes, p16 as a biomarker independent of HPV status or potential biomarker of radiation sensitivity in non-OPSCC needs further evaluation.

The clinically significant role of p16 expression as a surrogate marker of HPV infection for OPSCC is clearly established and appropriate. It is exemplified by its use as an integral biomarker for patient selection in clinical trials where there is a major effort to decrease the unnecessary toxicities of current treatment for HPV-positive patients without compromising survival. However, our data show that the use

of p16 IHC as a surrogate for HPV infection or as a prognostic biomarker in non-OPSCC requires further investigation before broad application in the clinical setting, and the role of p16 expression in radiation sensitivity warrants further investigation.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.
Employment or Leadership Position: None **Consultant or Advisory Role:** None **Stock Ownership:** None **Honoraria:** David Raben, Merck, Bristol-Myers Squibb **Research Funding:** None **Expert Testimony:** None **Patents, Royalties, and Licenses:** None **Other Remuneration:** Maura L Gillison, Roche

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GLOSSARY TERMS

immunohistochemistry: the application of antigen-antibody interactions to histochemical techniques. Typically, a tissue section is mounted on a slide and incubated with antibodies (polyclonal or monoclonal) specific to the antigen (primary reaction). The antigen-antibody signal is then amplified using a second antibody conjugated to a complex of peroxidase-antiperoxidase, avidin-biotin-peroxidase, or avidin-biotin alkaline phosphatase. In the presence of substrate and chromogen, the enzyme forms a colored deposit at the sites of antibody-antigen binding. Immunofluorescence is an alternate approach to visualize antigens. In this technique, the primary antigen-antibody signal is amplified using a second antibody conjugated to a fluorochrome. On ultraviolet light absorption, the fluorochrome emits its own light at a longer wavelength (fluorescence), thus allowing localization of antibody-antigen complexes.

p16: molecule that binds to cyclin-dependent kinase 4 and 6, thereby preventing their interaction with cyclin D. p16 (also known as $p16^{INK4}$) behaves as a negative regulator of proliferation and arrests cells in the G_0/G_1 phase of the cell cycle.

polymerase chain reaction (PCR): a method that allows logarithmic amplification of short DNA sequences within a longer DNA molecule.

pRb (Rb phosphorylation): phosphorylated form of *Rb*, the retinoblastoma susceptibility gene product. Phosphorylated *Rb* has important ramifications for cell cycle progression. In the phosphorylated state, *Rb* is unable to bind to the transcriptional factor E2F (which is also important for cell cycle regulation). This results in an excess of free E2F, which can then induce transcription of genes involved in cell cycle progression. Hence, phosphorylation of *Rb* allows cells to progress through the G_1 checkpoint into the S phase of the cell cycle. See *Rb*.

Appendix

Table A1. Characteristics of Patients With and Without p16 Expression Data

	RTOG 0129				RTOG 0234				RTOG 0522				Total			
	p16 Unknown (n = 203)		p16 Known (n = 85)		p16 Unknown (n = 34)		p16 Known (n = 95)		p16 Unknown (n = 124)		p16 Known (n = 142)		p16 Unknown (n = 361)		p16 Known (n = 322)	
Characteristic	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Age, years																
Mean	56.1		57.1		57.0		56.0		57.0		58.1		56.5		57.2	
SD	8.46		9.71		10.84		10.25		8.30		8.59		8.64		9.41	
Median	57		56		58		57		56.5		59		57		58	
Minimum to maximum	26 to 82		30 to 81		32 to 76		27 to 77		34 to 77		31 to 79		26 to 82		27 to 81	
Q1 to Q3	51 to 61		51 to 63		49 to 63		50 to 63		51 to 62		52 to 65		51 to 62		51 to 64	
Sex																
Male	170	83.7	61	71.8	25	73.5	67	70.5	101	81.5	116	81.7	296	82.0	244	75.8
Female	33	16.3	24	28.2	9	26.5	28	29.5	23	18.5	26	18.3	65	18.0	78	24.2
Zubrod performance status																
0	106	52.2	41	48.2	15	44.1	42	44.2	75	60.5	76	53.5	196	54.3	159	49.4
1	97	47.8	44	51.8	19	55.9	53	55.8	49	39.5	66	46.5	165	45.7	163	50.6
Smoking history	n = 144		n = 61		n = 31		n = 73		n = 96		n = 128		n = 271		n = 262	
Never smoked	16	11.1	5	8.2	8	25.8	17	23.3	4	4.2	7	5.5	28	10.3	29	11.1
Ever smoked	128	88.9	56	91.8	23	74.2	56	76.7	92	95.8	121	94.5	243	89.7	233	88.9
Smoking history, pack-years*	n = 144		n = 61		n = 31		n = 73		n = 96		n = 128		n = 271		n = 262	
Mean	40.0		43.2		24.6		33.8		39.7		42.6		38.1		40.3	
SD	31.66		28.42		27.44		29.96		30.86		28.74		31.20		29.18	
Median	37.5		40		17.5		33		34		40		35		40	
Minimum to maximum	0 to 137.5		0 to 111		0 to 114		0 to 120		0 to 162		0 to 135		0 to 162		0 to 135	
Q1 to Q3	14.925 to 58		24 to 56.25		0 to 42		1.5 to 51		20 to 52.5		20 to 60		15 to 53.75		17.6 to 60	
Primary site																
Oral cavity	30	14.8	12	14.1	27	79.4	68	71.6	0	0.0	0	0.0	57	15.8	80	24.8
Hypopharynx	38	18.7	20	23.5	5	14.7	7	7.4	28	22.6	34	23.9	71	19.7	61	18.9
Larynx	135	66.5	53	62.4	2	5.9	20	21.1	96	77.4	108	76.1	233	64.5	181	56.2
Clinical T stage																
T1	0	0.0	0	0.0	4	11.8	12	12.6	0	0.0	0	0.0	4	1.1	12	3.7
T2	29	14.3	10	11.8	9	26.5	28	29.5	23	18.5	35	24.6	61	16.9	73	22.7
T3	115	56.7	46	54.1	7	20.6	23	24.2	79	63.7	76	53.5	201	55.7	145	45.0
T4	59	29.1	29	34.1	10	29.4	32	33.7	22	17.7	31	21.8	91	25.2	92	28.6
TX	0	0.0	0	0.0	2	5.9	0	0.0	0	0.0	0	0.0	2	0.6	0	0.0
Unknown	0	0.0	0	0.0	2	5.9	0	0.0	0	0.0	0	0.0	2	0.6	0	0.0
Clinical N stage																
N0	69	34.0	30	35.3	7	20.6	15	15.8	34	27.4	35	24.6	110	30.5	80	24.8
N1	31	15.3	20	23.5	2	5.9	31	32.6	14	11.3	21	14.8	47	13.0	72	22.4
N2a	12	5.9	2	2.4	3	8.8	7	7.4	2	1.6	9	6.3	17	4.7	18	5.6
N2b	35	17.2	15	17.6	11	32.4	20	21.1	32	25.8	23	16.2	78	21.6	58	18.0
N2c	42	20.7	16	18.8	8	23.5	14	14.7	40	32.3	51	35.9	90	24.9	81	25.2
N3	14	6.9	2	2.4	1	2.9	2	2.1	2	1.6	3	2.1	17	4.7	7	2.2
NX	0	0.0	0	0.0	0	0.0	2	2.1	0	0.0	0	0.0	0	0.0	2	0.6
Unknown	0	0.0	0	0.0	2	5.9	4	4.2	0	0.0	0	0.0	2	0.6	4	1.2

Abbreviations: Q1, first quartile; Q3, third quartile; RTOG, Radiation Therapy Oncology Group; SD, standard deviation.
*Pack-year is defined as equivalent of smoking one pack of cigarettes per day for 1 year.

Table A2. Patient Characteristics by p16 Expression

Characteristic	RTOG 0129				RTOG 0234				RTOG 0522				Total			
	p16 Negative (n = 73)		p16 Positive (n = 12)		p16 Negative (n = 72)		p16 Positive (n = 23)		p16 Negative (n = 115)		p16 Positive (n = 27)		p16 Negative (n = 260)		p16 Positive (n = 62)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Age, years (P = .38)*																
Mean	57.2		56.4		56.3		55.0		58.4		56.8		57.5		56.1	
SD	9.86		9.13		10.70		8.85		8.48		9.08		9.53		8.89	
Median	55		59.5		58		55		59		58		58		57	
Minimum to maximum	30 to 81		34 to 66		27 to 77		36 to 69		31 to 79		42 to 71		27 to 81		34 to 71	
Q1 to Q3	51 to 63		51 to 62.5		50 to 63		49 to 63		52 to 65		47 to 63		52 to 64		50 to 63	
Sex (P = .87)†																
Male	52	71.2	9	75.0	52	72.2	15	65.2	92	80.0	24	88.9	196	75.4	48	77.4
Female	21	28.8	3	25.0	20	27.8	8	34.8	23	20.0	3	11.1	64	24.6	14	22.6
Zubrod performance status (P = .02)†																
0	33	45.2	8	66.7	31	43.1	11	47.8	56	48.7	20	74.1	120	46.2	39	62.9
1	40	54.8	4	33.3	41	56.9	12	52.2	59	51.3	7	25.9	140	53.8	23	37.1
Smoking history (P = .63)†	n = 51		n = 10		n = 55		n = 18		n = 101		n = 27		n = 207		n = 55	
Never smoked	4	7.8	1	10.0	14	25.5	3	16.7	4	4.0	3	11.1	22	10.6	7	12.7
Ever smoked	47	92.2	9	90.0	41	74.5	15	83.3	97	96.0	24	88.9	185	89.4	48	87.3
Smoking history: pack-years (P = .12)*‡	n = 51		n = 10		n = 55		n = 18		n = 101		n = 27		n = 207		n = 55	
Mean	45.9		29.7		31.9		39.9		45.1		33.1		41.8		34.7	
SD	29.51		17.39		28.15		35.08		29.00		26.13		29.38		27.96	
Median	40		31.875		33		33.85		44		35		40		33.75	
Minimum to maximum	0 to 111		0 to 52.5		0 to 110		0 to 120		0 to 135		0 to 90		0 to 135		0 to 120	
Q1 to Q3	25 to 70		18 to 41		0 to 50		4 to 67.5		25 to 61.5		8 to 50		20 to 60		10 to 51	
Primary site (P = .19)§																
Oral cavity	8	11.0	4	33.3	51	70.8	17	73.9	0	0.0	0	0.0	59	22.7	21	33.9
Hypopharynx	20	27.4	0	0.0	6	8.3	1	4.3	25	21.7	9	33.3	51	19.6	10	16.1
Larynx	45	61.6	8	66.7	15	20.8	5	21.7	90	78.3	18	66.7	150	57.7	31	50.0
Clinical T stage (P = .60)*																
T1	0	0.0	0	0.0	8	11.1	4	17.4	0	0.0	0	0.0	8	3.1	4	6.5
T2	8	11.0	2	16.7	21	29.2	7	30.4	29	25.2	6	22.2	58	22.3	15	24.2
T3	39	53.4	7	58.3	21	29.2	2	8.7	60	52.2	16	59.3	120	46.2	25	40.3
T4	26	35.6	3	25.0	22	30.6	10	43.5	26	22.6	5	18.5	74	28.5	18	29.0
Clinical N stage (P = .59)*																
N0	28	38.4	2	16.7	13	18.1	2	8.7	26	22.6	9	33.3	67	25.8	13	21.0
N1	18	24.7	2	16.7	22	30.6	9	39.1	18	15.7	3	11.1	58	22.3	14	22.6
N2a	2	2.7	0	0.0	4	5.6	3	13.0	7	6.1	2	7.4	13	5.0	5	8.1
N2b	11	15.1	4	33.3	16	22.2	4	17.4	20	17.4	3	11.1	47	18.1	11	17.7
N2c	12	16.4	4	33.3	10	13.9	4	17.4	41	35.7	10	37.0	63	24.2	18	29.0
N3	2	2.7	0	0.0	2	2.8	0	0.0	3	2.6	0	0.0	7	2.7	0	0.0
NX	0	0.0	0	0.0	1	1.4	1	4.3	0	0.0	0	0.0	1	0.4	1	1.6
Unknown	0	0.0	0	0.0	4	5.6	0	0.0	0	0.0	0	0.0	4	1.5	0	0.0

Abbreviations: Q1, first quartile; Q3, third quartile; RTOG, Radiation Therapy Oncology Group; SD, standard deviation.
*Wilcoxon rank-sum test comparing p16-negative and p16-positive total columns; NX and unknown were excluded.
†Fisher's exact test comparing p16-negative and p16-positive total columns.
‡Pack-year is defined as equivalent of smoking one pack of cigarettes per day for 1 year.
§Pearson's χ^2 test comparing p16-negative and p16-positive total columns.

Table A3. Patient Characteristics by High-Risk HPV ISH Status

Characteristic	RTOG 0129				RTOG 0234				RTOG 0522				Total			
	HPV Negative (n = 87)		HPV Positive (n = 6)		HPV Negative (n = 88)		HPV Positive (n = 15)		HPV Negative (n = 94)		HPV Positive (n = 7)		HPV Negative (n = 269)		HPV Positive (n = 28)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Age, years (P = .82)*																
Mean	57.3		56.2		55.4		56.7		59.1		55.0		57.3		56.2	
SD	9.19		12.19		10.68		9.79		8.29		6.53		9.51		9.34	
Median	57		60.5		57		59		60		57		58		58.5	
Minimum to maximum	30 to 81		34 to 66		27 to 77		30 to 67		36 to 79		42 to 62		27 to 81		30 to 67	
Q1 to Q3	51 to 62		51 to 65		49.5 to 63		49 to 65		52 to 65		52 to 59		51 to 63		51.5 to 63	
Sex (P = .25)†																
Male	62	71.3	6	100.0	60	68.2	12	80.0	78	83.0	6	85.7	200	74.3	24	85.7
Female	25	28.7	0	0.0	28	31.8	3	20.0	16	17.0	1	14.3	69	25.7	4	14.3
Zubrod performance status (P = .33)†																
0	43	49.4	4	66.7	38	43.2	6	40.0	45	47.9	6	85.7	126	46.8	16	57.1
1	44	50.6	2	33.3	50	56.8	9	60.0	49	52.1	1	14.3	143	53.2	12	42.9
Smoking history (P = .75)†	n = 61		n = 5		n = 69		n = 12		n = 88		n = 7		n = 218		n = 24	
Never smoked	5	8.2	0	0.0	18	26.1	1	8.3	4	4.5	1	14.3	27	12.4	2	8.3
Ever smoked	56	91.8	5	100.0	51	73.9	11	91.7	84	95.5	6	85.7	191	87.6	22	91.7
Smoking history: pack-years (P = .07)*‡	n = 61		n = 5		n = 69		n = 12		n = 88		n = 7		n = 218		n = 24	
Mean	44.6		25.6		32.7		36.8		44.4		16.7		40.8		28.6	
SD	29.31		16.66		30.59		24.64		27.57		16.18		29.42		22.07	
Median	40		18		32		37.85		42		15		40		22	
Minimum to maximum	0 to 111		10 to 52.5		0 to 120		0 to 78		0 to 135		0 to 45		0 to 135		0 to 78	
Q1 to Q3	25 to 60		17.5 to 30		0 to 50		18.75 to 55		27.15 to 60		1.35 to 30		18 to 58		12.5 to 45	
Primary site (P = .11)§																
Oral cavity	12	13.8	2	33.3	64	72.7	11	73.3	0	0.0	0	0.0	76	28.3	13	46.4
Hypopharynx	20	23.0	0	0.0	6	6.8	1	6.7	28	29.8	2	28.6	54	20.1	3	10.7
Larynx	55	63.2	4	66.7	18	20.5	3	20.0	66	70.2	5	71.4	139	51.7	12	42.9
Clinical T stage (P = .96)*																
T1	0	0.0	0	0.0	12	13.6	1	6.7	0	0.0	0	0.0	12	4.5	1	3.6
T2	9	10.3	2	33.3	24	27.3	6	40.0	26	27.7	2	28.6	59	21.9	10	35.7
T3	47	54.0	1	16.7	25	28.4	1	6.7	49	52.1	4	57.1	121	45.0	6	21.4
T4	31	35.6	3	50.0	27	30.7	7	46.7	19	20.2	1	14.3	77	28.6	11	39.3
Clinical N stage (P = .04)*																
N0	33	37.9	0	0.0	17	19.3	0	0.0	20	21.3	2	28.6	70	26.0	2	7.1
N1	20	23.0	2	33.3	27	30.7	6	40.0	14	14.9	1	14.3	61	22.7	9	32.1
N2a	2	2.3	0	0.0	5	5.7	2	13.3	8	8.5	1	14.3	15	5.6	3	10.7
N2b	14	16.1	0	0.0	20	22.7	2	13.3	20	21.3	0	0.0	54	20.1	2	7.1
N2c	16	18.4	3	50.0	11	12.5	5	33.3	32	34.0	3	42.9	59	21.9	11	39.3
N3	2	2.3	1	16.7	2	2.3	0	0.0	0	0.0	0	0.0	4	1.5	1	3.6
NX	0	0.0	0	0.0	2	2.3	0	0.0	0	0.0	0	0.0	2	0.7	0	0.0
Unknown	0	0.0	0	0.0	4	4.5	0	0.0	0	0.0	0	0.0	4	1.5	0	0.0

Abbreviations: ISH, in situ hybridization; Q1, first quartile; Q3, third quartile; RTOG, Radiation Therapy Oncology Group; SD, standard deviation.
*Wilcoxon rank-sum test comparing p16-negative and p16-positive total columns; NX and unknown were excluded.
†Fisher's exact test comparing p16-negative and p16-positive total columns.
‡Pack-year is defined as equivalent of smoking one pack of cigarettes per day for 1 year.
§Pearson's χ^2 test comparing p16-negative and p16-positive total columns.

Table A4. Anatomic Subsites Included As Nonoropharynx Squamous Cell Carcinoma

Subsite	No. of Patients	%
Oral cavity		
Oral tongue	40	12.4
Floor of mouth	19	5.9
Buccal mucosa	6	1.9
Lower gingiva	3	0.9
Upper gingiva	3	0.9
Retromolar trigone	6	1.9
Oral cavity, NOS	3	0.9
Larynx	3	0.9
Ventricular band	3	0.9
Arytenoid	7	2.2
Suprahyoid epiglottis	7	2.2
Infrahyoid epiglottis	6	1.9
Aryepiglottic fold	12	3.7
Vocal cords	20	6.2
Subglottis	4	1.2
Supraglottic larynx, NOS	98	30.4
Glottic larynx, NOS	23	7.1
Subglottic larynx, NOS	1	0.3
Hypopharynx		
Pyriform fossa	34	10.6
Postcricoid area	2	0.6
Posterior wall	7	2.2
Hypopharynx, NOS	18	5.6

Abbreviations: NOS, not otherwise specified.

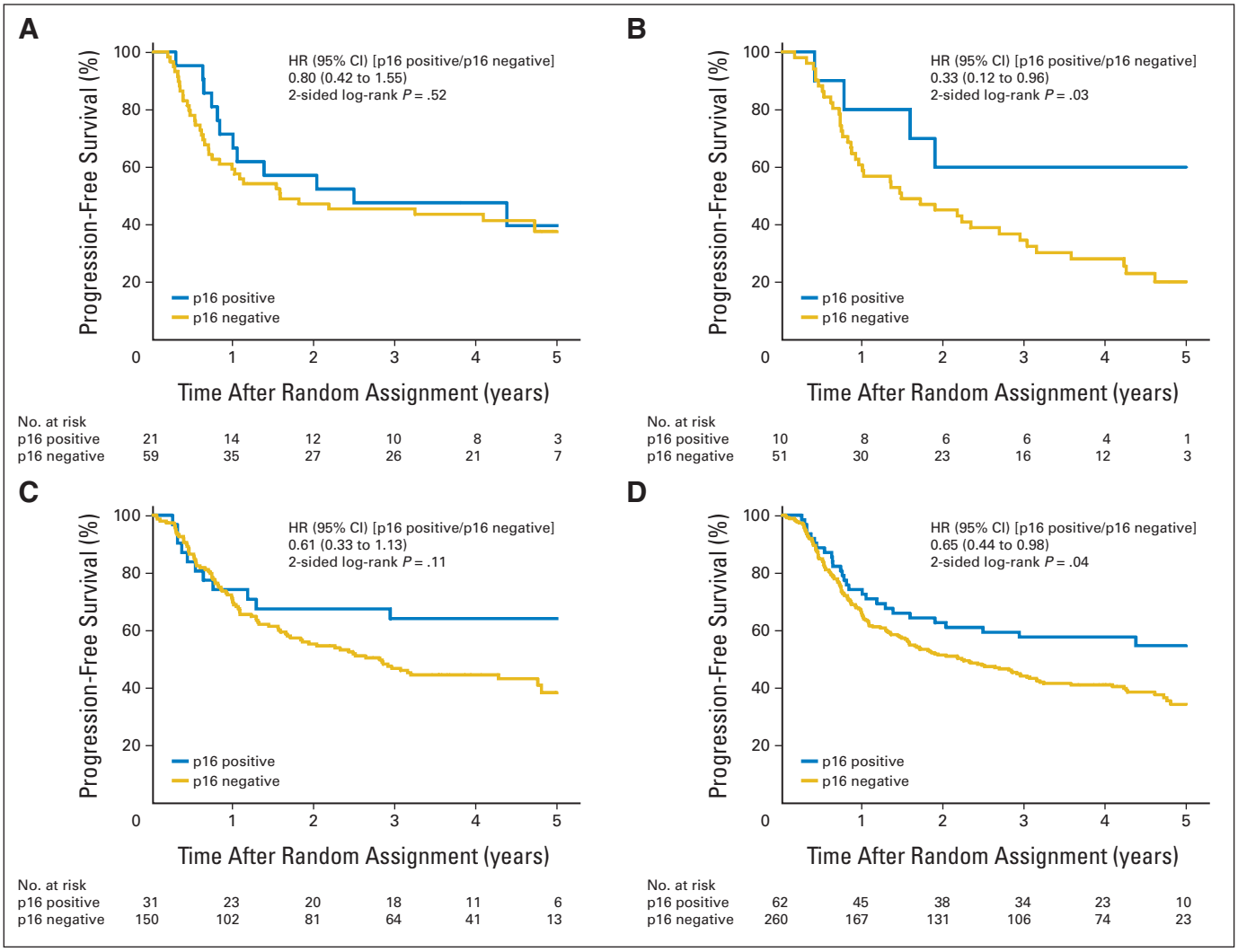

Fig A1. Progression-free survival by p16 expression for patients with (A) oral cavity, (B) hypopharynx, and (C) larynx tumors and (D) for these patients combined. HR, hazard ratio.

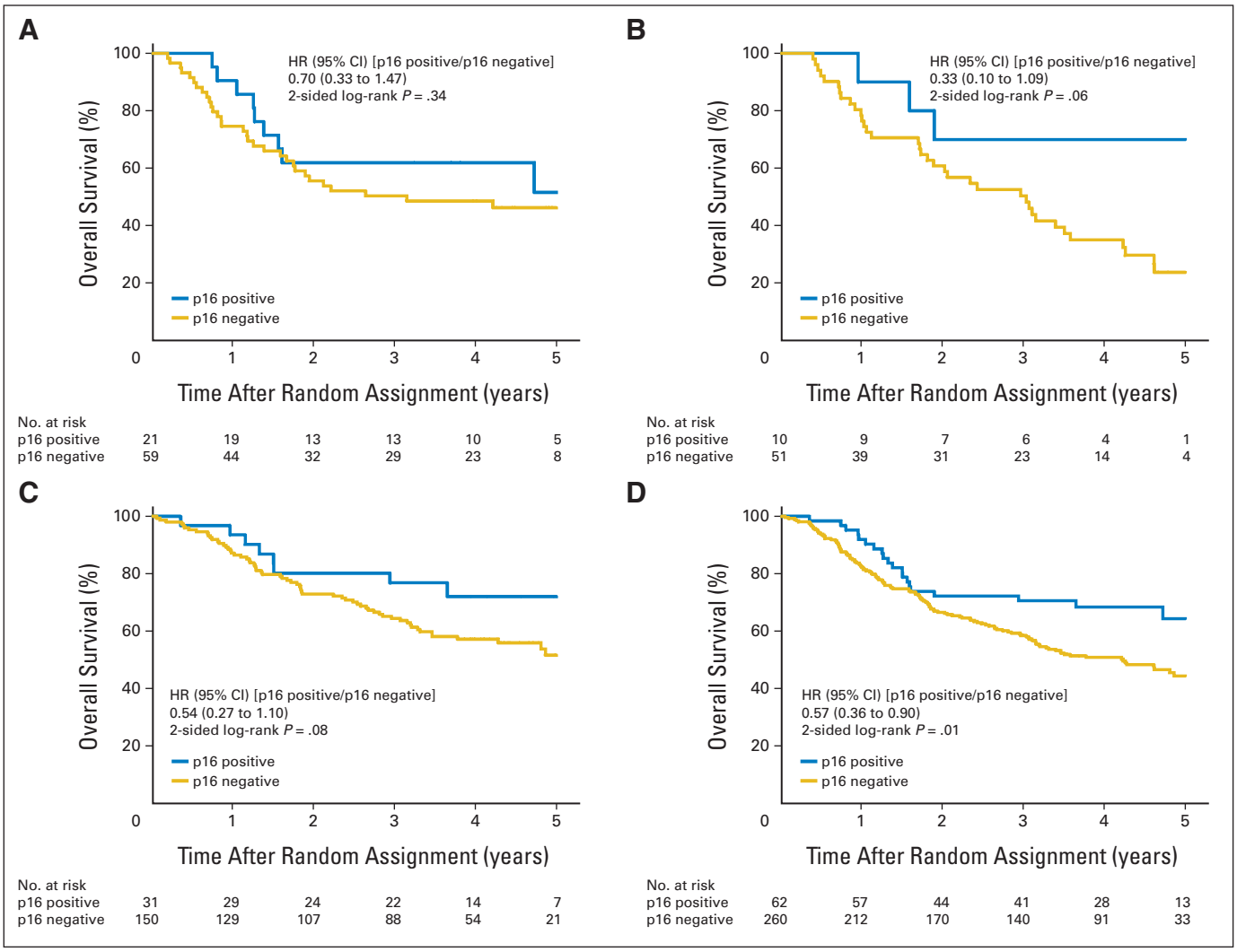

Fig A2. Overall survival by p16 expression for patients with (A) oral cavity, (B) hypopharynx, and (C) larynx tumors and (D) for these patients combined. HR, hazard ratio.