Combined P16 and human papillomavirus testing predicts head and neck cancer survival

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While its prognostic significance remains unclear, p16\textsuperscript{INK4a} protein expression is increasingly being used as a surrogate marker for oncogenic human papillomavirus (HPV) infection in head and neck squamous cell carcinomas (HNSCC). To evaluate the prognostic utility of p16 expression in HNSCC, we prospectively collected 163 primary tumor specimens from histologically confirmed HNSCC patients who were followed for up to 9.4 years. Formalin fixed tumor specimens were tested for p16 protein expression by immunohistochemistry (IHC). HPV type-16 DNA and RNA was detected by MY09/11-PCR and E6/E7 RT-PCR on matched frozen tissue, respectively. P16 protein expression was detected more often in oropharyngeal tumors (53%) as compared with laryngeal (24%), hypopharyngeal (8%) or oral cavity tumors (4%; \( p < 0.0001 \)). With respect to prognosis, p16-positive oropharyngeal tumors exhibited significantly better overall survival than p16-negative tumors (log-rank test \( p = 0.04 \)), whereas no survival benefit was observed for nonoropharyngeal tumors. However, when both p16 and HPV DNA test results were considered, concordantly positive nonoropharyngeal HNSCC had significantly better disease-specific survival than concordantly negative nonoropharyngeal tumors after controlling for sex, nodal stage, tumor site number, smoking and drinking [adjusted hazard ratio (HR) = 0.04, 0.01–0.54]. Compared with concordantly negative nonoropharyngeal HNSCC, \( p16(+)\)/HPV16(−) nonoropharyngeal HNSCC (\( n = 13 \), 7%) demonstrated no significant improvement in disease-specific survival when HPV16 was detected by RNA (adjusted HR = 0.83, 0.22–3.17). Our findings show that p16 IHC alone has potential as a prognostic test for oropharyngeal cancer survival, but combined p16/HPV testing is necessary to identify HPV-associated nonoropharyngeal HNSCC with better prognosis.

Human papillomavirus (HPV)-positive head and neck squamous cell carcinomas (HNSCC) represent a distinct set of tumors that exhibit better prognosis than their HPV-negative counterparts. While the majority of HPV-positive head and neck cancers originate in the oropharynx, a subset of nonoropharyngeal head and neck cancers also present with HPV. HPV DNA detected in these tumors is predominantly of genotype 16, a high-risk type also found in cervical cancer, and is often transcriptionally active, expressing the viral oncogenes E6 and E7.

The viral E7 product inactivates the retinoblastoma protein, which leads to perturbation in transcription factors including E2F,\textsuperscript{5,6} leading to overexpression of p16.\textsuperscript{5,6} P16\textsuperscript{INK4a} immunohistochemistry (IHC) is therefore increasingly being used as a surrogate for oncogenically active HPV infection to characterize HNSCC, particularly in the oropharynx. While some evidence shows that p16 overexpression in HNSCC is associated with improved survival and locoregional control,\textsuperscript{7} results have been inconsistent.\textsuperscript{8,9} While p16 overexpression may be useful as a surrogate marker for HPV infection in oropharyngeal SCC where HPV prevalence can be high, it has low positive predictive value (i.e., poorly discriminates false-positive from true-positive cases) in nonoropharyngeal HNSCC where HPV prevalence is much lower.\textsuperscript{10,11}
Current trials are assessing whether alternative treatment can be provided for HPV-associated oropharyngeal cancers. To improve clinical management, it is critical that clinical tests accurately identify HPV-driven and non-HPV associated HNSCC. The gold standard for HPV detection uses PCR-based methods that target either HPV DNA or RNA sequences, but these are labor intensive and not yet clinically approved. Using a combination of biomarkers involving p16 followed by HPV reflex testing may therefore help improve accuracy in patient prognosis. However, we have found that current options in clinical use, including in situ hybridization assays for HPV DNA detection, perform poorly. The purpose of this prospective study was to evaluate the prognostic utility of p16 protein expression alone and in combination with HPV16 detection in HNSCC.

**Material and Methods**

**Study population and samples**

The study cohort consisted of 158 prospectively enrolled patients with 163 primary HNSCC (including 5 with multiple primary tumors) treated with curative intent at Montefiore Medical Center (MMC) in New York. Following histologic confirmation, stage was determined based on the American Joint Committee on Cancer classification (sixth and seventh editions), and detailed clinical and pathologic data, including information on smoking history and alcohol consumption, were collected by medical interview. Institutional Review Boards at MMC and Albert Einstein College of Medicine approved the study protocol, and all patients provided written informed consent.

Tumor samples and tissues were collected by surgical resection or biopsy prior to therapy, and immediately snap frozen in liquid nitrogen. Frozen tissues were screened for the presence of tumor cells, and the remaining tumor tissue was homogenized prior to laboratory analyses.

**P16-INK4a IHC**

Immunohistochemical detecting p16-INK4a was performed by a single pathologist (YW) blinded to HPV status. A portion of each tumor was routinely fixed in 10% buffered formalin and embedded in paraffin. Paraffin sections 4 μm thick were placed on positively charged slides and heated at 60°C for 60 min. Sections were then deparaffinized and rehydrated through a series of xylene and graded alcohols (100, 95 and 75%). Endogenous peroxidase was blocked in 3% hydrogen peroxide for 10 min. Antigen from slides were retrieved with Dako Target Retrieval Solution, (Dako Cat.no.S1699), and immunoperoxidase staining was performed with an automatic slide stainer (Dako Autostainer Plus) using the Dako universal staining system. We used primary mouse monoclonal antibody against p16 (BD, Cat. no. 551154) in a dilution of 1:50 for 30 min at room temperature. After application of primary antibody, the slides were washed in buffered solution, and a secondary antibody (Dako Envision system- HRP labeled Polymer, antimouse, Cat. no. K400111) was applied for 30 min. DAB Substrate kit (Dako, Cat. no. K346811) was used with 3,3′-diaminobenzidine as chromogen. Slides were counterstained with Surgipath Hematoxylin (Cat. no. 01560), dehydrated through graded alcohols, cleared in xylene and coveredslipped with cytoseal 60 (Richard-Allen Scientific, Cat. no. 8310). Positive and negative controls consisted of uterine cervix with severe dysplasia, with and without primary antibody, respectively. Immunohistochemical expression for p16 was graded weak, moderate or strong according to nuclear and cytoplasmic staining intensity using clinically established criteria. P16 was scored positive when >50% of tumor cells presented with a strong nuclear stain. Tumors that did not meet this threshold of detection were classified as p16-negative. In sensitivity analyses, we separately assessed the survival patterns for p16-negative tumors with weak-to-moderate nuclear staining, and for tumors with cytoplasmic staining only classified as p16-negative.

**HPV DNA and HPV16 E6/E7 mRNA detection**

A detailed description of laboratory methods for HPV DNA detection by PCR and HPV16 RNA expression by reverse transcription PCR has been published elsewhere. Briefly, presence of HPV-DNA was assessed using MY09/MY11/HMB01-PCR system with Gold AmpliTaq that amplifies a conserved 450 base-pair segment in the L1-sequence of HPV. To assess for HPV16 mRNA expression, total RNA was extracted from the same or matched frozen tumor samples and tested for HPV16 transcripts using HPV specific oligonucleotide primers that span the 204–525 base-pair regions of the E6 and E7 oncogenes. HPV16 RNA-positive tumors were defined as those that expressed either E6 or E7 transcripts. Tumors were defined as RNA-negative.
if they only expressed GAPDH, whose primers were included in each assay as an RNA control. Samples with low RNA viral loads (i.e., cycle thresholds for E6 and E7 > 32) were considered HPV16 RNA negative. To assess the potential for contamination by batch effect, we coextracted DNA and RNA from formalin-fixed paraffin-embedded tissue blocks from samples that were discordant for DNA and RNA and retested them using real-time HPV16 DNA and RNA PCR protocols. Samples were recoded based on the retest results.

Statistical analyses
The study sample included only histologically confirmed invasive SCC of the oropharynx (base of tongue, tonsil, soft palate, oropharyngeal wall, uvula and oropharynx—not otherwise specified [NOS]), hypopharynx (posterior pharyngeal wall, pyriform sinus and hypopharynx-NOS), larynx (glottis, supraglottis-aryepiglottic fold, supraglottis-false vocal cord, supraglottis-arytenoid, supraglottis-epiglottis, supraglottis-NOS and larynx-NOS), and oral cavity (buccal, alveolar ridge, anterior tongue, floor of mouth, hard palate, inner lip mucosa and retromolar trigone). Cases were grouped into oropharyngeal and nonoropharyngeal HNSCC for purpose of analysis.

Differences in detection of p16 protein expression by clinical and demographic characteristics were examined by contingency tables and nonparametric tests. Clinical outcomes assessed included overall survival, disease-specific survival and locoregional recurrence. Overall survival was defined as time from diagnosis (in months) to death from all causes. Disease-specific survival was defined as the time to death from disease, and locoregional recurrence as the time to either local or regional recurrence of disease. Progression-free survival was also assessed, defined as the time to local or regional recurrence, distant metastasis, or disease-specific death.

To evaluate associations between combined p16 and HPV16 detection with survival, we generated Kaplan Meier plots and constructed Cox proportional hazards regression models adjusting for strong prognostic indicators and potential confounders. The potential for confounding was examined for all socio-demographic and clinical indicators: age, gender, race, ethnicity, smoking history (including comparisons: never vs. ever, former and by pack-years), alcohol consumption, tumor anatomic site, tumor stage, primary treatment modality, method of specimen procurement (biopsy vs. surgical or laser resection), Eastern Cooperative Oncology Group (ECOG) performance status and tumor location (bilateral vs. unilateral). Additionally, we confirmed that the proportional hazards assumption was met for all multivariable models. All statistical analyses were conducted with the SAS 9.3 statistical software package (Cary, NC), and all tests were two-sided.

Results
Figure 1 depicts a flowchart of all HNSCC tested for p16 IHC, HPV16 DNA and HPV16 RNA. Overall, a total of 163 primary HNSCC tumors were collected from 158 patients, including 51 (31%) from the oropharynx, 50 (30%) from the lip/oral cavity, 50 (30%) from the larynx and 12 (7%) from the hypopharynx. From these, 124 (76%) tumors were tested for HPV16 DNA and 155 (95%) for HPV16 RNA. When we
compared p16 with HPV16 positivity, there was moderate agreement across all HNSCC (kappa = 0.63 for p16/HPV16 DNA, 95% CI: 0.45–0.80; kappa = 0.64 for p16/HPV16 RNA, 95% CI: 0.50–0.79). Concordance was substantially higher in the oropharynx between p16 and HPV16 DNA (kappa = 0.73, 95% CI: 0.51–0.95), as well as between p16 and HPV16 RNA (kappa = 0.80, 95% CI: 0.64–0.97).

Less than 4% (n = 6/163) of all tumors tested by p16 IHC exhibited weak (n = 2, ≤10% cells), moderate (n = 1, ≤25% cells) or mixed weak/moderate p16 nuclear staining (n = 3, ≤20% cells). Approximately 4% of tumors (n = 7) showed only cytoplasmic staining and were classified as p16-negative. P16 protein expression was detected in 26% of tumors (n = 42), although detection was significantly higher among oropharyngeal tumors (53%) relative to the larynx (24%), hypopharynx (8%) and oral cavity (4%; p < 0.0001). Table 1 shows the prevalence of p16 across patient characteristics comparing the oropharynx to nonoropharyngeal HNSCC. Patients with p16-positive oropharyngeal tumors were more likely to be younger, have lower ECOG performance status scores, have higher nodal stage, be diagnosed with only one primary tumor and smoked or drank alcohol less than those with p16-negative tumors. By contrast, there were no significant differences in p16 expression across demographic and clinical characteristics for patients with nonoropharyngeal tumors.

We followed patients prospectively for up to 113 months from time of diagnosis (median follow-up = 67 months). Compared with patients with p16-negative oropharyngeal tumors, patients with p16-positive oropharyngeal tumors exhibited better overall survival (log-rank p-value = 0.04, Fig. 2a) and progression-free survival (log-rank p-value = 0.17, Fig. 2b). Comparing p16-positive with p16-negative oropharyngeal tumors, multivariable analyses showed that patients with p16 positive disease had a 93% lower hazard-risk of death (p = 0.01; Table 2). Similar patterns of association were observed when we considered disease-specific mortality and locoregional recurrence. However, no benefit in disease-specific survival or locoregional recurrence was observed when we restricted our analyses to nonoropharyngeal HNSCC sites. Sensitivity analyses revealed that p16-negative tumors with weak-to-moderate nuclear p16 staining or tumors with cytoplasmic p16 staining only, had similar survival patterns as compared to those with no p16 staining. Excluding these tumors from the analyses revealed no appreciable change in overall or progression free survival (data not shown).

When we evaluated survival in a subgroup of tumors with combined p16 and HPV16 detection, we observed a significant reduction in the hazard-risk of cancer death comparing positively concordant with negatively concordant HNSCC tumors after adjustment for sex, tumor subsite, nodal stage, tumor size, primary number, smoking and drinking (Figs. 3a and 3b). The association persisted even when restricted to nonoropharyngeal tumors (Figs. 3c and 3d), of which two cases were found to be concordantly positive: one in the hypopharynx and one in the larynx-supraglottis. While p16-positive/HPV16 DNA-negative nonoropharyngeal tumors showed some improvement in disease-specific survival as compared with tumors that were negative for both, the estimates did not reach statistical significance. No survival benefit was observed for p16-positive/HPV16 RNA-negative tumors as compared with tumors that were negative for both (Fig. 3d). Discordant tumors that were p16-negative/HPV16-positive, while rare in our study (N = 4 p16-negative/HPV16 DNA-positive and N = 2 p16-negative/HPV16 RNA-positive tumors), yielded no disease-specific mortality events; therefore, hazard ratios (HRs) were not estimable. Although not shown here, similar patterns of associations were observed with overall survival and locoregional recurrence as endpoints.

In the oropharynx, positively discordant tumors showed a ≥98% better overall survival (p < 0.01) compared with negatively concordant tumors in multivariable models (Table 3). There were four discordant tumors that were p16-positive/HPV-negative, one base of tongue and three tonsil cancers. There was one documented death in this subgroup, not attributable to cancer or tumor recurrence. HRs for these discordant cases were not estimable due to the limited sample size. Similar patterns of associations were observed for progression-free survival, although estimates were not statistically significant due in part to few recurrences and cancer-specific deaths (data not shown).

Discussion

With over 9 years of follow-up, our data showed an association between p16 expression and overall survival for cancers of the oropharynx. However, combined detection of p16 and HPV16 was superior in predicting survival than either method alone25 for oropharyngeal and nonoropharyngeal tumor sites. Our findings support emerging evidence from studies in other patient populations that highlight the importance of using multiple prognostic biomarkers to detect clinically relevant, transcriptionally active HPV in HNSCC.14,15,25,26

We found that the association between p16 protein expression and overall survival was restricted to oropharyngeal primary tumors. The survival advantage of p16 overexpression in oropharyngeal cancer appears to be due to its high correlation with high-risk HPV infection, as illustrated by the substantial concordance between p16 expression and HPV16 detection.27,28 Detection of p16 expression in tonsillar cancer, in particular, demonstrates strong correlation with HPV DNA detection and E6/E7 mRNA expression.29 However, we found this overlap did not extend to nonoropharyngeal tumors, where HPV prevalence was much lower, and p16 protein expression alone was not significantly associated with prognosis in these tumors.

When p16 expression was considered in combination with HPV16 status, we observed a ≥92% improvement in disease-specific survival for HNSCC positive for both p16 protein and HPV16, independent of head and neck cancer site. This is consistent with the literature. For example, a recent study of 179
### Table 1. Study population characteristics stratified by p16 status and stratified by tumor site in the overall population

| Patient Demographics | Oropharynx (N = 51) | Nonoropharynx (N = 112) | p-value<sup>1</sup> |
|----------------------|----------------------|--------------------------|----------------------|
| **Age at diagnosis in years** |                       |                          |                      |
| Mean (standard deviation) | 60 (8) | 65 (11) | 0.06 | 60 (10) | 63 (13) | 0.46 |
| Range | 46–76 | 40–84 | 41–77 | 22–91 |
| **Sex, n (%)** |                       |                          |                      |
| Men | 21 (78%) | 18 (75%) | 1.00 | 9 (60%) | 72 (74%) | 0.35 |
| Women | 6 (22%) | 6 (25%) | 6 (40%) | 25 (26%) |
| **Race<sup>2</sup> n (%)** |                       |                          |                      |
| White/Asian | 16 (62%) | 10 (45%) | 0.38 | 8 (67%) | 64 (72%) | 0.74 |
| Black/African American | 10 (38%) | 12 (55%) | 4 (33%) | 25 (28%) |
| **Ethnicity<sup>2</sup> n (%)** |                       |                          |                      |
| Non-Hispanic | 20 (77%) | 19 (79%) | 1.00 | 9 (64%) | 64 (70%) | 0.76 |
| Hispanic | 6 (23%) | 5 (21%) | 5 (36%) | 28 (30%) |
| **Smoking Status<sup>3</sup> n (%)** |                       |                          |                      |
| Current Smoker | 5 (19%) | 13 (54%) | 0.01<sup>4</sup> | 6 (40%) | 35 (36%) | 0.53<sup>3</sup> |
| Exsmoker | 16 (59%) | 9 (38%) | 8 (53%) | 48 (49%) |
| Never Smoker | 6 (22%) | 2 (8%) | 1 (7%) | 14 (14%) |
| **Drinking Status<sup>3</sup> n (%)** |                       |                          |                      |
| Current Drinker | 7 (26%) | 11 (46%) | 0.11<sup>5</sup> | 2 (13%) | 24 (25%) | 0.29<sup>3</sup> |
| Former Drinker | 4 (15%) | 4 (17%) | 3 (20%) | 21 (22%) |
| Never Drinker | 16 (59%) | 9 (38%) | 10 (67%) | 52 (54%) |
| **Anatomical Site<sup>4</sup> n (%)** |                       |                          |                      |
| Oropharynx | 27 (100%) | 24 (100%) | NA | NA |
| Oral Cavity | NA | NA | 2 (13%) | 48 (49%) | 0.01 |
| Larynx | NA | NA | 12 (80%) | 38 (39%) |
| Hypopharynx | NA | NA | 1 (7%) | 11 (11%) |
| **Overall Stage, n (%)** |                       |                          |                      |
| I–II | 5 (19%) | 8 (33%) | 0.34 | 4 (27%) | 22 (23%) | 0.75 |
| III–IV | 22 (81%) | 16 (67%) | 11 (73%) | 75 (77%) |
| **Nodal Stage n (%)** |                       |                          |                      |
| N0 | 5 (19%) | 8 (33%) | 0.02 | 6 (40%) | 46 (47%) | 0.52 |
| N1 | 0 | 4 (17%) | 1 (7%) | 15 (15%) |
| N2/3 | 22 (81%) | 12 (50%) | 8 (53%) | 36 (37%) |
| **Tumor Size n (%)** |                       |                          |                      |
| T1–T2 | 21 (78%) | 17 (71%) | 0.75 | 7 (47%) | 37 (38%) | 0.58 |
| T3–T4 | 6 (22%) | 7 (29%) | 8 (53%) | 60 (62%) |
| **Primary tumor site no., n (%)** |                       |                          |                      |
| First | 27 (100%) | 20 (83%) | 0.04 | 14 (93%) | 81 (84%) | 0.46 |
| Second to Fourth | 0 | 4 (17%) | 1 (7%) | 16 (16%) |
| **ECOG performance score<sup>2</sup> n (%)** |                       |                          |                      |
| 1 | 22 (96%) | 14 (70%) | 0.04 | 7 (64%) | 54 (66%) | 1.00 |
| 2–4 | 1 (4%) | 6 (30%) | 4 (36%) | 28 (34%) |
| **Initial treatment<sup>5</sup> n (%)** |                       |                          |                      |
| Surgery | 15 (56%) | 9 (38%) | 0.26 | 8 (57%) | 60 (65%) | 0.56 |
| Nonsurgical therapy | 12 (44%) | 15 (63%) | 6 (43%) | 32 (35%) |

<sup>1</sup>p-Value for 2-sided fisher exact test.

<sup>2</sup>Row numbers may not sum to column totals due to missing data.

<sup>3</sup>Smoking status was defined as never smoked, exsmoker (at time of diagnosis) and current smoker. Drinking status was defined as never drank alcohol regularly for more than one year, former or current drinker (at time of diagnosis). p-values for two-sided Cochrane-Armitage trend test.

<sup>4</sup>Oropharynx includes: base of tongue, tonsil, soft palate, oropharyngeal wall, uvula and oropharynx-NOS. Hypopharynx includes: posterior pharyngeal wall, pyriform sinus and hypopharynx-NOS. Larynx includes glottis, supraglottis-arypeiglottic fold and epiglottis. Oral cavity includes buccal, alveolar ridge, anterior tongue, floor of mouth, hard palate, inner lip mucosa and retromolar trigone.

<sup>5</sup>Primary nonsurgical therapy was defined as treatment with either primary radiotherapy with or without concomitant chemotherapy, including planned neck dissection and salvage surgery.
HNSCC found no significant improvement in overall survival with either HPV16 DNA or p16 immunohistochemical staining alone after 5 years of follow-up. However, positively concordant p16 and HPV16 E6/E7 measured in serology yielded a 70% improvement in overall survival when compared with negatively concordant tumors. In a prospective study of 647 HNSCC, p16-positive/HPV16 DNA-positive HNSCC showed a 59% improvement in disease-specific survival ($p < 0.01$) compared with those that were negatively concordant, controlling for age, gender, stage, treatment and smoking status.

Furthermore, we found that the strong association between combined p16/HPV16 detection and disease-specific survival persisted for nonoropharyngeal HNSCC. We have previously reported that HPV16 detection, either by DNA PCR or RNA RT-PCR, similarly does not significantly predict disease-specific survival. This is consistent with other studies that have reported no significant association with disease-specific survival when HPV16 was detected by E6/E7 RT-PCR. Retesting of discordant p16-negative/HPV16-positive tumors suggested these cases were initially misclassified in our data; a majority of these tumors (85%) were found to have low HPV16 RNA levels and were subsequently reclassified as negative for HPV16. Alternatively, others have suggested that p16-positive/HPV16-negative HNSCC likely represent a distinct subgroup arising from genomic interruption of the p53/pRb pathway.

While a growing consensus supports HPV16 RNA testing as the gold standard for HPV detection, we previously reported that overall survival is better with combined DNA and RNA HPV16 detection by PCR. In this study, we found no difference in survival benefit when we used

Figure 2. Kaplan Meier plots for p16-associated overall and progression-free survival in the oropharynx (panels a and b) and nonoropharynx (panels c and d).
combined p16/HPV16 DNA or RNA detection. Moreover, patients with tumors that tested HPV16-positive but p16-negative were relatively rare (3% and 1% of HNSCC tumors tested with HPV DNA and RNA, respectively) and had no cancer-related deaths. These findings support using combined p16/HPV DNA or RNA testing for HNSCC.

Our study has both strengths and limitations. Among the latter, we were not able to test all tumors for HPV16 DNA/RNA. However, sensitivity analyses showed no significant differences in clinical or demographic characteristics between patients with and without complete data on HPV16 DNA/RNA detection. Secondly, we had few numbers of nonoropharyngeal tumors to examine survival patterns by subsite. Larger studies are needed to confirm our findings given that HPV prevalence may differ across nonoropharyngeal HNSCC subsites.

Our study also had important strengths worth citing. First, we followed patients over a relatively longer period (up to 9.4 years).

Table 2. Adjusted HRs comparing positive versus negative p16 expression by tumor site

|                      | Oropharynx (N = 51) | Nonoropharynx (N = 112) | All HNSCC sites (N = 163) |
|----------------------|---------------------|--------------------------|---------------------------|
|                      | HR (95% CI)         | p-Value                  | HR (95% CI)               | p-Value                  | HR (95% CI)               | p-Value                  |
| Overall Survival     |                     |                          |                           |                          |                           |                          |
| p16 (positive vs. negative) | 0.07 (0.01 – 0.58) | 0.01                     | 0.67 (0.29 – 1.55)         | 0.34                     | 0.43 (0.20 – 0.92)         | 0.03                     |
| Disease Specific Survival |                     |                          |                           |                          |                           |                          |
| p16 (positive vs. negative) | 0.13 (0.01 – 2.96) | 0.20                     | 1.01 (0.32 – 3.15)         | 0.98                     | 0.71 (0.24 – 2.14)         | 0.55                     |
| Locoregional Recurrence |                     |                          |                           |                          |                           |                          |
| p16 (positive vs. negative) | 0.10 (0.01 – 1.60) | 0.10                     | 1.00 (0.28 – 3.63)         | 1.00                     | 0.59 (0.18 – 1.92)         | 0.38                     |

1HRs adjusted for age, sex, nodal stage, tumor size, primary tumor site number, smoking, drinking and tumor subsite (for nonoropharyngeal SCC).

Figure 3. Adjusted HRs for disease-specific survival by p16 and HPV expression (HPV16 DNA and E6/E7) in all HNSCC sites combined (panels a and b) and in the nonoropharynx (panels c and d). HRs adjusted for sex, nodal stage, tumor size, tumor subsite, primary tumor site number, smoking and drinking.
In contrast, for nonoropharyngeal HNSCC, we would recommend testing all p16-positive cases using a PCR-based assay for HPV given the poor performance of p16 IHC in nonoropharyngeal HNSCC, and risks associated with under-treatment. Larger clinical studies; however, are needed to confirm our findings.

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### Table 3. Adjusted HRs for overall survival in the oropharynx by p16/HPV16 testing

| Combined p16/HPV detection | p16/DNA detection | p16/RNA detection |
|----------------------------|-------------------|-------------------|
| Deaths (n)                 | HR (95% CI)       | p-value           |
| 10/17                      | 1.00 (referent)   |                   |
| 1/3                        | 2                 |
| 0/2                        | 2                 |
| 1/15                       | 0.02 (<0.01–0.23) | <0.01             |

1. HRs adjusted for sex, nodal stage, tumor size, primary tumor site number, smoking and drinking.
2. Cannot be assessed.

[Correction needed: In some cases, the text may contain errors or inconsistencies. Please consult the original source for the most accurate information.]
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