High-Risk Human Papillomavirus Detection in Oropharyngeal, Nasopharyngeal, and Oral Cavity Cancers
Comparison of Multiple Methods

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IMPORTANCE Human papillomaviruses are now recognized as an etiologic factor in a growing subset of head and neck cancers and have critical prognostic importance that affects therapeutic decision making. There is no universally accepted gold standard for high-risk HPV (hrHPV) assessment in formalin-fixed, paraffin-embedded (FFPE) tissue specimens, nor is there a clear understanding of the frequency or role of hrHPV in sites other than oropharynx.

OBJECTIVE To determine the optimal assessment of hrHPV in FFPE head and neck tumor tissue specimens.

DESIGN, SETTING, PARTICIPANTS In the setting of a large Midwestern referral center, assessment of hrHPV by p16 immunohistochemical staining, in situ hybridization, and polymerase chain reaction (PCR)-MassArray (PCR-MA), with L1 PGMY-PCR and sequencing to resolve method discordance, was conducted for 338 FFPE oropharyngeal, nasopharyngeal, and oral cavity tumor tissue specimens. Relative sensitivity and specificity were compared to develop a standard optimal test protocol. Tissue specimens were collected from 338 patients with head and neck cancer treated during the period 2001 through 2011 in the departments of Otolaryngology, Radiation Oncology, and Medical Oncology.

INTERVENTION Patients received standard therapy.

MAIN OUTCOMES AND MEASURES Optimal hrHPV identification, detection, and activity in head and neck cancers.

RESULTS Using combined PCR-MA with L1 PGMY-PCR and sequencing for conclusive results, we found PCR-MA to have 99.5% sensitivity and 100% specificity, p16 to have 94.2% sensitivity and 85.5% specificity, and in situ hybridization to have 82.9% sensitivity and 81.0% specificity. Among HPV-positive tumors, HPV16 was most frequently detected, but 10 non-HPV16 types accounted for 6% to 50% of tumors, depending on the site. Overall, 86% of oropharynx, 50% of nasopharynx, and 26% of oral cavity tumors were positive for hrHPV.

CONCLUSIONS AND RELEVANCE PCR-MA has a low DNA (5 ng) requirement effective for testing small tissue samples; high throughput; and rapid identification of HPV types, with high sensitivity and specificity. PCR-MA together with p16\textsuperscript{INK4a} provided accurate assessment of HPV presence, type, and activity and was determined to be the best approach for HPV testing in FFPE head and neck tumor tissue specimens.

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The role of carcinogenic high-risk human papillomaviruses (hrHPV) in the etiology of head and neck cancer has been increasing over the past 20 years. At our institution, 80% to 90% of oropharyngeal cancers are HPV positive, and evidence for hrHPV in head and neck squamous cell carcinoma of other sites is also increasing. Generally, HPV-positive oropharyngeal cancers exhibit better responses to treatment than do HPV-negative tumors. A recent trial conducted at our institution using concurrent platinum-taxol-based chemotherapy and intensity-modulated radiation therapy resulted in 3-year progression-free survival of 88% among patients with oropharynx cancer with stage 3 and 4 disease. However, a recent study from Belgium reported that survival among HPV-positive patients with oral cavity cancer was worse than their HPV-negative counterparts. Similarly, among patients with nasopharynx cancer treated at our institution, those with HPV-positive nasopharyngeal cancers had poorer outcome than Epstein-Barr virus (EBV)-positive patients (Stenmark et al, unpublished data, 2013).

Many reports indicate that HPV-positive tumors with transcriptionally active viral oncogenes are those most likely to respond well to treatment. In contrast to low-risk HPV types such as HPV6 and HPV11, which also infect mucosal epithelia but rarely cause cancer, the hrHPV types HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 73 have all been implicated in oncogenesis. This difference between low-risk HPV and hrHPV types is due in part to the nature of the E6 and E7 viral oncogenes that exhibit alternate splicing in hrHPV, resulting in transforming capacity. Thus, for precision medicine, it is important to assess not only the presence of HPV16 but also other hrHPV types. This will be essential to accurately determine the most effective treatment option for each patient based on their individual tumor characteristics. Optimally, viral oncogene activity is determined using high-quality tumor RNA to identify alternate transcripts linked to transformation or assessing HPV E6 and E7 indirectly by detection of patient antibodies to E6 and E7. However, availability of fresh-frozen tumor tissue or access to serologic assays is rare, whereas fixed tumor from the diagnostic biopsy is more readily accessible. Therefore, it is essential to have robust and accurate testing methods using formalin-fixed, paraffin-embedded (FFPE) materials to complement the histopathologic and clinical staging data to arrive at the optimal therapeutic plan.

Multiple methods of HPV detection and assessment are widely used, but the optimal testing method has yet to be clearly defined. Immunohistochemical detection of highly expressed p16INK4a is a widely used surrogate method for the presence of HPV in a tumor. This biomarker is indicative of hrHPV E7 oncogene expression, which up-regulates p16 and promotes entry into the cell cycle. However, p16 can be up-regulated by mechanisms other than HPV, leading to false-positive assessment of HPV in the sample. In head and neck cancers, p16 is also one of the most frequently lost genes and as such could provide a false-negative evaluation result for the presence of HPV. Direct detection of HPV DNA in tumor cells by in situ hybridization (ISH) is also widely used in pathology departments and has the advantage of identifying the presence and location of the viral DNA within tumor cells, but this method lacks sensitivity. Polymerase chain reaction (PCR)-based methods are highly advantageous because they require little DNA, but these often lack the ability to detect multiple high-risk types or to identify the high-risk type present, a shortcoming shared by both p16 assessment and ISH.

Because many HPV-positive tumors respond well to therapy, there are national and local efforts to significantly decrease treatment intensity for patients with these tumors to spare them from unnecessary treatment morbidity. However, even with very aggressive concurrent chemoradiation therapy, a subset of 20% to 30% of patients with HPV-positive oropharyngeal cancer progress either locally or with distant metastases. It has been suggested that the HPV-positive tumors that are driven primarily by the HPV viral oncogenes are the most likely to have a good response to treatment. Thus, there is a growing need for reliable and rapid tests for detection of transcriptionally active HPV in head and neck cancers to select patients for the most appropriate treatment based on their own tumor characteristics. In this study we compared 3 commonly used HPV assessment tools: HPV PCR-MA for 15 hrHPV types, HPV ISH for 12 high-risk types, and p16 immunohistochemical staining. We then used consensus L1 PGMY-PCR and sequencing to resolve discordant results for tumors that were HPV-negative by PCR-MA but p16 positive or ISH positive. From our results we propose an optimal HPV detection and identification algorithm.

Methods

Patient Specimens
All patients provided written informed consent to study their tissue under a study approved by the Institutional Review Boards of the University of Michigan Medical School. Tumor specimens from 338 patients with advanced stage head and neck cancer enrolled in the Head and Neck Cancer SPORE (Specialized Programs of Research Excellence) were obtained and evaluated, including 212 oropharyngeal, 18 nasopharyngeal, and 108 oral cavity cancers. Tumor cores (FFPE) from pretreatment biopsies and/or posttreatment recurrences (when available) were used to construct tissue microarrays for ISH and immunohistochemical staining. Also, FFPE tumor cores were taken from each tumor block at the time of array construction for genomic DNA extraction.

p16INK4a Immunohistochemical Staining
Staining for p16INK4a was performed per supplier protocol (CINtec p16INK4a Histology Kit; mtm Laboratories). Antibody binding was scored by an experienced head and neck pathologist (J.B.M.), using a continuous scale for the proportion of tumor cells demonstrating nuclear and cytoplasmic p16 staining. Percentage scored was divided into a quartile scale of 1 to 4 (1 was...
Results

The 338 tumors were tested and compared by at least 2 of the 3 methods as summarized by tumor site in Table 1 and Table 2. As expected, the most frequently HPV-positive tumors were from oropharynx; with 173 of 208 (83%) positive by PCR-MA, 170 of 205 (83%) positive by p16, and 120 of 164 (73%) positive by ISH. Taken together, 183 of 212 (86%) oropharynx tumors were HPV positive by 1 or more method, and 29 of 212 (14%) oropharynx tumors were HPV negative by all tests performed on those samples (minimum of 2 tests each). The predominant HPV type determined by PCR-MA in oropharynx was HPV16 alone (162 of 173 [94%]). However, 6% of oropharynx tumors contained other high-risk types including HPV18 (n = 2), HPV33 (n = 3), HPV35 (n = 3), and HPV39 (n = 1), and 2 cases contained multiple HPV types—1 positive for HPV16, 35, and 66 and 1 positive for HPV16 and 33 (Table 1 and Table 2). The mass spectrum for the oropharyngeal tumor that contains both HPV16 and HPV33 is illustrated in Figure 1.

Of the 18 nasopharynx tumors, 9 (50%) were p16 positive and 8 (44%) were PCR-MA-positive. Only 1 of 18 was tested by ISH, and it was negative by ISH but positive by both other assays. The hrHPV types identified in the 8 PCR-MA-positive nasopharynx tumors included HPV16 (n = 3), HPV18 (n = 2), HPV39 (n = 1), and HPV59 (n = 2) (Table 1 and Table 2).

The oral cavity tumors were less frequently HPV positive, with only 28 of 108 (26%) tumors HPV positive by 1 or more methods and 80 (74%) tumors HPV negative by all tests (minimum of 2 tests). The hrHPV types identified in the oral cavity tumors were HPV16 (n = 4), HPV31 (n = 1), HPV35 (n = 1), HPV39 (n = 1), HPV59 (n = 1), and HPV66 (n = 1), and there were 2 cases with multiple infections, 1 containing HPV16 and 35 and 1 containing HPV39 and 58 (Table 1 and Table 2).

Of the 330 tumors from all sites tested by HPV PCR-MA, 191 (58%) were HPV positive and 140 (42%) were HPV negative. Of the 329 tumors tested by p16 staining, 199 (60%) were p16 positive and 130 (40%) were p16 negative. Of the 184 tumors tested by HPV ISH, 125 (68%) were HPV positive and 140 (42%) were HPV negative (Table 3).

There were 167 of 338 tumor specimens tested by all 3 methods; 105 were HPV positive by all methods, 28 were negative by all methods, and 34 samples were discordant by at least 1 method (Figure 2A and Table 3). The remaining 171 of 338 samples were tested by only 2 of the 3 methods. Of these, 57 (49 by PCR-MA and p16, 5 by ISH and PCR-MA, and 3 by p16 and ISH) were HPV positive by the 2 methods used, 89 were negative by the 2 methods used, and 25 samples were discordant (Figure 2B and Table 2).
All 25 tumors that were HPV negative by HPV PCR-MA but p16 positive or ISH positive (9 cases shown circled in Figure 2A and 16 cases shown circled in Figure 2B) were further analyzed by consensus PCR using optimized PGMY09/PGMY11 primers. Of these, all 17 of the oral cavity tumors that were negative by HPV PCR-MA but p16 positive or ISH positive remained HPV negative when tested with L1 PGMY-PCR. Of the 8 oropharynx cancers that were negative by HPV PCR-MA but p16 positive or ISH positive, 1 was found to contain HPV DNA using L1 PGMY-PCR. That tumor was p16 positive and ISH positive and was found to harbor HPV16 as determined by Sanger sequencing of the consensus LI PGMY-PCR product. The single nasopharynx cancer that was p16 positive but HPV negative by PCR-MA was also HPV negative by HPV L1 PGMY-PCR. Across the 3 sites, 169 tumors contained only HPV16; of these tumors, 163 had p16 data (157 [96%] were p16 positive and 6 [4%] were p16-negative). Twenty-two tumors contained other hrHPV types, 3 in combination with HPV16. Of these, 5 of the 22 (23%) failed to express p16 (1 tumor with HPV16 and HPV35, 1 with HPV35, 1 with HPV39, 1 with HPV39 and HPV58, and 1 with HPV68) (Table 1 and Table 2).

When evaluating the performance of the assays using combined PCR-MA with L1 PGMY-PCR and sequencing as the definitive assay, the HPV PCR-MA had a sensitivity of 99.5% and a specificity of 100%, p16 assay had a sensitivity of 94.2% and a specificity of 85.5%, and the ISH assay had a lower sensitivity of 82.9% and a specificity of 81.0% (Table 4).

### Discussion

The association of hrHPV with oropharynx cancer is now well established. Recently, hrHPV has also been implicated in a subset of nasopharyngeal carcinomas in white North Americans and in other head and neck cancer sites, including oral cavity cancers. The goal of this study was to carefully assess a large number of head and neck squamous cancers from 3 different sites (oropharynx, oral cavity, and nasopharynx) using p16INK4a staining, HPV ISH, and HPV PCR-MA on the same samples to determine the true incidence of hrHPV involvement in the tumors and to assess the relative sensitivity and specificity of each detection method. This study revealed that among these tumor sites at a large Midwestern referral center, more than 80% of oropharynx cancers, approximately half of nasopharyngeal cancers, and 10% of oral cavity cancers contain hrHPV.
In the oropharynx, 95% of the HPV-positive tumors contained HPV16, including 2 tumors that also contained 1 or 2 additional hrHPV types. Five percent of the HPV-positive oropharynx cancers contained other high-risk types (HPV18, HPV23, HPV35, and HPV39). However, in the nasopharynx and oral cavity, more than half of the HPV-positive tumors contained non-HPV16 high-risk types. The presence of both HPV16 and other hrHPV types in the absence of EBV in nasopharyngeal cancer is a strong indictment of HPV as a causal factor in a subset of nasopharynx cancers. This is reinforced by the strong concordance of p16 positivity with HPV and with the observation that neither EBV-positive nor HPV and EBV-double-negative tumors express p16.

In oropharynx cancer, HPV-positive tumors have a more favorable outcome than HPV-negative tumors. However, little is known about the effect of HPV on outcome in other sites in the head and neck. Studies from Belgium have reported both a high incidence (44%) of hrHPV-positive oral cancers and a very poor prognosis for these tumors compared with HPV-negative oral cavity tumors. Similar results were reported from Taiwan. We found a much lower proportion of hrHPV-positive oral cavity tumors (10%), and among these we observed a higher rate of HPV heterogeneity, with 40% containing only HPV16 and 60% that had other hrHPV types. Our proportion of HPV-positive oral cavity cases is relatively small, and whether these represent a separ-
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In conclusion, our findings support the use of a combination of p16-positive and HPV-positive results (correlation coefficient = 0.999996 \( P < .001 \)). This indicates that the virus is transcriptionally active in nearly all HPV-containing tumors, suggesting that it is unlikely that the HPV is strictly an incidental passenger. Therefore, these tumors cannot be excluded from consideration. Furthermore, a significant subset of p16 and HPV-positive oropharynx tumors (20%-30%) recur or progress with distant metastatic spread, yet the reason for this is unknown. It may be that non-HPV16 types are responsible for the outlier tumors, or perhaps these tumors driven by other hrHPV types are responding well to therapy. We already know that many of the recurrent and/or metastatic cancers in our set contain HPV16, suggesting that tumors driven by non-HPV16 types are as likely to respond to current therapies as the HPV16-driven tumors. Accordingly, it is important to include the other types and to carry out larger studies to determine if tumors driven by non-HPV16 types can be assigned to reduced intensity treatments.

In the present study, p16 expression status was determined in the majority of tumors evaluated, and there was a significant correlation between p16-positive and HPV-positive results (correlation coefficient = 0.999996 \( P < .001 \)). This indicates that the virus is transcriptionally active in nearly all HPV-containing tumors, suggesting that it is unlikely that the HPV is strictly an incidental passenger. Nevertheless, of 186 tumors for which an HPV type was identified and p16 staining was carried out, 175 (94%) were p16 positive and 11 (6%) were p16 negative. Whether this represents a subset of 6% in which HPV is not a driving mechanism or whether this subset has incurred a mutation, deletion, or methylation event affecting CDKN2A is a subject for further investigation.

Many groups have surveyed oropharyngeal tumors for HPV, and some have examined multiple head and neck tumor sites and have used a variety of detection methods. The present study represents one of the largest series of head and neck tumors from different sites evaluated by multiple assay methods for the presence of hrHPV. The PCR-MA assay has features that make it the optimal test in our hands. The minimum requirement for input DNA is very low; 5 ng is adequate for evaluation. The assay has high sensitivity and specificity and identifies each hrHPV type using specific primers, probes, and competitors, and it focuses on the E6 region to confirm that this transforming oncogene is present in the sample. Evaluation of the cyclin-dependent kinase inhibitor protein, p16INK4A, is a valuable diagnostic addition to the PCR-MA assay because it typically represents the transcriptional activity of the E7 oncogene. In our series, there was 1 oropharynx tumor that was negative by HPV PCR-MA but p16 positive and ISH positive that was later confirmed to contain HPV16 by L1 PGMY-PCR and sequencing. We speculate that the PCR-MA assay missed this single case owing to rearrangement of the viral genome that affected part of the E6 oncogene. More commonly, p16 is overexpressed in a subset of tumors in the absence of hrHPV. Other mechanisms of p16 overexpression include mutation of Rb, amplification of cyclinD1, and overexpression of E2F family members. In this series of tumors, reanalysis of discordant cases revealed that 24 of 25 tumors that were negative by HPV PCR-MA but p16 positive remained HPV negative by consensus L1 PGMY-PCR.

In situ hybridization for hrHPV has high specificity among known HPV-positive tumors but has comparatively low sensitivity and can miss HPV-containing tumors as assessed by other methods. Reanalysis of ISH-PCR-MA discordant cases revealed that all 4 tumors that were negative by HPV PCR-MA but ISH positive remained HPV negative by consensus L1 PGMY-PCR.

In conclusion, our findings support the use of a combination of p16 immunohistochemical staining and HPV PCR-MA analysis as the optimal assessment for HPV detection, typing, and viral oncogene activity in FFPE tissue biopsy specimens. For discordant p16-positive/PCR-MA-negative tumors, we recommend L1 PGMY-PCR and sequencing.

**Table 4. Assay Performance Using Combination PCR-MA/PGMY-PCR and Sequencing (PCR/SEQ) as the Definitive Assay**

<table>
<thead>
<tr>
<th>Test Performance</th>
<th>PCR/SEQ+</th>
<th>PCR/SEQ−</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV PCR-MAa</td>
<td>191 (TP)</td>
<td>0 (FP)</td>
<td>191</td>
</tr>
<tr>
<td>PCR-MA+</td>
<td>1 (FN)</td>
<td>138 (TN)</td>
<td>139</td>
</tr>
<tr>
<td>PCR-MA−</td>
<td>192</td>
<td>138</td>
<td>330</td>
</tr>
<tr>
<td>p16 IHCb</td>
<td>179 (TP)</td>
<td>20 (FP)</td>
<td>198</td>
</tr>
<tr>
<td>p16+</td>
<td>11 (FN)</td>
<td>118 (TN)</td>
<td>129</td>
</tr>
<tr>
<td>p16−</td>
<td>189</td>
<td>138</td>
<td>327</td>
</tr>
<tr>
<td>HPV ISHc</td>
<td>116 (TP)</td>
<td>8 (FP)</td>
<td>124</td>
</tr>
<tr>
<td>ISH+</td>
<td>24 (FN)</td>
<td>34 (TN)</td>
<td>58</td>
</tr>
<tr>
<td>ISH−</td>
<td>140</td>
<td>42</td>
<td>182</td>
</tr>
</tbody>
</table>

Abbreviations: FN, false negative; FP, false positive; HPV, human papillomavirus; ISH, in situ hybridization; PCR-MA, polymerase chain reactive-MassArray; TN, true negative; TP, true positive.

a Sensitivity = TP/(TP + FN) = 191/192 = 99.5%. Specificity = TN/(FP + TN) = 138/138 = 100%.

b Sensitivity = TP/(TP + FN) = 178/189 = 94.2%. Specificity = TN/(FP + TN) = 118/138 = 85.5%.

c Sensitivity = TP/(TP + FN) = 116/140 = 82.9%. Specificity = TN/(FP + TN) = 34/42 = 81.0%.
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REFERENCES


