Anatomic Pathology / PCR for HPV in Oropharyngeal Squamous Carcinoma

Polymerase Chain Reaction Detection of HPV in Squamous Carcinoma of the Oropharynx

Elin S. Agoston, PhD,1 Stephen J. Robinson,2 Karishma K. Mehra, MD,1 Chandler Birch,1 Dana Semmel, MD,1 Jelena Mirkovic, PhD,3 Robert I. Haddad, MD,4 Marshall R. Posner, MD,4 David Kindelberger, MD,1 Jeffrey F. Krane, MD, PhD,1 Joshua Brodsky,1 and Christopher P. Crum, MD1

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Abstract

Human papillomavirus (HPV) testing is routinely performed on oropharyngeal carcinomas. We compared the Access Genetics (Minneapolis, MN) polymerase chain reaction (PCR) assay (AGPCR), DNA-DNA in situ hybridization (ISH; Ventana, Tucson, AZ), and HPV-16 E7 PCR amplification in consecutively accessioned oropharyngeal cancers. We tested 126 cases by both PCR methods; 102 were positive by either for a maximum positive rate (MPR) of 81.0%. Relative to the MPR, the sensitivities of AGPCR and E7 PCR were 90.2% and 72.5%, respectively. Of 17 AGPCR + cases tested by ISH, 14/14 unequivocally positive/negative were concordant. All cases (97/97) positive by either PCR assay were positive for p16. There was no relationship between level of histologic differentiation and HPV status. ISH and AGPCR have comparable performance for the detection of HPV in oropharyngeal carcinomas. PCR is a suitable and economical assay that is comparable to ISH in sensitivity and may provide logistical advantages relative to ISH for assessing HPV status in oropharyngeal malignancies. However, it is imperative that appropriate sensitivity controls be in place for such assays.

Squamous cell carcinoma of the oropharynx is strongly linked to human papillomavirus (HPV) infection.1-10 While rates of smoking, once thought to be a leading risk factor, have dropped, rates of tonsillar cancer increased by 3% annually from 1998 through 2003.11 HPV-related oropharyngeal cancer is now an accepted and well-defined entity that has its own clinicopathologic characteristics.10 This highlights the potential contribution of HPV in the etiology of this disease and the increasing requirement for efficient clinical HPV-16 testing in this population. Detection of this virus is also associated with poorly differentiated tumors and improved prognosis.6,12-22 Differences in cell of origin and in mechanism of cell-cycle disruption in HPV-associated and non–HPV-associated tonsillar cancers have been implicated as the underlying cause for this difference in outcome.1,23 Thus, HPV testing is frequently requested when these tumors are found because the patients often respond well to chemoradiation.13,20 In addition, the test can be used to identify the site of origin when metastatic carcinoma is detected in a cervical lymph node.24,25

HPV-16 is the most prevalent viral subtype and, together with p16 immunostaining, is found in up to 82% of cases.23,26 Controversy exists as to whether HPV testing should be performed with a polymerase chain reaction (PCR) or in situ hybridization (ISH)-based technology. Moreover, the possibility that a probe set targeting the L1 region might miss cases in which this region has been deleted by viral integration has previously raised concerns that probes targeting the early (E6 or E7) region should be used to improve sensitivity.27

The aim of this study was to evaluate 3 approaches to detecting HPV in oropharyngeal tissues: (1) PCR with...
generic L1 primers (Access Genetics [Minneapolis, MN] PCR [AGPCR]), (2) PCR with early (E7) HPV-16–specific primers (E7PCR), and (3) DNA-DNA ISH. The comparisons made include the following: (1) HPV test result vs level of histologic differentiation (by report), (2) HPV test result vs p16 immunostaining, (3) E7 vs L1 primer set, and (4) L1 primer set vs ISH.

Materials and Methods

PCR Detection of HPV

Oropharyngeal biopsy specimens from patients undergoing surgery at Brigham and Women’s Hospital, Boston, MA, were analyzed for HPV using E7PCR and AGPCR. PCR was performed on DNA prepared from formalin-fixed, paraffin-embedded tissue samples. One 8-μm section was scraped and incubated with digestion buffer (50 mmol/L tris(hydroxymethyl)aminomethane [Tris], pH 8.0; 1 mmol/L EDTA, 0.5% polyborate-20, and 0.2 mg/mL Proteinase K) overnight at 62°C and then incubated with Chelex (Bio-Rad, Hercules, CA), boiled, and centrifuged. The supernatant was used directly in PCR. The primers 16E7A and 16E7B were used for E7PCR as previously described. AGPCR was used performing primers and reagents purchased from Access Genetics. Typing was achieved by digestion of the PCR product with the following restriction enzymes: PstI, RsaI, and HaeIII. All PCR products were resolved on 1-mm, 5% acrylamide gels, stained with ethidium bromide, and visualized by UV transillumination.

Immunohistochemical Studies for p16

Immunohistochemical studies were performed on oropharyngeal samples alongside negative and positive control slides using the EnVision Plus/horseradish peroxidase system (DAKO, Carpinteria, CA). Briefly, to inactivate endogenous peroxidase, paraffin-embedded sections were incubated in hydrogen peroxide and absolute alcohol for 30 minutes. Antigen retrieval was then performed using pressure cooker pretreatment. Tissue sections were subsequently incubated with a primary antibody (p16 clone, G175-405, DAKO) for 40 to 60 minutes at 25°C. Following Tris-buffered saline rinses, the tissue was incubated using the Envision Plus secondary antibody for 30 minutes, followed by diaminobenzidine for 5 minutes. Patterns of cytoplasmic and/or nuclear reactivity were evaluated. Patterns of immunoreactivity were scored as negative (patchy or weak staining) and positive (strong staining involving more than 50% of the tumor cells).

In Situ Hybridization

The Ventana platform for ISH was used for HPV detection in 17 cases. Briefly, formalin-fixed, paraffin-embedded tissue sections were incubated with the HPV family 16 probe cocktail (Ventana Medical Systems, Tucson, AZ). This formulation contains probes to the following HPV genotypes: 16, 18, 31, 33, 35, 39, 51, 52, 56, 58, and 66. Visualization was performed using the ISH iVIEW Plus Detection Kit (Ventana Medical Systems). This kit uses indirect detection of antigen, beginning with a rabbit antidinitrophenol primary antibody, followed by a biotinylated secondary antibody and, last, streptavidin-conjugated alkaline phosphatase as the chromogenic enzyme.

Histologic Grading

Histologic grade was obtained from the pathology report and recorded as well, moderate, or poorly differentiated squamous cell carcinoma.

Comparison of Methods and Data Analysis

For the purposes of comparison, the maximum positive rate (MPR) for HPV detection was positivity by the L1 or E7 primer or both. Each HPV test and p16 immunostaining was compared with the MPR. ISH and AGPCR were compared in a separate analysis.

Results

A group of 141 cases of oropharyngeal cancer was tested for HPV at Brigham and Women’s hospital using the commercially available AGPCR method, which incorporates restriction fragment length polymorphism viral typing when possible. A subset of these cases (n = 126) was also tested for HPV-16 and HPV-18 using primers specific for the E7 region of those subtypes. A subset of 17 from the 141 cases was analyzed by ISH and AGPCR. A subset of the cases (n = 102) with available sections was tested by both PCR methods; 97 were also stained for p16 by immunohistochemical studies. In 82 cases, the HPV test result and level of histologic differentiation were compared (by report).

HPV Assay Sensitivities

Table II illustrates a comparison of each HPV test with the MPR, defined as a positive by either method. The sensitivities of E7PCR and AGPCR were 72.5% (74/102) and 90.2% (92/102), respectively. Of the 92 cases positive by AGPCR, 75 (82%) were HPV-16+, 1 (1%) was HPV-58+, and 16 (17%) were positive but the intensity of the PCR products was too faint to precisely ascertain the HPV type. When AGPCR and ISH were compared, 14 of 17 revealed unequivocal results by both assays (positive or negative). All 14 were concordant. In 2 cases, the ISH assay was faintly positive (a slight bluish blush to the tumor cell nuclei) and AGPCR was negative. In a third case, the AGPCR was weakly positive and could not be classified further owing to the small amount of reaction product; the
ISH was negative. All but one ISH+ case had diffuse nuclear staining, suggesting that at least a portion of the viral DNA detected was episomal in origin [Image 1].

**Immunostaining for p16**

All of the cases scoring positive for HPV by any of the methods were strongly positive for p16 [Image 2]. The sensitivity of p16 immunostaining was 100%, whereas specificity was low at 38% relative to the MPR.

**HPV Status vs Tumor Differentiation**

HPV status did not correlate closely with reported tumor differentiation, ie, classifying a tumor as moderate or poorly differentiated did not influence HPV status ($P = .765; \chi^2$).

**Discussion**

HPV testing is integral in determining the etiology of malignant tumors of the oropharynx, identifies a subset of patients who will have a better response to chemoradiation, and is now routinely performed when this diagnosis is made. ISH is strongly recommended by some, however, PCR-based HPV tests have been in use for more than 20 years and have the added advantage of providing type-specific information. One potential limitation is sensitivity, particularly the possibility that primers targeting the L1 region may miss HPV sequences that are genomically integrated and have lost the capsid-encoding sequences, which are not necessary for tumorigenesis. However, there is little evidence that this phenomenon is responsible for an inordinate number of false-negatives. Still, because HPV L1 PCR can be performed in any molecular diagnostic laboratory and is potentially more cost-effective than ISH, it is important to determine the sensitivity of this approach relative to others for detecting HPV in oropharyngeal tissues.

Compared with an MPR (positivity by AGPCR or E7PCR) for HPV, the L1 primers detected 92 of 102, for a sensitivity of 90.2%. This sensitivity is significant in the context of 2 other variables. The first is the variability in DNA quality imposed by formalin-fixed, paraffin-embedded

![Image 1](https://example.com/image1.png) ![Image 2](https://example.com/image2.png) ![Image 3](https://example.com/image3.png)

**Image 1** In situ hybridization for human papillomavirus (HPV) nucleic acids in 2 oropharyngeal carcinomas. **A**, A diffuse nuclear staining pattern is consistent with the accumulation of episomal sequences (hematoxylin counterstain, ×400). Assessment of coexisting integrated sequences is limited. **B**, Punctate staining is characteristic of integrated HPV DNA (hematoxylin counterstain, ×200). **C**, At higher magnification, multiple dotted intranuclear signals are seen (hematoxylin counterstain, ×400).

**Table 1** Performance of HPV Testing Methods Relative to the MPR for 126 Total Cases Tested by Both Methods

<table>
<thead>
<tr>
<th>MPR+*</th>
<th>AGPCR+</th>
<th>Sensitivity</th>
<th>E7PCR+</th>
<th>Sensitivity</th>
<th>p16+</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>102 (81.0)</td>
<td>92/102</td>
<td>90.2</td>
<td>74/102</td>
<td>72.5</td>
<td>97/97</td>
<td>100</td>
<td>38</td>
</tr>
</tbody>
</table>

AGPCR, Access Genetics (Minneapolis, MN) polymerase chain reaction; E7PCR, early HPV-16 PCR; HPV, human papillomavirus; MPR, maximum positive rate.

* No. (%) of cases positive by E7PCR or AGPCR (ie, MPR).
The detection rate of HPV. Much of the HPV detected in lost in a minority of cases and may not significantly influence DNA. Thus, it appears that the capsid-encoding region (L1) is tion of signal, in keeping with the presence of episomal HPV sequences. All but 1 ISH+ case displayed a distribu-
cation of this gene, while a reasonable benchmark for adequacy in globin standard is approximately 300 bp, and amplification weight to the HPV target (450 bp). The AGPCR internal "housekeeping gene" standard that is identical in molecular
malignancy (×400).

Intense nuclear and cytoplasmic p16 immunostaining was characteristic of the cases in this study. Note the cytoplasmic staining only in the stromal inflammatory cells (center), which is not specific for malignancy (×400).

material. Essentially, 90.2% were positive by AGPCR when the globin amplification was confirmed in the tissue studied. This level of sensitivity (overall 92/126 [73.0%]) is comparable to or exceeds that seen with other studies of oropharyngeal malignancies, irrespective of the test used. However, 1 parameter that must always be addressed, and 1 that is not in the AGPCR system, is the use of an internal “housekeeping gene” standard that is identical in molecular weight to the HPV target (450 bp). The AGPCR internal globin standard is approximately 300 bp, and amplification of this gene, while a reasonable benchmark for adequacy in well-preserved DNA samples, might overestimate the quality of DNA sample if DNA degradation has taken place, leading to false negatives. We have encountered this problem previously.

The second variable is E7 positivity, which, in this study, was not as sensitive relative to the MPR (72.5%). This sensitivity was improved to 77.1% when non–HPV-16 or unknown HPV types were excluded. Nevertheless, the lower sensitivity for E7 argues against loss of L1 HPV sequences in a signifi-
cant number of HPV– (by fluorescence ISH, ISH, and PCR) tonsillar cases stained positive for p16.15,18,23,30 Because a small subset raises the possibility that the AGPCR method is missing some HPV+ cases. Nevertheless, this figure has been corroborated by other independent studies in which 7% to 17% of the HPV– (by fluorescence ISH, ISH, and PCR) tonsillar cases stained positive for p16.15,18,23,30 Because a small lack of concordance with p16 has been verified by others, irrespective of the test used, the relative advantage conferred by relying on HPV testing must be scrutinized not within the realm of a given HPV type, but by determining if p16+/HPV– tumors are a distinct clinicopathologic entity.

Positivity of p16 is a powerful correlate of improved survival in oropharyngeal cancers. Moreover, although expres-
sion of p16 is tightly linked to HPV-16, there is evidence that p16 is an independent and (like HPV-16) a positive predictor of survival. Although additional studies may be required, it is conceivable that p16 positivity could be as reliable as HPV in predicting outcome, justifying p16 staining as a preliminary screening test for these tumors or the only assay, if HPV testing is not available. Similarly, it could be argued that type-specific testing (for HPV-16) is not critical given the high rate of HPV-16 in these tumors. However, the advantage of typing is to confirm the presence of HPV-16 or other highly oncogenic HPV types, which may be helpful when interpreting weaker amplification signals that could be rare oral contamin-
mants of little pathogenetic significance.

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DNA in cervical cancers has been documented previously, particularly in cases with HPV-16. How oropharyngeal cancers maintain a high viral load in the epithelial state and the relevance of this observation to disease mechanism and outcome are unclear. Moreover, the significance of episomal vs integrated HPV DNA and whether ISH will segregate these 2 molecular states is unknown. A potential advantage of ISH is the precise localization of the HPV sequences to the tumor cells. However, in our experience and that of others, the fre-

quency of incidentally detected HPV in the oropharynx (in the absence of neoplasia) is very low (C.P. Crum, R. Haddad, and M. Posner, unpublished data, 2009). It has been shown that the mechanism of the oncogenic HPV protein E7 is to bind and inactivate the retinoblastoma tumor suppressor protein, pRb. This, in turn, leads to increased levels of p16, considered a surrogate marker of HPV infection. The cases in which p16 is up-regulated and no HPV DNA is detected may be due to alternative mechanisms of cell cycle disruption. In most reports pertaining to SV40, Kaposi sarcoma–associated herpesvirus, and Epstein-Barr infection as tumorigenic initiators, p16 is methylated or otherwise silenced or downregulated. An important question that arose in this study was whether p16 immunohistochemical studies should be used diagnostically as a surrogate marker of HPV infection in oropharyngeal biopsy specimens. Of the 97 cases by PCR and immunohistochemical studies, 10 were p16+ that tested HPV– by PCR. At roughly 10% of the samples, this relatively small subset raises the possibility that the AGPCR method is missing some HPV+ cases. Nevertheless, this figure has been corroborated by other independent studies in which 7% to 17% of the HPV– (by fluorescence ISH, ISH, and PCR) tonsillar cases stained positive for p16.15,18,23,30 Because a small lack of concordance with p16 has been verified by others, irrespective of the test used, the relative advantage conferred by relying on HPV testing must be scrutinized not within the realm of a given HPV type, but by determining if p16+/HPV– tumors are a distinct clinicopathologic entity.

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Currently, the standard of care at most institutions does not involve altering treatment plans with respect to HPV status, but as the evidence for improved outcome in HPV positive oropharyngeal tumors continues to build, this may change. Indeed, testing for HPV/p16 status in oropharyngeal cancer will become a requirement in head and neck cancer clinical trials and a stratification factor for therapy. It is thus imperative that a uniform and practical method for HPV detection is available. The level of laboratory sophistication, availability of hardware for PCR- or ISH-based assays, and specimen volume will dictate which approach is more practical. However, we have shown that the PCR testing method is a valid method of HPV testing formalin-fixed, paraffin-embedded samples of oropharyngeal carcinomas and is comparable with others reported in the literature.

From the 1Division of Women’s and Perinatal Pathology, Department of Pathology; and 4Department of Medical Oncology, Head and Neck Oncology Program, Dana-Farber Cancer Institute, the Department of Medicine, Brigham and Women’s Hospital, Boston, MA; 4Boston University, Boston; and 5Tufts University Medical School, Boston.

Address correspondence to Dr Crum: Division of Women’s and Perinatal Pathology, Dept of Pathology, Brigham and Women’s Hospital, 75 Francis St, Boston, MA 02115.

References


