Incorporation of Cervista Human Papillomavirus 16/18 Assay Into Algorithms for Classifying Human Papillomavirus Status in Formalin-Fixed, Paraffin-Embedded Head and Neck Squamous Carcinoma Specimens

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Context.—Human papillomavirus (HPV) DNA in situ hybridization (ISH) assay and p16 immunohistochemistry (IHC) are used to determine high-risk HPV status in formalin-fixed, paraffin-embedded (FFPE) tissues in oropharyngeal squamous cell carcinoma (SCC). Although high sensitivity and specificity for HPV can be obtained by combined p16 IHC and HPV DNA ISH, the occasional discrepancy between these assays has prompted evaluation of Cervista HPV assays in FFPE tissue from patients with oropharyngeal SCC.

Objective.—To compare the efficacy of Cervista HPV 16/18 and Cervista HPV HR assay to that of HPV DNA ISH assay and p16 IHC in FFPE tissue in head and neck squamous cell carcinoma of oropharyngeal origin.

Design.—Archived FFPE tissue from 84 patients with SCC of oropharyngeal origin and available HPV DNA ISH and p16 IHC test results were tested with the Cervista HPV 16/18 assay and further verified by polymerase chain reaction (PCR)–based HPV16/18 genotyping tests in cases with discrepancy.

Results.—Of the 84 specimens, 75% (63 of 84) were positive and 16% (13 of 84) had discrepant or equivocal findings by p16 IHC and HPV DNA ISH testing. Use of Cervista HPV assays, either to clarify discrepant/equivocal findings or as confirmation after initial p16 IHC/HPV DNA ISH tests, identified 81% (68 of 84) of HPV-positive cases without equivocal HPV results. Five of 13 cases with discrepancy or equivocal HPV DNA ISH results tested positive for HPV16 or HPV18 by Cervista HPV 16/18 assay, which was further confirmed by PCR-based HPV 16/18 genotyping.

Conclusions.—The Cervista HPV assays are a reasonable alternative to HPV DNA ISH in determining HPV status in FFPE tissue specimens from patients with oropharyngeal SCC.

Prior infection with high-risk human papillomavirus (hrHPV), predominantly the HPV16 type, has been recognized as an etiologic factor in most oropharyngeal squamous cell carcinomas (SCCs) and a small proportion of nonoropharyngeal SCCs of the head and neck.1–3 High-risk HPV-associated oropharyngeal SCC, specifically affecting the tonsil or base of tongue, is a distinct subtype that is different from non–HPV-associated oropharyngeal SCC in carcinogenesis, clinical characteristics, and response to therapy.3,4 Patients with hrHPV-positive SCC of the tonsil or base of tongue have a higher 5-year disease-specific survival rate than their counterparts with an HPV-negative tumor.1–7 Clinical trials are being conducted to evaluate whether patients with HPV16-positive oropharyngeal SCC can be spared components of intensive therapy to reduce therapy-related morbidity without jeopardizing therapeutic efficacy.

In the United States, the incidence of hrHPV–associated oropharyngeal SCC has increased dramatically in the past 2 decades.2,4,9 Documentation of hrHPV status, especially HPV16, in patients with oropharyngeal SCC is becoming critical for clinical management and selection for clinical trials. Recently, the 8th edition of the American Joint Committee on Cancer Staging Manual, effective on January
In patients with oropharyngeal SCC, HPV testing is performed most frequently in tissue specimens acquired by biopsy of the primary tumor or needle biopsy of a metastatic lymph node. p16 immunohistochemistry (IHC) testing is widely used to determine HPV status of oropharyngeal SCCs and has become a standard of care for such patients. However, because p16 IHC is less specific than other HPV detection methods, including HPV in situ hybridization (ISH) assay, many advocate for a confirmatory test in tissue specimen biopsies by using HPV DNA ISH assay, which detects 13 pooled types of hrHPV (16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68, 70), to confirm p16 IHC results. The HPV DNA ISH assay has high specificity and consequently is an excellent choice for a confirmatory test, though it is limited by its relatively low sensitivity and sometimes equivocal results.12,13

HPV testing by polymerase chain reaction (PCR)–based assays in oropharyngeal tissue has been reported,13 but the lack of a standard protocol for these assays limits their application. Published studies using commercially available HPV assays in oropharyngeal SCC tissue specimens are limited.14

However, it is not clear whether HPV status can be determined by Cervista HPV assays in formalin-fixed, paraffin-embedded (FFPE) tissue specimens from patients with oropharyngeal SCC.

To evaluate the sensitivity and specificity of the Cervista HPV assays in head and neck tissue specimens, we compared the results for Cervista HPV 16/18 and HPV HR assays (Hologic Inc, Bedford, Massachusetts) in fine-needle–aspirated specimens to determine the status of HPV16/18 and other hrHPV types in patients with oropharyngeal SCC. Using the Cervista HPV assays in fine-needle–aspirated specimens has become the standard of care in our institution to determine HPV status in such patients. This minimally invasive protocol efficiently provides accurate, critical information supporting treatment recommendations and prognosis for individual patients.30

DNA Extraction for HPV Testing

FFPE tissue sections were prepared from paraffin blocks (5 μm) with the last section stained with hematoxylin-eosin, which was evaluated to determine the quality of the specimen. Five unstained tissue sections were used for DNA extraction. Briefly, the FFPE tissue sections were deparaffinized by xylene (>3) for 10 minutes each, followed by an ethanol wash (>3) to remove paraffin. Deparaffinized tissue from slides were scraped and placed in a 1.5-mL microcentrifuge tube. DNA extraction was performed by using the Qiagen DNeasy Blood & Tissue kit (Cat No. 69506, Qiagen, Valencia, California) following the manufacturer’s instructions.
Cervista HPV assays, oligonucleotides targeting the human histone 2 genes (H2be, HIST2HcBE) were present in each of the oligonucleotide mixtures to serve as an internal control for the detection of cellular DNA.

**PCR-Based HPV Assays**

To confirm the HPV status in cases for which Cervista HPV and HPV DNA ISH assay results were discrepant, we performed PCR-based HPV testing by using HPV16 or HPV18 type-specific primers and the consensus HPV primers (GP5+/GP6+), described elsewhere.16 HPV16 or HPV18 was detected by using type-specific primers of HPV16 (HPV16.1/HPV16.2) or HPV18 (HPV18.1/HPV18.2) (Millipore Sigma, St Louis, Missouri) as described previously.16 Briefly, a 25-ng aliquot of genomic DNA was added to a PCR master mixture containing 1X PCR buffer (100 mM Tris-HCL, 500 mM KCL, pH 8.3), 3 mM MgCl2, 200 mM concentrations of each deoxynucleoside triphosphate, 200 nM concentrations of each primer, and 1 unit of AmpliTaq Gold DNA polymerase (Life Technologies, Carlsbad, California). PCR was performed with a Bio-Rad Thermal Cycler (Bio-Rad Laboratories Inc, Hercules, California) under the following cycling conditions: 10 minutes at 94°C, followed by 30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C, and 5 minutes at 72°C in the final cycle (35 cycles).

HPV DNA was detected by using consensus primers within the L1 open-reading frame (GP5+/GP6+) (Millipore Sigma) as described previously.16 Briefly, a 25-ng aliquot of genomic DNA was added to a PCR master mixture containing 1X PCR buffer (100 mM Tris-HCL, 500 mM KCL, pH 8.3), 3.5 mM MgCl2, 200 mM concentrations of each deoxynucleoside triphosphate, 200 nM concentrations of each primer, and 1 unit of AmpliTaq Gold DNA polymerase (Life Technologies). PCR was performed with a Bio-Rad Thermal Cycler under the following cycling conditions: 5 minutes at 94°C, 1 minute at 94°C, 2 minutes at 55°C, 1.5 minutes at 72°C, and 7 minutes at 72°C in the final cycle. From the second to the 15th cycles, the annealing temperature was programmed to decrease 1°C for each cycle from 55°C to 40°C, followed by an additional 30 cycles with annealing temperature of 40°C.

The specimens were visualized with ethidium bromide staining on a 4% low-melt agarose gel. A positive and a negative control as well as a blank control were used for each reaction.

**Statistical Analysis**

The results of the Cervista HPV assays were compared to the results of the HPV DNA ISH and p16 IHC assays for each specimen. The final HPV status of each case was determined as follows: (1) HPV-positive, p16 IHC+/HPV DNA ISH+, Cervista HPV 16/18+, and/or Cervista HPV HR+; (2) HPV-negative, p16 IHC-/HPV DNA ISH−, Cervista HPV 16/18−, and/or Cervista HPV HR−; and (3) cases with discrepant results between the p16 IHC and HPV DNA ISH or equivocal HPV DNA ISH results, the final HPV status to be determined by PCR-based HPV16/18 genotyping and/or GP5+/GP6+ HPV testing. Descriptive statistics were generated. p16 IHC, HPV DNA ISH, and Cervista HPV testing results were compared with the final HPV status for sensitivity, specificity, positive predictive value, negative predictive value, false-positive rates, and false-negative rates. The McNemar test was used to compare the relative efficacy between HPV DNA ISH and Cervista HPV assays. The computations were carried out by using SAS version 8.0 software (SAS Institute, Cary, North Carolina).

**RESULTS**

The demographic, clinical, and pathologic characteristics of the 84 patients are summarized in Table 1. The patients’ ages ranged from 32 to 85 years (mean, 58 years; median, 58 years). Of the 84 cases, 75 of 84 (89%) were positive for p16 overexpression. Using HPV DNA ISH as the confirmatory test, we considered 63 of 84 tumors (75%) as clearly HPV positive and 8 of 84 tumors (10%) as clearly HPV negative, with 13 of 84 (16%) having discordant or equivocal results including 10 of 84 cases (12%) with equivocal HPV DNA ISH results and 3 of 84 cases (4%) with p16 IHC−/positive/HPV DNA ISH−/negative results.

Using Cervista HPV assays, the HPV status was confirmed in 63 cases with p16 IHC+/HPV DNA ISH+ and 8 cases with p16 IHC−/HPV DNA ISH−/equivocal HPV DNA ISH results. Of the 13 cases, 5 were reclassified to be HPV positive including 4 HPV16 cases and 1 HPV18 case. Eight cases were confirmed to be negative for HPV16/18 by Cervista HPV 16/18 assay (Figure; Table 2).

Type-specific PCR tests for HPV16 or HPV18 were positive in 5 of 13 cases with discordant p16 IHC/HPV DNA ISH−/HPV DNA ISH+ results and confirmed all 5 Cervista HPV 16/18−/positive cases. The remaining 8 cases were negative for HPV16/18 by type-specific PCR and hrHPVs by consensus primer-mediated PCR tests. The negative PCR-based HPV testing results are also consistent with the negative Cervista HPV 16/18 and Cervista HPV HR test results (Table 2).

Cervista HPV 16/18 assay identified 68 of 84 tumors (81%) as HPV positive and 16 tumors as HPV negative. Of the 68 Cervista HPV-positive cases, HPV16 was the predominant HPV genotype (62, 91%), followed by HPV18 (5, 7.4%) and non-16/18 HPV high-risk type (1, 1.5%).

### Table 1. Demographic and Clinicopathologic Characteristics of Patients With Oropharyngeal Squamous Carcinoma (n = 84)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total No.</th>
<th>p16+ No. (%)</th>
<th>HPV DNA ISH/p16, No. (%)</th>
<th>Cervista HPV+, No. (%)</th>
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</thead>
<tbody>
<tr>
<td>Total</td>
<td>84</td>
<td>75 (89)</td>
<td>63 (75)</td>
<td>68 (81)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>72</td>
<td>66 (92)</td>
<td>55 (76)</td>
<td>59 (82)</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>9 (75)</td>
<td>8 (67)</td>
<td>9 (75)</td>
</tr>
<tr>
<td>Age, y ≤55</td>
<td>35</td>
<td>31 (89)</td>
<td>23 (65)</td>
<td>26 (74)</td>
</tr>
<tr>
<td>&gt;55</td>
<td>49</td>
<td>44 (90)</td>
<td>40 (82)</td>
<td>42 (86)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Non-Hispanic white</td>
<td></td>
<td>68 (88)</td>
<td>60 (78)</td>
<td>64 (83)</td>
</tr>
<tr>
<td>Others</td>
<td>7</td>
<td>7 (100)</td>
<td>3 (43)</td>
<td>4 (57)</td>
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<tr>
<td>T categoryc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T0–T2</td>
<td>59</td>
<td>55 (93)</td>
<td>47 (80)</td>
<td>50 (85)</td>
</tr>
<tr>
<td>T3–TX</td>
<td>25</td>
<td>20 (80)</td>
<td>16 (64)</td>
<td>18 (72)</td>
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<td>N categoryc</td>
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<tr>
<td>N0–N1</td>
<td>12</td>
<td>11 (92)</td>
<td>9 (75)</td>
<td>9 (75)</td>
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<tr>
<td>N2–N3</td>
<td>72</td>
<td>64 (89)</td>
<td>54 (75)</td>
<td>59 (82)</td>
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<td>TNM stagec</td>
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<td></td>
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<tr>
<td>1–3</td>
<td>11</td>
<td>10 (91)</td>
<td>8 (73)</td>
<td>8 (73)</td>
</tr>
<tr>
<td>4</td>
<td>73</td>
<td>65 (89)</td>
<td>55 (75)</td>
<td>60 (82)</td>
</tr>
</tbody>
</table>

Abbreviations: HPV, human papillomavirus; ISH: in situ hybridization.

a The efficacy of HPV DNA ISH+/p16 IHC+: the same as that of HPV DNA ISH.

b Hologic Inc, Bedford, Massachusetts.

* One patient TX, 1 patient NX, and 2 patients TNM stage X.
In 75 cases with p16 IHC+ results, HPV DNA ISH was positive in 63 (84%) and Cervista HPV was positive in 68 (91%). With combined p16 IHC/HPV DNA ISH and confirmatory Cervista HPV 16/18/PCR-based HPV test results, the final HPV status was summarized to be positive in 68 cases and negative in 16 cases. There were no significant differences in HPV-positive rates among demographic or clinical subgroups (Table 1). The marginal homogeneity of HPV DNA ISH and Cervista HPV efficacy in p16 IHC+ cases was evaluated by using the McNemar test and no significant difference was observed between the 2 groups ($P = .07$).

Based on the final HPV status (Figure), the sensitivity, specificity, positive predictive values, negative predictive values, false-positive rates, and false-negative rates for p16 IHC, HPV DNA ISH, and Cervista HPV assays are presented in Table 3. These results indicate that p16 IHC has a higher sensitivity (100.0%) than HPV ISH (92.7%), whereas HPV ISH has a higher specificity (100%) than p16 IHC (56.3%) in determining HPV status in oropharyngeal SCC. p16 IHC yielded a false-positive rate of 9.3% and the HPV DNA ISH assay yielded false-negative results of 7.4% in oropharyngeal SCC.

**DISCUSSION**

This evaluation of Cervista HPV assays when used in conjunction with p16 IHC and HPV ISH assays in FFPE tissue specimens of head and neck SCC...
origin demonstrates that the Cervista HPV assays can be helpful in clarifying discrepant p16 IHC/HPV DNA ISH or equivocal HPV ISH results. Furthermore, Cervista assays can be incorporated into an HPV testing algorithm as the confirmatory test following initial p16 IHC testing, along with HPV DNA ISH to clarify any discrepant results. We conclude that the Cervista HPV assays are a valid choice to determine HPV status in FFPE specimens of oropharyngeal SCC.

In our institution, FFPE tissue specimens from patients with oropharyngeal SCC are subjected first to p16 IHC assay to determine hrHPV status, with HPV DNA ISH as a confirmatory assay. Immunohistochemistry staining for p16 in conjunction with HPV testing has been recommended and used as a prognostic marker in patients with oropharyngeal carcinoma. The advantage of p16 IHC is its high sensitivity for detecting oropharyngeal carcinomas associated with HPV. However, p16 IHC results can be positive in HPV-negative oropharyngeal SCC, nonoropharyngeal SCCs, and other non–head and neck carcinomas. As previously reported, combined p16 IHC and HPV ISH assays can increase the specificity and the accuracy of HPV status determination for patients with oropharyngeal carcinoma. In our study cohort, the sensitivity of combined p16 IHC/HPV DNA ISH for HPV status was 92.7%. With a substantially improved sensitivity for HPV status by adding Cervista HPV 16/18 testing, we suggest here that adding Cervista HPV testing assay to p16 IHC and HPV DNA ISH testing can further refine testing algorithms.

The advantage of the HPV DNA ISH assay in FFPE specimens of oropharyngeal SCC is that the ISH assay is a signal amplification–based method. The FFPE specimens can be processed and evaluated by a process similar to that used for immunostaining in routine practice. In tissue sections, HPV DNA ISH signal located in the nuclei of the tumor cells that allows direct visualization of HPV signals is reliable evidence for confirming true positivity of hrHPV in carcinoma. However, there are limitations to using HPV DNA ISH, specifically, HPV DNA ISH assays in FFPE specimens. The sensitivity of HPV DNA ISH can be affected by the degree of HPV integration in the carcinoma, such that highly integrated HPV may generate false-negative HPV results. More importantly, interpretation of HPV DNA ISH results in FFPE can be challenging. For example, a sparse ISH signal located outside of nuclei could be interpreted incorrectly as a positive result. Although in our study cohort, no false-positive result by HPV DNA ISH testing was observed, we observed 10 equivocal HPV DNA ISH results (12%) in our cohort. We speculate that an equivocal HPV ISH interpretation could be made with bias because of a positive p16 IHC result. Of the 10 cases with equivocal HPV DNA ISH results, 9 had positive p16 immunostaining results. This indicates that interpretation of the HPV DNA ISH test result with bias of the p16 IHC result may be the cause of equivocal or even false-positive results. Our observations suggest that cases with equivocal HPV DNA ISH results and cases with discrepancy between p16 immunostaining and HPV DNA ISH can be resolved by additional testing with the Cervista HPV assays (Figure; Table 2). In recent years, HPV RNA ISH (RNAgene HPV) has been used in FFPE tumor tissue to determine HPV status in oropharyngeal SCC. The advantage of using HPV RNA ISH is that it is more clinically relevant to detect the transcriptionally active form of HPV. HPV RNA ISH assay was reported to have a higher sensitivity than HPV DNA ISH assays, and the potential to be used as a single test to determine HPV status. Since published data for the efficacy of HPV RNA ISH are limited, more clinical studies are needed to define the efficacy of the HPV RNA ISH assays in routine clinical settings for patient care.

The PCR-based HPV16/18 testing assay, usually targeting E6 or E7 of HPV16 or HPV18, is highly sensitive in detecting HPV DNA. Practically, however, single institution–designed PCR-based HPV testing has not been widely adopted in the United States. The reasons probably include the lack of a standard protocol for PCR assays and concern that cross-contamination with other HPV-positive specimens will lead to false-positive results. Use of commercially available HPV testing assays in FFPE oropharyngeal carcinoma tissue specimens has been reported. Kerr et al reported using the Cobas HPV assay (Roche Diagnostics, Indianapolis, Indiana) to detect hrHPVs in FFPE specimens of head and neck SCC. The authors reported a high concordance between Cobas HPV and HPV DNA ISH assays for hrHPV status (92%) in this setting. In our study, a concordance of 86% (72 of 84) between Cervista HPV and HPV DNA ISH was observed. The potential advantage of using a commercially available HPV assay is the standardization of the assay, producing HPV results that are comparable over different laboratories. More importantly, use of HPV testing assays already approved by the US Food and Drug Administration for Papanicolaou (Pap) cytology specimens would allow processing of both Pap cytology specimens and non-gynecologic specimens on a single HPV testing platform, which would simplify the quality assurance and improve the efficiency of HPV testing.

Another advantage of using a commercially available HPV assay such as the Cobas HPV or Cervista HPV 16/18 assay is the HPV genotyping information provided. In our cohort, the hrHPV type was predominantly HPV16 (91%, 62 of 68). Kerr et al reported a similar HPV16–positive rate (88%, 28 of 32). However, they reported 12% of non-16/18 hrHPV (4

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**Table 3. Efficacy of p16 IHC, HPV DNA ISH, and Cervista HPV Assays<sup>a</sup> According to the Final HPV Status**

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>PPV, %</th>
<th>NPV, %</th>
<th>FN, %</th>
<th>FP, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16 IHC</td>
<td>100.0</td>
<td>56.3</td>
<td>90.7</td>
<td>100.0</td>
<td>0.0</td>
<td>9.3</td>
</tr>
<tr>
<td>HPV DNA ISH&lt;sup&gt;b&lt;/sup&gt;</td>
<td>92.7</td>
<td>100.0</td>
<td>100.0</td>
<td>76.2</td>
<td>7.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Cervista HPV assays&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Abbreviations: FN, false negative; FP, false positive; HPV, human papillomavirus; IHC, immunohistochemistry; ISH, in situ hybridization; NPV, negative predictive value; PPV, positive predictive value.

<sup>a</sup> Hologic Inc, Bedford, Massachusetts.

<sup>b</sup> The efficacy of HPV DNA ISH+/p16 IHC+: the same as that of HPV DNA ISH; the efficacy of Cervista HPV/p16 IHC+: the same as that of Cervista HPV assays.

<sup>c</sup> HPV DNA ISH equivocal considered negative.
of 32), higher than the 1.5% (1 of 68) rate that we observed, and no HPV18-positive cases, while we observed 7% (5 of 68) of HPV18 positivity. The differences of HPV genotypes between the 2 studies may reflect differences in the study cohorts or in the patient populations represented by the 2 cohorts. Since most of the SCGs of the tonsil and base of tongue were positive for HPV16 in our study, we recommend using Cervista HPV 16/18 testing as the first-line testing assay, with Cervista HPV HR testing reserved for cases with a negative HPV16/18 result.

Our study identified no false-positive results for the Cervista HPV assays. Kerr et al14 reported 3 cases of 62 (5%) with false-positive results for the Cobas HPV assay.14 The Cobas HPV assay, when used for cervical Pap cytology, is programmed to have a cutoff to eliminate Pap specimens with low HPV copy numbers that may be clinically insignificant for predicting high-grade cervical intraepithelial neoplasia. The fact that the Cervista HPV assays are signal amplification assays, while the Cobas HPV assay is a target amplification assay, may result in differences in analytic sensitivity and specificity between the 2 assays. A direct comparison study would be needed to address the comparative testing efficacy of these 2 commercially available HPV assays.

The limitation of the study includes a small cohort as well as the cases with 2 HPV DNA ISH assays, although we did not observe significant differences between the 2 HPV DNA ISH assays for HPV positivity or equivocal cases. In summary, the Cervista HPV assays are comparable in sensitivity and specificity to combined HPV ISH and p16 IHC in determining HPV status in FFPE tissue specimens from patients with oropharyngeal carcinoma. Cervista HPV assays also provide HPV16/18 genotyping information. Furthermore, Cervista HPV assays resolve equivocal HPV ISH testing results or discrepancies between p16 IHC and HPV DNA ISH test results and can be incorporated into a HPV testing algorithm with p16 IHC/HPV DNA ISH assays.

References