Detection of Human Papillomavirus Using Hybrid Capture 2 in Oral Brushings From Patients With Oropharyngeal Squamous Cell Carcinoma

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Abstract

Detection of high-risk (HR) human papillomavirus (HPV) in oropharyngeal squamous cell carcinoma (SCC) has important prognostic implications; patients exhibit improved survival compared with patients with HPV–SCC. Oral brushing and rinsing samples were obtained from patients with oropharyngeal, oral cavity, or hypopharyngeal SCC and tested for HR-HPV using Hybrid Capture 2 (HC2; QIAGEN, Valencia, CA). HR-HPV in situ hybridization (ISH) was performed on biopsy tissue samples from the same patients.

Oral cytologic samples from 16 SCCs were tested by HC2. Biopsy tissue samples were available for ISH in 11 cases. Five oropharyngeal SCCs were HR-HPV+ by ISH and HC2 (oral brushing). Of the oropharyngeal SCCs, 2 were positive by HC2 (oral brushing) and negative or equivocal by ISH. We found that 2 oral cavity carcinomas and 2 hypopharyngeal carcinomas were negative by HC2. One hypopharyngeal cancer was positive by ISH. All oral rinsing samples were negative by HC2. HC2 may be an effective method of determining HR-HPV status in patients with oropharyngeal SCC.

Squamous cell carcinoma (SCC) of the head and neck is a significant cause of cancer mortality. In the United States, it is estimated that 36,540 new cases of oral and pharyngeal malignancies will be diagnosed in 2010, and approximately 7,880 patients will die of this disease.1 While the incidence of head and neck SCC related to exposure to such carcinogens as tobacco and alcohol has stabilized in recent years, the incidence of head and neck SCC attributed to high-risk (HR) human papillomavirus (HPV) is increasing.2-4 Patients with HPV-associated SCC have a clinical profile distinct from patients with tobacco- and alcohol-related SCC. The former tend to be younger, with tumors localized specifically to the oropharynx.5,6 As many as 70% of cases of oropharyngeal SCC may be associated with HR-HPV; the overwhelming majority of these cases are attributable to HPV type 16.3,4,7 The patients have a greater response to radiation therapy and improved overall survival compared with patients with non-HPV–associated tumors, even in cases with locally advanced disease.8-11

Numerous methods of testing for HR-HPV in fresh frozen or formalin-fixed, paraffin embedded (FFPE) tissue samples from head and neck SCC are available, including in situ hybridization (ISH), polymerase chain reaction (PCR)-based assays, and immunohistochemical testing for p16 expression. Each method has its own comparative advantage in terms of sensitivity, specificity, or level of technical difficulty. The Hybrid Capture 2 (HC2; QIAGEN, Valencia, CA) test, which is an in vitro nucleic hybridization assay, is used for HR-HPV testing in cervical cytologic specimens but has not been routinely implemented in testing for HR-HPV in head and neck SCC. An obvious potential advantage of using HC2 is that it is performed on cytologic specimens, which can be procured using minimally invasive techniques. A noninvasive method
for determining HR-HPV status could facilitate appropriate treatment planning early in the management of oropharyngeal SCC, even before surgical sampling of the lesion. Oral rinsing samples might also represent a screening technique for patients at risk for HPV-related oropharyngeal cancer.

In the present study, we evaluated the effectiveness of using HC2 to test for HR-HPV in oral brushing and oral rinsing samples from patients with oropharyngeal SCC. Results were compared with those obtained by testing for HR-HPV on biopsy material using HR-HPV ISH.

Materials and Methods

The study was approved by the institutional review board at the University of Utah, Salt Lake City.

Case Selection and Sample Procurement

The study included patients referred to the Huntsman Cancer Hospital, University of Utah, for evaluation of an oropharyngeal lesion clinically “suspicious” for or recently diagnosed as SCC. Also included was a subset of patients with presumed or known SCC involving the oral cavity or hypopharynx. Patients who had undergone surgery or radiation for treatment of the lesion were excluded. Cytologic samples (oral brushing and oral rinsing) were obtained from each patient. An oral brushing sample was obtained by brushing the visible lesion with a brush-tipped instrument designed for this purpose and sold commercially (Cytosmear cytology brush, 6.875”, No. 4653B, PurFybr, Munster, IN). The brush material was then transferred to a vial of Preservcyt solution (Hologic, Marlborough, MA) for HR-HPV testing using HC2. In cases in which a visible mucosal lesion was not identified, a brushing of the mucosa overlying the known mass was obtained. Patients produced an oral rinsing by gargling tap water and then spitting into a paper cup. The sample was then transferred to Preservcyt solution. Cases with sufficient biopsy material and/or material from the subsequent surgical resection were also tested for HR-HPV using ISH on FFPE tissue samples.

HPV Testing With the HC2 Assay

HR-HPV testing was performed on 4 mL of each cytologic specimen (an oral brushing and an oral rinsing sample from each patient) using the HC2 assay, according to the manufacturer’s instructions (QIAGEN). The assay uses an RNA probe mixture derived from 13 cancer-associated HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66). The resultant chemiluminescent signal is measured in relative light units (RLUs). Each result is reported as a ratio of RLUs to a cutoff value based on 3 positive control samples that are supplied with the assay kit. The manufacturer has established an RLU/cutoff ratio of 1.0 or more as a positive result for scoring cervical cytologic samples. In this study, test results were scored as positive or negative using the same threshold.

HPV Testing by ISH

ISH testing for HR-HPV DNA was performed on 4-μm-thick FFPE tissue sections using the INFORM HPV III Family 16 (B) Probe cocktail (Ventana Medical Systems, Tucson, AZ). This dinitrophenyl-labeled probe cocktail has an affinity to 12 HR-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66). The assay was performed according to the manufacturer’s guidelines, using an automated slide staining system (Ventana Medical Systems). Nuclear staining (diffuse or punctate) in tumor cells was considered a positive result for HPV.

Results

Oral cytologic samples from 16 patients were obtained and tested for HR-HPV by HC2 during a 1.5-year period. In all but 1 case (in which only an oral brushing sample was obtained), an oral brushing and an oral rinsing sample were obtained and tested. SCC was confirmed by examination of formalin-fixed biopsy material in each case. Tissue was available for HR-HPV ISH in 11 (69%) of 16 cases; the remaining cases had insufficient tissue remaining in the block or could not be procured from outside institutions for ISH testing. Results of HR-HPV testing in cases evaluated by both methods are summarized in Table I.

For 7 patients with oropharyngeal carcinoma, specimens were available for HR-HPV testing by both methods. In 5 of these cases, the biopsy sample was HR-HPV+ (by ISH) and the oral brushing sample was positive (by HC2); these cases include 1 in which a visible mucosal lesion was not identified. Samples from 2 patients with oropharyngeal carcinoma tested positive for HR-HPV by HC2 analysis of oral brushing samples but had an HR-HPV negative or equivocal result by ISH. There were no patients with oropharyngeal carcinoma who tested positive for HR-HPV by ISH and negative by HC2.

Both methods were used to test samples from 2 patients with an oral cavity carcinoma and 2 patients with carcinoma involving the hypopharynx. None of these patients were positive for HR-HPV by HC2 testing. One of the cases of hypopharyngeal cancer that lacked a visible mucosal lesion was positive for HR-HPV by ISH. All of the oral rinsing specimens were negative for HR-HPV by HC2 testing.

Discussion

HPV infection has been shown to be an important etiologic agent for oropharyngeal SCC. Moreover, HPV-associated oropharyngeal SCCs seem to differ significantly...
from tobacco-related carcinomas in terms of age of occurrence, sexual practices, and stage at diagnosis. It is important to note that HPV-associated oropharyngeal SCCs seem to be associated with improved survival, which is likely due, in part, to improved response to radiotherapy. However, the use of tobacco may negatively impact the behavior of HPV-associated tumors, making them less responsive to therapy.

Studies have demonstrated that the HPV types associated with SCC of the oropharynx are the high-risk types similar to those seen in the cervix. The involved types are HPV-16 (87%), HPV-18 (3%), and HPV-33 (11%). PCR-based analysis of exfoliated cells obtained by oral rinsing using 10 mL of normal saline has been shown to be effective in the identification of HPV-infected cells associated with oropharyngeal SCCs. Currently, HC2 is the dominant method for the detection of HR-HPV subtypes in cervical dysplasia. We postulated that HC2 would be an effective technique for recognition of HR-HPV–related oropharyngeal SCCs. HC2 is an accurate, specific, and relatively sensitive technique for the recognition of HR-HPV in exfoliated cell specimens. The present study was performed to test the sensitivity and specificity of HC2 for the detection of HPV-related oropharyngeal SCC using cell samples obtained by oral rinsing and direct brushing of clinical lesions. If HC2 testing of oral rinsing specimens was sufficiently accurate in recognizing HPV-related oropharyngeal SCCs, the method could serve as an inexpensive screening test usable in dentist and physician offices for the evaluation of patients at risk for these neoplasms.

In our series of 7 patients with oropharyngeal carcinoma, brushing specimens were HC2 positive in all 4 cases that were HR-HPV positive by ISH. The single case in which the HC2 result was negative but the HR-HPV ISH result was positive was an SCC of the hypopharynx. This is a location usually unassociated with HPV+ carcinomas. Moreover, HC2 detected HR-HPV in an additional case of oropharyngeal SCC in which HR-HPV ISH was equivocal and another case in which the HR-HPV ISH result had been negative. HR-HPV ISH was positive in only 4 (57%) of 7 cases. Thus, HC2 seems to be more sensitive for the detection of HR-HPV in brushing specimens than is ISH, with HC2 detecting HPV in 6 (86%) of 7 oropharyngeal SCCs tested.

Smith et al reported that PCR and direct DNA sequencing was able to detect HR-HPV in 20% of oral cavity and oropharynx SCCs by studying exfoliated cells in oral rinsing samples. In our study of 16 patients with clinically suspected SCC of the oral cavity, hypopharynx, or oropharynx, HC2 did not detect any cases positive for HR-HPV, even though HR-HPV was detected by HC2 or HR-HPV ISH in 7 cases using brushing specimens. This is likely due to an insufficient number of exfoliated cells being collected in the rinse fluid (as opposed to directly sampling the gross lesion by brushing), producing a specimen too dilute to reach the threshold of HPV detection by HC2. Thus, HC2 analysis of oral rinsing specimens does not seem to be useful for detecting HPV-related oropharyngeal SCCs.

The discrepancy in effectiveness between these 2 exfoliative cytologic methods of procuring sufficient tissue for HPV detection has important clinical implications related to screening. Although HC2 testing of oral rinsing samples may not be a viable screening tool for oropharyngeal cancer, HC2 testing on oral mucosal brushing specimens may merit further evaluation as a possible screening method. In our study, HC2 testing was positive on an oropharyngeal brushing specimen from a patient who did not have a visible mucosal lesion (Table 1); the specimen was procured simply by brushing the unremarkable-appearing oropharyngeal mucosa. This patient had a biopsy-proven oropharyngeal SCC that tested positive for HR-HPV by ISH. The potential feasibility of HC2 testing of oropharyngeal brushing specimens as a screening tool for SCC in patients without a suspicious mucosal lesion remains to be determined in larger studies.

### Table 1

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Site of Carcinoma</th>
<th>Visible Mucosal Lesion</th>
<th>HR-HPV ISH</th>
<th>HC2 Oral Brushing</th>
<th>HC2 Oral Rinsing</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Oral cavity</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Hypopharynx</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Oropharynx</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>4</td>
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<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>Oropharynx</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>ND†</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
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<td>+</td>
<td>–</td>
</tr>
<tr>
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<td>Hypopharynx</td>
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<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>Oropharynx</td>
<td>+</td>
<td>Equivocal†</td>
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<td>–</td>
</tr>
<tr>
<td>11</td>
<td>Oral cavity</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

HC2, Hybrid Capture 2; HPV, human papillomavirus; HR, high–risk; ISH, in situ hybridization; +, yes or positive; –, no or negative.

* Oral rinsing sample not obtained.
† Extensive necrosis and rare staining cells, interpreted as “equivocal.”
Our study is, of course, limited by its small sample. However, the results demonstrate that HC2 analysis of brushing specimens may be a useful technique for assessment of HPV status in oropharyngeal SCC. There are several potential advantages of HC2 over other assays for HR-HPV testing, which may ultimately be demonstrated in larger studies comparing methods. The wide availability of HC2 testing and its lower cost may make this test more feasible than ISH- and PCR-based tests. Ample evidence exists to implicate the overexpression of p16 as a reliable surrogate marker of HR-HPV; however, successful immunohistochemical and immunocytochemical testing for p16 is dependent on the presence of well-preserved cells, a limitation that has less of an adverse impact on DNA-based assays, including HC2. As the demand for HPV testing in the context of oropharyngeal SCCs continues to increase, it will be essential for laboratories to implement HPV testing methods that provide a meaningful result for clinicians and that are practical for laboratory use. Different specimen types (FFPE vs exfoliative samples) may necessitate different testing modalities. Our results suggest that HC2 is an effective method for HR-HPV testing in oropharyngeal brushing specimens that merits further evaluation and comparison with other methods.

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References


