Cytologic Evaluation of p16 Staining in Head and Neck Squamous Cell Carcinoma in CytoLyt Versus Formalin-Fixed Material

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BACKGROUND: The management of high-risk human papillomavirus (HR-HPV)–related oropharyngeal head and neck squamous cell carcinomas (HNSCCs) are distinct from HNSCC linked to smoking and alcohol use. HR-HPV–positive HNSCC frequently presents as a cervical lymph node metastasis. Because fine-needle aspiration (FNA) is often the initial diagnostic procedure, evaluating HR-HPV status in cytology specimens is important. The overexpression of p16 is a surrogate for HR-HPV; however, the evaluation of p16 in FNAs remains controversial. METHODS: From September 2015 to December 2016, cytopathologists performed 25 FNAs of neck lymph nodes that were suspicious for HR-HPV–positive HNSCC. Initial passes produced smears for on-site evaluation and CytoLyt material. Additional passes were formalin-fixed. A CytoLyt cell block (CCB) and a formalin-fixed cell block (FFCB) were prepared, and p16 immunocytochemistry was performed. RESULTS: In 24 of 25 cases, the FFCB had diffuse (≥70% of cells), strong nuclear/cytoplasmic p16 staining. In all 24 of these cases, HR-HPV was detected by in situ hybridization. The corresponding CCB had weak-to-moderate p16 staining in <70% of cells (range, 5%-60% of cells) in 17 cases, 4 had weak-to-moderate diffuse staining, and 4 were acellular. The percentage of p16-positive cells was significantly higher with FFCB than with CCB (formalin: 94% ± 2%; CytoLyt, 38% ± 7%; 2-tailed, paired Student t test; P < .001; Fisher exact test, P < .001). CONCLUSIONS: The fixative used had a drastic impact on p16 staining, which explained the staining variability reported in the literature. FFCBs show a diffuse staining pattern, which correlates with HR-HPV status, whereas CCBs show a weaker and inconsistent staining pattern, which is more difficult to interpret. Cancer Cytopathol 2019;127:750-756. © 2019 American Cancer Society.

KEY WORDS: cytology; human papillomavirus (HPV); p16; squamous carcinoma.

INTRODUCTION

High-risk human papillomavirus (HR-HPV)–related oropharyngeal head and neck squamous cell carcinomas (HNSCCs) have different demographics and prognosis compared with non–HR-HPV–related HNSCC, which is usually linked to smoking and alcohol use. In HR-HPV–positive oropharyngeal HNSCC, the patients tend to be younger, of higher socioeconomic standing, and often have no history of tobacco or alcohol abuse.1 The primary tumor is commonly located in the oropharynx, and the patients often have metastatic disease to the neck lymph nodes at initial presentation. HR-HPV–positive oropharyngeal HNSCCs are associated with prolonged survival and are more radiosensitive than HR-HPV–negative HNSCCs because of an intact apoptotic response to radiation.1,2 Consequently, a combination of chemotherapy and radiation is the preferred definitive treatment in lieu of surgical resection in HR-HPV–positive oropharyngeal HNSCC. Therefore, the accurate
evaluation of HR-HPV status is critical in the management of these patients. The National Comprehensive Cancer Network (NCCN) and the College of American Pathologists (CAP) recommends p16 and/or HR-HPV testing as part of the workup of all cervical metastatic squamous cell carcinomas with a known oropharyngeal primary not previously tested for HR-HPV, a suspected oropharyngeal primary, or an occult primary.3,4 Frequently, p16 is used as a surrogate marker for HR-HPV infection.5 Currently, a squamous lesion is considered to be associated with HR-HPV when there is overexpression of the p16 protein, which is defined as ≥70% strong nuclear and cytoplasmic staining by immunohistochemistry in formalin-fixed, paraffin-embedded tissue biopsy specimens.6 In fine-needle aspiration (FNA) specimens, the CAP makes no recommendation against any specific methodology for HR-HPV testing. When the results are negative, however, they recommend testing should be performed on tissue if it becomes available.4 Because HR-HPV–positive oropharyngeal HNSCC often presents as a cervical lymph node metastasis from an occult primary, FNA is often the initial diagnostic procedure. Unlike surgical pathologic material, the current literature does not support a reproducible, universal recommendation for determining what extent of p16 staining correlates with positive HR-HPV status in FNA specimens. In addition, the collection and preparation of cytologic material for a cell block can drastically vary depending on the laboratory, with many laboratories using material collected in alcohol instead of formalin.7 Given the different effects alcohol and formalin could potentially have on the p16 protein, we sought to evaluate whether the discrepant results observed in the literature are because of different fixation techniques.

**MATERIALS AND METHODS**

At the Memorial Sloan Kettering Cancer Center, from September 2015 to December 2016, cytopathologists performed 25 consecutive FNAs of cervical neck lymph nodes on patients who either had a previous diagnosis of oropharyngeal HNSCC with unknown p16 status or were determined to have HNSCC at the time of the procedure by the performing cytopathologist. The 25 patients were comprised of 22 men and 3 women, and their average age was 60 years (range, 47-76 years) (Table 1). The FNA was typically performed under ultrasound guidance using a 1-inch, 25-gauge needle attached to a 10-mL syringe. The initial passes were performed for Diff-Quik–stained and ethanol-fixed smears, and the needles were rinsed inCytoLyt (Hologic Inc). The performing cytopathologist would perform rapid on-site evaluation of the Diff-Quik–stained smears to determine adequacy and obtain a preliminary result. Once a diagnosis of metastatic HNSCC was rendered, and an area of the lymph node with sufficient viability was identified, the cytopathologist would perform 1 or 2 more passes targeting this area. These passes were then allowed to clot before being transferred into formalin for fixation. From the collected material, a CytoLyt cell block (CCB) and a formalin-fixed cell block (FFCB) were made as recently described.8 Both the CCB and the FFCB were cut prospectively, and heat-induced epitope retrieval was used to prepare the unstained slides. Immunocytochemical (ICC) staining was then performed on each using a mouse antihuman monoclonal p16 antibody (clone E6H4; antibody concentrate, 1 µg/mL; Ventana Medical Systems Inc). This process was followed regardless of whether the tumor had basaloid morphology typical of HR-HPV–positive oropharyngeal HNSCC or was overtly keratinizing. Once this was complete, each specimen was evaluated for the percentage of tumor cell staining as well as the strength of both cytoplasmic and nuclear staining. The percentage of

| TABLE 1: Patient Demographics |
|-------------------------------|
| **Patient** | **Age at Dx, y** | **Sex** | **Smoking Hx, Pack-Years** | **Alcohol Use** |
| 1 | 57 | Man | Never | Social |
| 2 | 51 | Man | 2.5 | Social |
| 3 | 58 | Woman | 36 | 9-y Abstinent |
| 4 | 69 | Man | 7 | Social |
| 5 | 47 | Man | 0.5 | Heavy |
| 6 | 62 | Man | Never | Social |
| 7 | 57 | Man | Never | Social |
| 8 | 65 | Man | Never | Never |
| 9 | 59 | Man | Never | Social |
| 10 | 50 | Man | Never | Social |
| 11 | 57 | Man | ~30 Cigars/y | Social |
| 12 | 67 | Man | 12 | Heavy |
| 13 | 72 | Man | Never | Social |
| 14 | 58 | Man | 40 | Social |
| 15 | 76 | Man | 10 | Social |
| 16 | 53 | Woman | Never | Occasional |
| 17 | 53 | Man | Never | Social |
| 18 | 61 | Man | Never | Social |
| 19 | 56 | Woman | 15 | Daily |
| 20 | 73 | Man | Never | Daily |
| 21 | 55 | Man | 15 | Social |
| 22 | 65 | Man | 10 | Social |
| 23 | 62 | Man | Never | Daily |
| 24 | 49 | Man | Never | Heavy |
| 25 | 62 | Man | Never | Occasional |

Abbreviations: Dx, diagnosis; Hx, history.
positive tumor staining and staining intensity were carefully estimated, as is routinely done in our clinical practice, by the same cytopathologist to ensure consistency.

Chart review of all patients was performed retrospectively to determine the patient’s smoking and alcohol use, how they were treated, and clinical outcomes. Direct evaluation of HR-HPV using in situ hybridization (clones 16, 18, 26, 31, 33, 35, 39, 41, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82, and E6/E7 messenger RNA; Ready to Use; Advanced Cell Diagnostics) targeting the transcripts of E6 and E7 messenger RNA was performed in all cases retrospectively.

Statistical analyses were performed using the statistical software package SPSS version 22.0 (IBM Corporation). For each fixation condition, the percentage of positive tumor cells was compared using a 2-tailed, paired Student t test. In addition, p16 immunopositivity was determined using the same criteria applied to the surgical specimen (ie, ≥70% of tumor cells showing nuclear and cytoplasmic staining, as defined by the CAP guideline). The rate of p16 immunopositivity was computed and compared using the Fisher exact test. P values < .05 were considered to be statistically significant.

RESULTS

The FFCBs displayed a consistent staining pattern for p16 by ICC, with 24 of the 25 specimens being diffusely positive (moderate-to-strong cytoplasmic and nuclear staining in ≥70% of cells) (Fig. 1). Retrospectively, we performed HR-HPV in situ hybridization on all 24 cases that showed diffuse p16 staining. In all cases, HR-HPV was detected (Fig. 2). HR-HPV in situ hybridization performed on the 1 case that had only focal p16 staining was noncontributory because of insufficient tumor remaining on deeper levels. The CCB showed a more variable staining pattern, but, in all cases, p16 stained a lower percentage of cells and with less intensity than the formalin-fixed counterpart (Fig. 3). Only 4 cases showed weak-to-moderate p16 staining in ≥70% of cells, whereas 6 showed weak-to-moderate staining in ≤10% of tumor cells. Eleven cases stained between 10% and 69% of cells, and, in 4 cases, the CCB was inadequate for evaluation because of scant cellularity (Table 2, Fig. 4). We attempted to stain 2 of the CCB cases with the p16 antibody excluding heat-induced epitope retrieval, but there was no recovery of antigenicity. Only 11 cases had surgical material available for p16 staining, and all showed concordant results with the formalin-fixed material.

The percentage of p16-positive tumor cells was significantly higher in FFCB versus CCB specimens (mean ± standard errors of mean: formalin, 94% ± 2%; CytoLyt, 38% ± 7%; 2-tailed, paired Student t test; P < .001).

By using a cutoff value of ≥70% of tumor cells with p16 staining to determine p16 immunopositivity, as...
Figure 2. Photomicrographs show (A) an H&E-stained sample of squamous cell carcinoma, (B) diffuse p16 staining on a formalin-fixed cell block, and (C) positivity for high-risk human papillomavirus by in situ hybridization performed on a formalin-fixed cell block.

Figure 3. (A,D,G) Three representative cases of squamous cell carcinoma with a side-by-side comparison of (B,E,H) p16 staining on the CytoLyt cell block (CCB) and (C,F,I) the formalin-fixed cell block (FFCB), respectively.
suggested by the CAP guideline, the rate of p16 positivity was significantly different between CytoLyt-fixed samples (positive rate: 19%; 4 of 21 samples) and formalin-fixed samples (positive rate: 100%; 24 of 24 samples; Fisher exact test; \( P < .001 \)).

Seventeen of the 25 patients received a combination of chemotherapy and radiation, 4 underwent surgical resection of disease followed by chemotherapy and radiation, 1 underwent surgical resection and received postoperative radiation only, 1 underwent surgical resection alone, and 2 were not treated at our hospital. Of the 23 patients who received treatment at Memorial Sloan Kettering Cancer Center, 2 developed lung metastasis 9 months after completing initial treatment, and 1 locally recurred; 2 died of disease; and 20 had no evidence of disease (range, 25-38 months from the completion of treatment). Of the 17 patients treated with only chemotherapy and radiation, 16 had no evidence of disease after completing treatment, and 1 recurred with lung metastasis after 9 months (Table 3). The overall response to therapy, especially the response to chemotherapy and radiation, is consistent with HR-HPV–positive HNSCC.

### DISCUSSION

Patients with HR-HPV–associated oropharyngeal HNSCC often present with only palpable cervical lymphadenopathy and have no history of malignancy. In patients who have this presentation, the initial clinical differential diagnoses include reactive lymph node, lymphoma, metastatic papillary carcinoma, a branchial cleft cyst, and metastatic HNSCC, among others. In this setting, FNA is often performed because it has the ability to determine the cause of the lymphadenopathy. In the setting of metastatic HNSCC, rapid on-site evaluation is important because it provides an opportunity to make the diagnosis at the time of the procedure and properly triage material for ancillary testing, such as p16 ICC. Ultrasound-guided FNA (US-FNA) has many advantages in this setting, although the lymph nodes are palpable. Frequently, these lymph nodes are cystic or necrotic and US-FNA enables the performing cytologist to target areas that appear solid or viable. Most importantly, once an area of sufficiently viable tumor is identified, US-FNA allows for accurate targeting of that area to ensure designated passes for a cell block will contain viable tumor. In addition, it is also less invasive than core-needle and excisional biopsies and is associated with fewer complications. Establishing the diagnosis of HR-HPV–positive HNSCC by US-FNA allows proper clinical management of the patient, even when the primary site cannot be identified. In our study, 5 of 25 patients (20%) underwent surgical exploration of the base of tongue and/or tonsils in an attempt to locate the primary site but had no carcinoma detected in any of the resections or biopsies. Given that the primary tumor is located within the oropharynx in the majority of HR-HPV–positive HNSCCs, the oral cavity and oropharynx are covered by the radiation field in these cases.

Although establishing HR-HPV status in HNSCC is critical for patient management, the use of FNA material to make this assessment is not fully accepted. Various authors have reported on the role of p16 ICC in cytology specimens with conflicting results. Different studies have used different percentages of p16 immunoreactivity to correlate with HR-HPV status, whereas others have suggested that cytologic material cannot reliably be used. For instance, Xu et al and Jalaly et al reported weaker staining for p16 on cytology specimens and observed that staining as little as 10% or 15% of tumor

### TABLE 2. Evaluation of p16 Staining and Human Papillomavirus

| Patient | % p16 Staining in CCB | % p16 Staining in FFCB | High-Risk HPV by mRNAISH |
|---------|-----------------------|------------------------|--------------------------|
| 1       | 5 w                   | 95 s                   | Positive                 |
| 2       | 50 w                  | 99 s                   | Positive                 |
| 3       | 100 w                 | 100 s                  | Positive                 |
| 4       | 25 m                  | 75 s                   | Positive                 |
| 5       | NC                    | 5 m                    | Positive                 |
| 6       | 10 w                  | 90 s                   | Positive                 |
| 7       | 25 m                  | 90 m-s                 | Positive                 |
| 8       | 5 w                   | 90 m-s                 | Positive                 |
| 9       | 10 w                  | 95 s                   | Positive                 |
| 10      | 30 m                  | 70 m                   | Positive                 |
| 11      | 10 w                  | 90 s                   | Positive                 |
| 12      | 50 w-m                | 99 s                   | Positive                 |
| 13      | 5 m                   | 95 m-s                 | Positive                 |
| 14      | 50 m                  | 99 s                   | Positive                 |
| 15      | NC                    | 90 s                   | Positive                 |
| 16      | 5 w                   | 90 m-s                 | Positive                 |
| 17      | NC                    | 99 s                   | Positive                 |
| 18      | 30 w                  | 95 s                   | Positive                 |
| 19      | 70 s                  | 95 s                   | Positive                 |
| 20      | 40 w-s                | 100 m-s                | Positive                 |
| 21      | 90 m                  | 100 s                  | Positive                 |
| 22      | 90 m                  | 100 s                  | Positive                 |
| 23      | 15 w-m                | 99 s                   | Positive                 |
| 24      | 25 w-m                | 100 s                  | Positive                 |
| 25      | 60 m                  | 99 s                   | Positive                 |

Abbreviations: CCB, CytoLyt cell block; FFCB, formalin-fixed cell block; HPV, human papillomavirus; m, medium; mRNAISH, messenger RNA in situ hybridization; NC, noncontributory; s, strong; w, weak.
cells, respectively, had a high concordance with HR-HPV–related disease.\textsuperscript{2,11} Begum et al reported strong p16 staining on viable tumor cells while observing diminished or absent staining in degraded tumor cells. Grimes et al observed strong and diffuse staining for p16 in patients with HR-HPV–related disease.\textsuperscript{9,12} Upon review of these studies, we noted that the effect of processing on the immunoreactivity, including type of fixative used, had not been standardized. Although immunostains are generally validated using formalin-fixed, paraffin-embedded tissue, several cytology laboratories use material previously fixed in an alcohol-based medium before cell block processing.\textsuperscript{7} The type of fixative can cause decreased antigenicity of certain antibodies, including other nuclear markers such as MIB1 and ER as well as cytoplasmic stains such as S100.\textsuperscript{16-18} This decrease in antigenicity might be attributed to differences in the effect of each fixative on the cells obtained. Formalin fixes specimens through the formation of intramolecular and intermolecular cross-links, whereas alcohol removes water from the free carboxyl, hydroxyl, amino, amido, and imino groups of the proteins, resulting in protein coagulation and tissue

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{chart.png}
\caption{This chart illustrates the comparison of p16 staining in tumor cells in CytoLyt and formalin-fixed material.}
\end{figure}

\begin{table}[h]
\centering
\caption{Treatment and Clinical Outcomes of Patients}
\begin{tabular}{llll}
\hline
Patient & Initial Treatment & Status & Disease-Free Interval, mo \\
\hline
1 & Surgical resection + cisplatin + Rx (30 Gy) & NED & 41 \\
2 & Cisplatin + Rx (60 Gy) & NED & 38 \\
3 & Cisplatin + Rx & NED & 38 \\
4 & Cisplatin + Rx (70 Gy) & NED & 36 \\
5 & Cisplatin + Rx (70 Gy) & Recurred with lung metastasis after 9 mo & \\
6 & Cisplatin + Rx (60 Gy) & NED & 35 \\
7 & Surgical resection + cisplatin + Rx (30 Gy) & Recurred locally, DOD & \\
8 & Cetuximab + Rx (70 Gy) & NED & 34 \\
9 & Surgical resection + cisplatin + Rx & Recurred with lung metastasis after 9 mo, DOD & \\
10 & Cisplatin + Rx (60 Gy) & NED & 29 \\
11 & Surgical resection + cisplatin + Rx (30 Gy) & NED & 29 \\
12 & Cisplatin + Rx (70 Gy) & NED & 29 \\
13 & Cisplatin + Rx (70 Gy) & NED & 27 \\
14 & Cisplatin + Rx (70 Gy) & NED & 28 \\
15 & None & DOD & \\
16 & Cisplatin + Rx (70 Gy) & NED & 28 \\
17 & Surgical resection + post-op RT & NED & 26 \\
18 & Cisplatin + Rx (70 Gy) & NED & 27 \\
19 & Cisplatin switched to carboplatin + Rx (70 Gy) & NED & 26 \\
20 & Cisplatin + Rx (70 Gy) & NED & 26 \\
21 & Cisplatin + Rx & NED & 25 \\
22 & Cisplatin + Rx & NED & 25 \\
23 & Surgical resection & NED & 27 \\
24 & Cisplatin + Rx (70 Gy) & NED & 25 \\
25 & None & Lost to follow-up & \\
\hline
\end{tabular}
\footnotesize{Abbreviations: DOD, dead of disease; Gy, grays; NED, no evidence of disease; post-op, postoperative; Rx, radiation.}
\end{table}
shrinkage.\textsuperscript{19} When comparing the studies in which the fixation material was specified, Xu et al and Jalaly et al observed low-to-weak p16 expression in HR-HPV–positive HNSCC in alcohol-fixed material, whereas Begum et al reported diffuse staining in formalin-fixed material. These findings support our current results.\textsuperscript{2,9,11}

**Conclusion**

It has been demonstrated that CytoLyt decreases the degree of immunostaining of certain antibodies; however, to our knowledge, its effect on the p16 immunostain has not been reported.\textsuperscript{13} The reason that suboptimal immunoreactivity with this antibody is particularly important is that, unlike most diagnostic antibodies in pathology, which are not quantitative, the interpretation of p16 depends on quantitative cutoff values. In the current study, we found that the fixative used had a drastic impact on p16 staining, and we believe this explains the staining variability reported in the literature. The expression of p16 in cytologic material collected in formalin showed a diffuse staining pattern, which correlated with HR-HPV status, whereas CCBs showed a weaker and inconsistent staining pattern, which is more difficult to interpret.

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**CONFLICT OF INTEREST DISCLOSURES**

The authors made no disclosures.

**AUTHOR CONTRIBUTIONS**

Darren J. Buonocore: Conceptualization, methodology, investigation, resources, writing—original draft, and writing—review and editing.

Evan Fowle: Investigation and writing—original draft.

Oscar Lin: Methodology, investigation, resources, writing—review and editing, funding acquisition, and supervision.

Bin Xu: Writing—review and editing.

Nora Katabi: Writing—review and editing.

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