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Real-time PCR HPV genotyping in fine needle aspirations of metastatic head and neck squamous cell carcinoma: Exposing the limitations of conventional p16 immunostaining

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ABSTRACT

Background: Given the propensity for HPV-positive head and neck squamous cell carcinoma (HPV-HNSCC) to metastasize to cervical lymph nodes, fine needle aspiration (FNA) plays an important diagnostic role in their initial detection. Indeed, there is now an unwavering commitment to HPV testing of FNAs even in the absence of clear methodologic guidelines and threshold criteria. A particular difficulty pertains to the interpretation of p16 staining.

Design: Data was collected for 210 patients with suspected regionally metastatic HNSCC that had undergone FNA as part of standard clinical care. Initial HPV screening was performed on cell blocks with real-time PCR using primers targeting L1 of high-risk HPV types. Additional genotyping was performed on HPV-positive cases. The results were compared to p16 staining and subsequent excisions when available.

Results: Of the 207 samples with sufficient DNA, 175 (85%) were HPV positive. HPV-16 was the most commonly detected genotype (90%). Of the HPV-positive cases, the primary site was the oropharynx (n = 154, 88.0%), supraglottic larynx (n = 2, 1.1%), nasal cavity (n = 1, 0.6%), hypopharynx (n = 1, 0.6%) or unknown (n = 17, 9.7%). On comparison with 31 paired surgical excisions, HPV status was concordant in all cases (100% correlation). Of 142 HPV-positive cases with matching p16 stains, p16 staining was reported as positive (n = 85, 60%), focal (n = 27, 19%), negative (n = 24, 17%) or non-contributory (n = 6, 4%); and only 33% reached the standard threshold limit (i.e. 70%) for HPV positivity.

Conclusion: For patients with metastatic HNSCC, real-time PCR of FNAs reliably reflects HPV status, and is superior to conventional p16 immunostaining.

Introduction

The human papillomavirus (HPV), particularly type 16, has been established as an important cause over 80% of those head and neck squamous cell carcinomas (HNSCCs) arising in the oropharynx [1]. The identification of HPV in oropharyngeal squamous cell carcinoma (OPSCC) identifies a distinct form of HNSCC that is highly responsive to various forms of therapy and is associated with a favorable clinical outcome [2–5]. Accordingly, there has been a move among cancer care organizations towards mandatory HPV testing for all patients with OPSCC [6,7]. Recently, the College of American Pathologists, based on its comprehensive review of the medical literature, has called for mandatory testing of all OPSCCs, lymph node metastasis of a suspected oropharyngeal primary, and metastatic HNSCC of unknown primary origin [6]. Further, p16 has been advocated by the CAP and other influential organizations as the method of choice for determining HPV status [6,7]. P16 immunostaining is highly accurate, widely available to most diagnostic laboratories, and is easy to interpret in tissue specimens [8]. As a surrogate for high risk HPV, P16 staining is regarded as tantamount to HPV infection when there is at least 70% nuclear and

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cytoplasmic expression with at least moderate to strong intensity [6].

Although consensus groups have made considerable progress toward implementing a standardized HPV detection strategy, these tissue-based methodologies may not be appropriate for cytology-based materials. For FNA specimens, (1) tumor sampling may be inadequate – particularly for HPV metastasis where cystic degeneration and low cellularity is a common problem; (2) computational analysis (e.g. percent tumor staining) may not be applicable where tumor cells are dispersed individually and as small cell clusters; and (3) the intensity of p16 staining may be diminished as a result of cellular degradation [9,10]. Based on these limitations, the CAP recommends HPV testing of lymph node metastases, but is currently unable to provide clear direction regarding the most appropriate detection platform and threshold criteria [6].

The inability to reliably determine HPV status of FNA samples of lymph node metastases is highly problematic. Among patients with HPV-positive OPSCC, approximately 85% develop lymph node metastases, and approximately 50% of these patients are first diagnosed based upon an enlarged cervical lymph node [11–13]. In these patients, FNA is often used to establish HPV status in an effort to discern site of tumor origin, obtain a critical staging parameter, predict patient outcome, direct therapy, and select patients for clinical trials. In effect, the fundamental role of oncologic pathology encompassing tumor classification, staging, prognostication and therapy is contingent on accurate HPV status determination. The purpose of this study was to: 1) assess a PCR-based approach for its reliability in determining HPV status of FNA samples, and 2) critique the widely employed p16 immunostaining strategy.

Methods

Patient and tumor

Routine HPV testing was introduced for all OPSCCs diagnosed at Mount Sinai Hospital in June 2012. HPV testing of lymph nodes for suspected metastatic HNSCCs became common practice at this same time. To identify all cases evaluated for HPV status, the pathology database (Power Path) was searched for all HNSCCs and suspected lymph node metastases tested from June 2012 to July 2018. The medical records were reviewed to determine patient demographics including gender and age, and clinic-pathologic features including specimen type (e.g. FNA, biopsy, resection), tumor type, anatomic site of tumor origin, p16 staining, and HPV status including genotype.

Cytology specimens:

Twenty-five and twenty-seven gauge needles were used to collect the samples. After smearing, the needles were washed in Cytolyt® solution media which was later centrifuged and processed for cell block preparation. Thromboplastin-plasma cell block technique was used to process the cases until August 2017 when the processing lab switched to a histo-gel cell block technique. The cell blocks were reviewed to confirm the presence of tumor cells for subsequent p16 immunohistochemistry and HPV molecular studies.

Human papillomavirus DNA extraction and quantification

The Maxwell 16 formalin-fixed paraffin-embedded Tissue LEV DNA Kit (Promega, Madison, WI) was used for DNA extraction from formalin-fixed paraffin-embedded tissue sections, in accord with the manufacturer's instructions. The concentrations and quality of extracted DNAs were measured by a NanoDrop ND-2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Human papillomavirus DNA genotyping

HPV genotyping was performed in a stepwise fashion. An initial HPV screening is performed by consensus PCR by amplifying the HPV L1 region using consensus primers GP5+ and GP6+ and the LightCycler 480 High Resolution Melting Master kit. The L1 region is conserved in over 27 different genotypes, including common low-risk and high-risk HPV types found in cervical and head and neck cancer [14]. If HPV DNA is amplified in the sample, a High Resolution Melting Curve Analysis (HRMCA) is performed on the LightCycler 480 instrument to confirm the presence of HPV. Positive samples are reflexed for HPV16/18 genotyping using HPV 16 and HPV 18 specific PCR.

For type specific detection of HPV, real-time PCR by LightCycler 480 instrument is utilized to amplify E6 regions of HPV16 and 18. Amplicons are detected by two specific pairs of fluorescence hybridization probes, consisting of four different short oligonucleotides that hybridize to an internal sequence of the amplified fragments of HPV16 and 18 during the annealing phase of the amplification cycle. Two probes are labeled at the 5′-end with LightCycler-Red 640 for HPV16 or LightCycler-Red 670 for HPV18, and to avoid extension, modified at the 3′-end by phosphorylation. The other probes are labeled at the 3′-end with fluorescein. After specific hybridization to the template DNA, two probes of each target come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores. The emitted fluorescence of LC-Red 640 or LC-Red 670 is then measured by the LC480 Instrument.

In the event HPV16/18-specific PCR was negative, Sanger Sequencing was performed to detect other HPV genotypes after amplification with GP51 and GP61 primer pairs. HPV subtypes other than HPV16/18 were determined by aligning sequences in GenBank.

Results

The clinical and pathologic findings are summarized in Table 1. 218 FNAs were evaluated by real-time PCR. Ten (4.6%) of the samples were insufficient for further analysis due to insufficient tumor in the cell blocks, and the remaining 208 with sufficient DNA formed the basis of this study. These samples were from 202 patients. 173 (86%) were from males and 29 (14%) were from females, ranging in age from 24 years to 90 years (mean, 61). 177 (85%) of the FNA samples were HPV positive and 32 (15%) were HPV negative. For the HPV positive cases, the sites of tumor origin were the oropharynx (n = 152), larynx (n = 1), nasal cavity (n = 1) and nasopharynx (n = 1). Six of the tumors were very large involving multiple sites including the larynx, hypopharynx and oropharynx. In 16 cases, the site of origin was unknown. For the HPV negative cases, the sites of tumor origin were the oropharynx (n = 11), larynx (n = 6), oral cavity (n = 4), hypopharynx (n = 2), facial skin (n = 2), major salivary glands (n = 2), nasopharynx (n = 1) and lung (n = 1). In 1 of the HPV-negative cases the primary site was not identified, and in 1 case the suspected metastatic HNSCC was diagnosed on ultimately diagnosed as a branchial cleft cyst.

HPV type 16 was the most commonly detected genotype (n = 158, 89.3%). The other high risk genotypes (n = 19, 10.7%) included 35 (n = 12, 6.7%), 33 (n = 3, 1.7%), 18 (n = 2, 1.1%), 45 (n = 1, 0.6%) and 59 (n = 1, 0.6%). By anatomic site, HPV 16 comprised 93% of the HPV positive cases arising in the oropharynx, 43% of cases arising in non-oropharyngeal sites, and 81% of cases where the primary site was unknown.

To help establish the fidelity of HPV assessment across different tumor samples from the same patient, we compared HPV status for 31 patients who had also undergone HPV testing of their subsequent surgical excisions (i.e. excisional biopsies and resections). There was 100% concordance for both the HPV positive (n = 28) and HPV negative (n = 3) sample pairs (Fig. 1). Moreover, there was 100% concordance in identifying genotypes (type 16, n = 25; type 35, n = 2; type 18, n = 1) when comparing the FNA and excision sample pairs. There was

Table 1
Summary of pathologic features for metastatic carcinomas to cervical lymph nodes evaluated by real time PCR HPV genotyping.

	HPV status	
	Positive n = 177 (85%)	Negative n = 31 (15%)
HPV genotypes		
16	158 (89.2%)	N/A
35	12 (6.7%)	N/A
33	3 (1.7%)	N/A
18	2 (1.1%)	N/A
45	1 (0.6%)	N/A
59	1 (0.6%)	N/A
Site of tumor origin		
oropharynx	152	11
larynx	1	6
oral cavity	0	4
sinonasal tract	1	0
nasopharynx	1	1
hypopharynx	0	2
trans-oropharynx*	6	0
unknown	16	1
facial skin	0	2
major salivary glands	0	2
lung	0	1
branchial cleft cyst	0	1
Reported p16 status (n = 165)		
positive	85 (60%)	2 (9%)
negative	24 (17%)	13 (57%)
focally positive	27 (19%)	3 (13%)
non-contributory	6 (4%)	5 (22%)
Matching resections (n = 28)		
concordant	28 (100%)	3 (100%)
discordant	0 (0%)	0 (0%)

* *trans*-oropharynx origin defined as advanced stage carcinomas that spanned multiple anatomic sites including the oropharynx, larynx and hypopharynx.

also consistency in HPV status when multiple FNA samples were collected over different time points in the setting of tumor recurrence. Five patients with HPV-16 positive lymph node metastases and 1 patient with an HPV negative lymph node metastasis yielded concordant results when FNAs of their recurrent tumors were again tested for HPV status.

The cytopathology reports were reviewed to verify p16 staining status as documented in the clinical records. P16 immunostaining was performed in 165 cases. Of 142 HPV-positive cases with matching p16 stains, p16 staining was reported as positive (n = 85, 60%), focally positive (n = 27, 19%), negative (n = 24, 17%) or non-contributory (n = 6, 4%). Of the 23 HPV-negative cases with matching p16 stains, p16 staining was reported as positive (2, n = 9%), focally positive (3, n = 13%), negative (13, n = 57%), or non-contributory (n = 5, 22%).

The p16 stains were then retrieved and reviewed to determine the percentage, distribution and intensity of p16 staining (Table 2). Of the 131 p16 stained slides that were available for review, 95 (73%) of the cell blocks contained cohesive cell clusters of viable tumor cells. In the remaining 36 (27%) cases, the cells were individually dispersed and showed extensive degradation. P16 staining patterns were then compared to HPV status as determined by real time PCR. Forty-three of the 83 (52%) HPV-positive carcinomas where tumor was present as cohesive cellular fragments met threshold p16 staining criteria (moderate to strong cytoplasmic and nuclear staining in 70% or more tumor cells) as an HPV surrogate. In the 31 HPV-positive metastases where the tumor cells were individually dispersed and degenerated, p16 staining reached tissue threshold criteria in only 9 (29%) of the FNAs (Fig. 2). For the HPV-negative cases, 1 of 17 (6%) reached the p16 staining threshold of 70%. Using tissue-based staining thresholds, the positive predictive value of p16 staining as a surrogate for HPV was 98%, but its negative predictive value was only 21%. Some investigators have advocated for using any staining as a surrogate for HPV in FNA specimens. Using this revised approach for grading p16 staining of cytology material, 99% of the HPV positive cases were p16 positive, but 35% of the HPV negative cases were also p16 positive. The positive predictive value of p16 staining as a surrogate for HPV was 94%, and its negative predictive value was 48%.

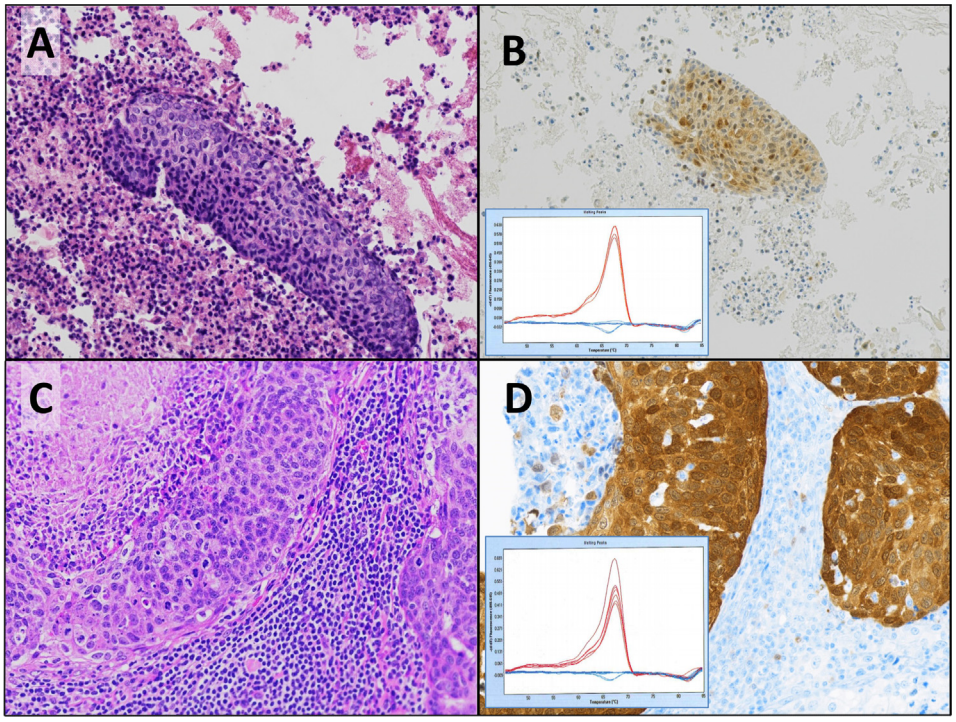

Fig. 1. This FNA from a metastatic HPV-related squamous cell carcinoma shows cohesive nests of viable tumor cells (A, hematoxyline and eosin) that retain some staining for p16 (B, p16 immunostain). Its matched tonsillar resection shows a squamous cell carcinoma (C, hematoxylin and eosin) that is strongly p16 positive (D, p16 immunostain). HPV DNA amplified from both tumors are HPV16 positive (inserts, HPV16 melting curves: positive SIHA control - large red peak; cases – smaller red peaks; and HPV16 negative controls - blue curves). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
P16 immunostaining of cell blocks for patients with metastatic HNSCC of known HPV status* (n = 129).

P16 staining	HPV positive (n = 112)		HPV negative (n = 17)	
	Viable cohesive cell clusters (n = 82)	Dispersed degenerated cells (n = 30)	Viable cohesive cells (n = 13) clusters	Dispersed degenerated cells (n = 4)
Tissue criteria				
< 70%	39 (48%)	21 (70%)	12 (92%)	4 (100%)
≥70%	43 (52%)	9 (30%)	1 (8%)	0 (0%)
Any staining				
0–100%	76 (93%)	24 (80%)	4 (31%)	2 (50%)

* HPV status as determined by real time PCR analysis of matched cell block; HNSCC, head and neck squamous cell carcinoma.

Discussion

The College of American Pathologists has recently mandated HPV testing of all lymph node metastasis of a suspected oropharyngeal primary and metastatic HNSCCs of unknown primary origin [6]. At the same time, specific recommendations regarding appropriate detection methods and threshold criteria are not provided for cytology samples [6] – a significant shortcoming given that for most patients with HNSCC, important diagnostic and therapeutic decisions hinge on the ability to accurately determine HPV status of aspirated lymph node metastases. Most pathologists resort to p16 immunostaining largely based on the assumption that tissue based practices can be reliably applied to FNAs of lymph node metastases. Holmes et al. [15] demonstrated a high correlation of p16 staining when comparing preoperative lymph node samples and subsequent surgical specimens, but this study mainly utilized core tissue biopsies as the substrate for p16 staining. Use of cell block constructed from FNAs is quite a different matter, where accurate HPV detection is more likely to be threatened by scant cellularity, cell degradation and other factors. Several studies have challenged the assumption that tissue-based criteria can be seamlessly applied to these FNA samples. In these studies, false negative rates soar until staining thresholds are downwardly adjusted from 70% [16] to 15% [17], to 10% [10], to any positive staining [9]. Unlike the situation for resection specimens, the p16 staining threshold for cytology material remains a moving target.

Our findings confirm the unreliability of p16 immunostaining of cell blocks constructed from FNAs of metastatic HNSCCs. Based on a review of the cytopathology reports, only 60% of the patients with histologically confirmed metastatic HPV-HNSCC were correctly classified based on p16 staining of their FNAs. The other 40% were either misclassified as HPV negative (17%) or unclassified based on focal staining (19%) or non-contributory staining (4%). Cellular degradation has been implicated as a large contributor to absent or diminished staining in metastatic HPV-HNSCC, and this may have been a significant factor in our high false negative rate. Twenty-seven percent of the HPV-OPSCCs showed extensive degradation with the complete absence of viable cohesive cell clusters, and in these samples only 29% of the HPV-positive cases met tissue threshold criteria for a positive HPV test (Table 1). P16 staining was found to be inconsistent even in those cell blocks with cohesive clusters of viable tumor cells. Only 52% of these samples met tissue threshold criteria as an HPV surrogate. Recognizing the problem of diminished p16 staining in cytology material of metastatic HNSCCs, some have advocated lowering the bar - even to the point of accepting any p16 staining as an HPV surrogate marker. Using this approach, we found that the false negativity could be reduced from 54% to 13%, but at the cost of elevating the false positive rate from 6% to 35%. This elevation of the false negative rate is unacceptable when the clinical context demands optimal specificity. Where the clinical stakes are truly high, such as selection of patients for “de-escalation” or therapeutic vaccine trials based on HPV-status, suboptimal specificity

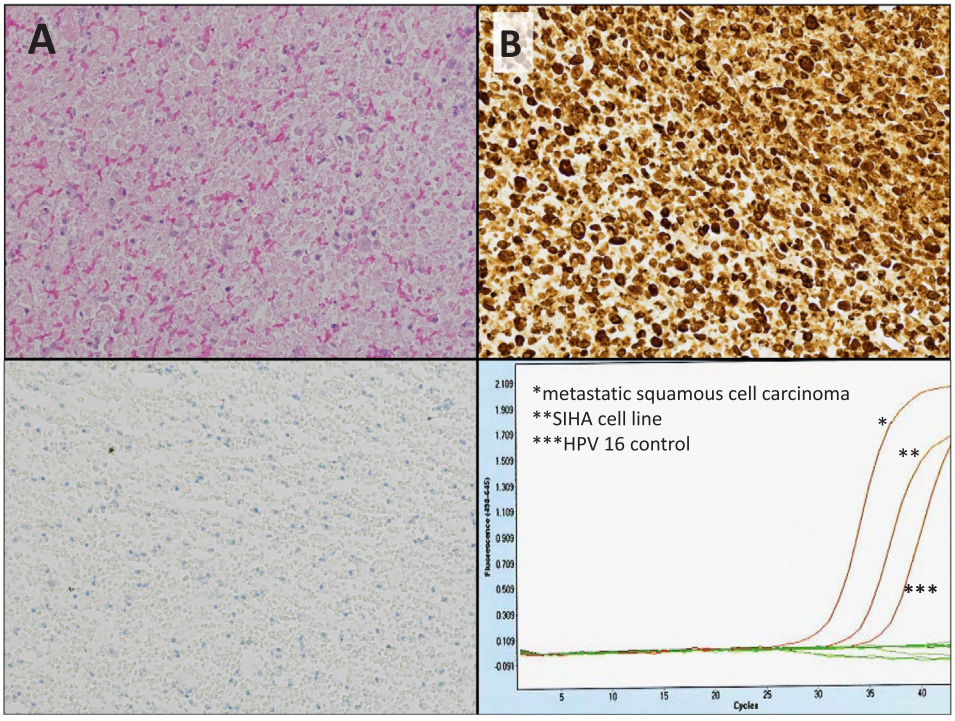

Fig. 2. In this FNA of metastatic squamous cell carcinoma, the tumor cells are present as dispersed degenerated cells (A, hematoxylin and eosin). There epithelial nature is confirmed by a cytokeratin immunostaining (B, CK5/6 immunostain). Even though the tumor cells do not overexpress p16 (C, p16 immunostain), there is HPV16 amplification using a type16-specific primer (D, including positive controls SIHA cell line** and HPV16 DNA***; and HPV16 negative controls shown as green curves). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

runs the risk of inappropriate treatment of patients with HPV-unrelated cancers.

Historically, PCR-based approaches to HPV testing detection assays have been notoriously untrustworthy. Most studies relied on non-quantitative PCR-based detection methods, but these methods were highly disabled by a susceptibility to viral contaminants and low specificity. In a systematic review of PCR-based HPV incidence studies, Isayeva et al. [18] noted highly variable detection rates with upper limits of HPV positivity reaching 100% in laryngeal carcinomas, 74% in oral cavity carcinomas, and 70% in oral cytology samples from individuals without cancer. Since 2012, our institution has used a multistep real-time PCR approach that: (1) utilizes a general consensus probe to discern the presence of virus across more than 23 HPV genotypes including both common and uncommon high risk types [14], (2) requires viral amplification within a set cycle threshold to discern clonally present HPV (versus viral contaminants and passenger virus), (3) includes a housekeeping gene (i.e. actin) as an internal control to ensure sufficient sample cellularity, (4) applies both DNA melting point analysis and type specific hybridization to confirm the presence of HPV 16 and 18, and 5) employs Sangers sequencing to confirm and type all HPV positive cases caused by non-16 and 18 HPV genotypes. The rigorousness of this tissue-based approach was not compromised when applied to cell blocks constructed from FNAs. Indeed, there was complete agreement (100% concordance) in HPV status and HPV genotype when the FNA results were compared to the corresponding surgical pathology results, and when multiple FNA results from the same patient were compared across different time points. Moreover, the method was much less susceptible to cell degradation compared to p16 immunostaining.

Testing of cytology samples for HPV and other potentially useful biomarkers will undoubtedly strain access to this valuable resource and limit implementation unless current practices are modified to accommodate new applications. This quandary is highlighted in one retrospective analysis of FNAs where only one-third of the aspirate preparations retained material (e.g. cell blocks) for additional studies, and sufficient cellular material for HPV analysis was present in only two-thirds of these cell blocks [9]. Unlike p16 immunostaining and other tissue-based methods such as in situ hybridization, our real-time PCR method did not rely on the presence of viable tissue fragments. Detection rates did not significantly vary in those samples that were highly degraded compared to those that contained viable cohesive cell fragments (88% vs. 86%, $p = 1.00$, Fischer's exact) (Fig. 2). This finding suggests that our real time PCR approach is adaptable to a liquid phase platform. In liquid phase assays, cytology samples are directly transferred into a liquid phase media sidestepping the construction of cell blocks. Several liquid phase assays (e.g. Hybrid Capture 2, Qiagen, Gaithersburg, MD; Cervista, Hologic Inc, Bedford, MA; Roche Cobas, Roche Molecular Systems, Pleasanton, CA) clinically validated for HPV detection in cervical cytology specimens have showed promise in feasibility studies of FNAs from patients with metastatic HNSCC. To date, the impact of these studies has been mitigated by relatively small case numbers.

In our experience of cytology samples, HPV 16 was identified in 90% of the HPV positive metastases, followed by types 35, 33, 18, 45 and 59. This order of frequency mirrors the genotype distribution reported in tissue-based studies of primary HPV-related OPSCCs [19,20]. This capacity to discriminate HPV genotypes in cytology samples may represent a breakthrough in advancing the understanding of HPV-OPSCC and optimizing patient care. First, HPV genotyping is necessary to draw meaningful associations between relevant clinic-pathologic profiles and HPV type. As few studies to date have made any effort to distinguish between HPV subtypes, virtually everything known about the clinical behavior of HPV-related oropharyngeal squamous cell carcinoma is dominated by HPV16-related cases. But assumptions regarding the equivalency of HPV across different genotypes may not be valid. The few studies that have evaluated clinical outcomes as a function of HPV genotype for patients with HPV-related OPSCC have suggested that non-16 variants are associated with worse outcomes [19–22]. These studies have been exclusively tissue-based, and have largely relied on population-based cancer registries [20,21]. Single institution experiences have been too underpowered to draw meaningful conclusions [19]. The application of an HPV genotyping strategy to FNA samples could facilitate the accrual of the large prospective multi-institutional cohorts that will be necessary to more fully understand the impact of HPV genotype variants on clinical outcomes and epidemiologic trends. Second, HPV genotyping could better inform the emerging antigen-specific immunotherapeutic approaches to HPV-related OPSCC. As one important example, current therapeutic vaccinations which are almost exclusively focused on targeting HPV16 antigens may be shown to be less effective for the small but potentially important group of HPV-OPSCCs caused by a non-16 type. Whether dealing with immunotherapy or "de-intensification" protocols, HPV genotyping may permit an even more individualized approach to the treatment of patients with HPV-related OPSCC.

Patients commonly enter oncologic care based on a neck mass diagnosed as metastatic HNSCC by cytopathology assessment. For these patients who have not yet had a tissue diagnosis of the carcinoma at its primary site, the use of cytology material as a substrate for HPV assessment must reliably clarify HPV tumor status in a way that would direct the search for site of origin, predict clinical outcome, and guide therapy all while abrogating the need for tissue acquisition of the primary tumor via a surgical procedure. Our findings indicate that whether p16 staining thresholds are set rigidly high or promiscuously low, p16 staining of FNA samples is unreliable in meeting these expectations. On the other hand, we have found that a real-time PCR approach is: (1) highly accurate, (2) resistant to cellular degradation and the absence of viable tissue fragments, and (3) allows for HPV characterization that may more clearly refine the behavior of HPV-HNSCC as a function of HPV genotype.

Conflict of interest

None declared.

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