Detection of Human Papillomavirus-16 in Fine-Needle Aspirates to Determine Tumor Origin in Patients with Metastatic Squamous Cell Carcinoma of the Head and Neck

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Abstract **Purpose:** Patients with head and neck squamous cell carcinoma (HNSCC) often clinically present with metastases to regional lymph nodes. Fine-needle aspiration of neck masses is routinely used to establish the presence of metastatic carcinoma and in turn to initiate a subsequent workup to determine the site of tumor origin. Human papillomavirus (HPV) 16 is an important etiologic agent for HNSCCs that arise from the oropharynx but less so for tumors from non-oropharyngeal sites. HPV16 detection thus provides a strategy for localizing an important subset of HNSCCs, but this approach has not been applied to fine-needle aspiration specimens.

Experimental Design: We did *in situ* hybridization for HPV16 on 77 consecutive aspirated neck masses diagnosed as metastatic squamous cell carcinoma. P16 immunohistochemistry was also done because p16 overexpression may serve as a surrogate marker of HPV-associated HNSCC.

Results: HPV16 was detected in 13 of the 77 (17%) aspirates. By site of origin, HPV16 was detected in 10 of 19 metastases from the oropharynx but in none of 46 metastases from other sites (53% versus 0%; $P < 0.0001$). HPV16 was not detected in 2 branchial cleft cysts misdiagnosed as metastatic squamous cell carcinoma, but it was detected in 3 of 10 metastases from occult primary tumors. P16 expression was associated with the presence of HPV16: 12 of 13 HPV16-positive metastases exhibited p16 expression, whereas only 4 of 62 HPV16-negative metastases were p16 positive (92% versus 6%; $P < 0.0001$). P16 expression also correlated with site of tumor origin: 13 of 19 oropharyngeal metastases were p16 positive, whereas only 1 of 46 non-oropharyngeal metastases was p16 positive (68% versus 2%; $P < 0.0001$).

Conclusions: HPV16 status can be determined in tumor cells aspirated from the necks of patients with metastatic HNSCC. Its presence is a reliable indicator of origin from the oropharynx.

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Oncogenic human papillomavirus (HPV), particularly type HPV16, has been established as a causative agent for ~25% of head and neck squamous cell carcinoma (HNSCC; refs. 1–3). As the role of HPV16 in head and neck tumorigenesis becomes better understood, these HPV16-related cancers are becoming increasingly recognized as a biologically distinct subgroup of HNSCC with a characteristic clinical profile. Accordingly, detection of HPV16 may have several practical clinical applications. For example, HPV16 DNA can be detected in the saliva and serum of patients with HNSCC and may have clinical usefulness for cancer screening and cancer surveillance (4–7). The presence HPV16 in HNSCC has been correlated with improved survival and may serve as a useful biomarker of prognosis (1, 8–10). Additionally, patients with HPV-induced HNSCC may benefit from future HPV-targeted treatment strategies (e.g., therapeutic HPV vaccines; ref. 11).

Another distinctive feature of HPV16-related HNSCC is its strong tendency to arise from the oropharynx. Accordingly, HPV DNA detection may be exploited to pinpoint primary tumor origin in patients who present with metastatic HNSCC (12, 13). Discerning site of tumor origin for patients with HNSCC is not always straightforward. Indeed, most patients with HNSCC already have metastatic spread to regional lymph nodes at the time of presentation (14), 13% of patients present with a neck mass as the first and only clinical manifestation (15), and 3% to 9% of the primary tumors elude detection despite a thorough clinical, radiographic, endoscopic, and histopathologic evaluation (16). We previously did HPV16 analysis on surgically excised cervical lymph nodes harboring metastatic HNSCC and found that HPV16 is only detected in those tumors arising from the oropharynx (17). This finding points to HPV16 as an attractive biomarker for discerning tumor origin and sets the stage for the application of this strategy to cells aspirated from neck masses during the initial stages of the clinical evaluation.

Patients and Methods

Cases. Study approval was obtained from The Johns Hopkins Medical Institutions Internal Review Board. The cytopathology files of

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The Johns Hopkins Medical Institutions were searched for consecutive patients from 1995 to 2005 diagnosed with metastatic squamous cell carcinoma based on a fine-needle aspirate of a neck mass. As this was a retrospective study requiring the availability of unstained slides, cases were included only if initial processing of the aspirate included preparation of a cell block whereby aspirated material was spun into a cellular pellet for fixation in formalin and embedment in paraffin. A H&E-stained slide was made from each cell block to confirm the presence of squamous cells. Cases were then scored for the abundance (scant versus satisfactory) and integrity (degenerated versus intact) of the squamous cells. Cellularity was considered scant if only rare individual squamous cells or squamous cell clusters were present for analysis. Specimens were considered degenerated if the squamous cells had acidophilic cytoplasm and pyknotic nuclei with loss of chromatin detail (Fig. 1).

Medical records were reviewed to document the primary site of tumor origin. The primary site was defined by histopathologic confirmation of squamous cell carcinoma in a mass detected on physical examination by a head and neck surgeon.

In situ *hybridization.* HPV detection in formalin-fixed and paraffin-embedded cell blocks was done using the *in situ* hybridization catalyzed signal amplification method for biotinylated probes (DAKO GenPoint, Carpinteria, CA; ref. 18). This catalyzed signal amplification system permits visualization of single copies of HPV16 in infected cells (19). Briefly, 5-micron tissue sections underwent deparaffinization, heat-induced target retrieval in citrate buffer, and digestion using proteinase K (Roche Diagnostics, Indianapolis, IN). Slides were subsequently hybridized with a biotinylated HPV16 type–specific probe (DAKO) and, in selected cases, with a wide-spectrum HPV probe–specific for subtypes 6, 11, 18, 31, 33, 35, 45, 51, and 52.

Signal amplification was done by consecutive application of streptavidin–horseradish peroxidase complex, biotinyl tyramide, and streptavidin–horseradish peroxidase complex. Visualization of positive hybridization signals was done by incubation with the chromogenic substrate 3,3′-diaminobenzidine. Cases were considered positive if hybridization signals visualized as punctate dots were present in the nuclei of the squamous cells. Fine-needle aspirates from metastatic HPV16-positive and HPV16-negative tonsillar carcinomas served as positive and negative controls, respectively.

Immunohistochemistry. Five-micron sections were deparaffinized. Antigen retrieval was done using heat-induced epitope retrieval with 10 mmol/L citrate buffer. Sections were incubated with a mouse monoclonal antibody against p16 (MTM Laboratories, Heidelberg, Germany) at a 1:500 dilution. The p16 antibody was visualized using the avidin-biotin-peroxidase technique (DAKO LSAB kit, DAKO Cytomation, Carpinteria, CA).

When dealing with p16 staining in tissue sections of HNSCC, the pattern of p16 expression is generally observed to be dichotomous (10).

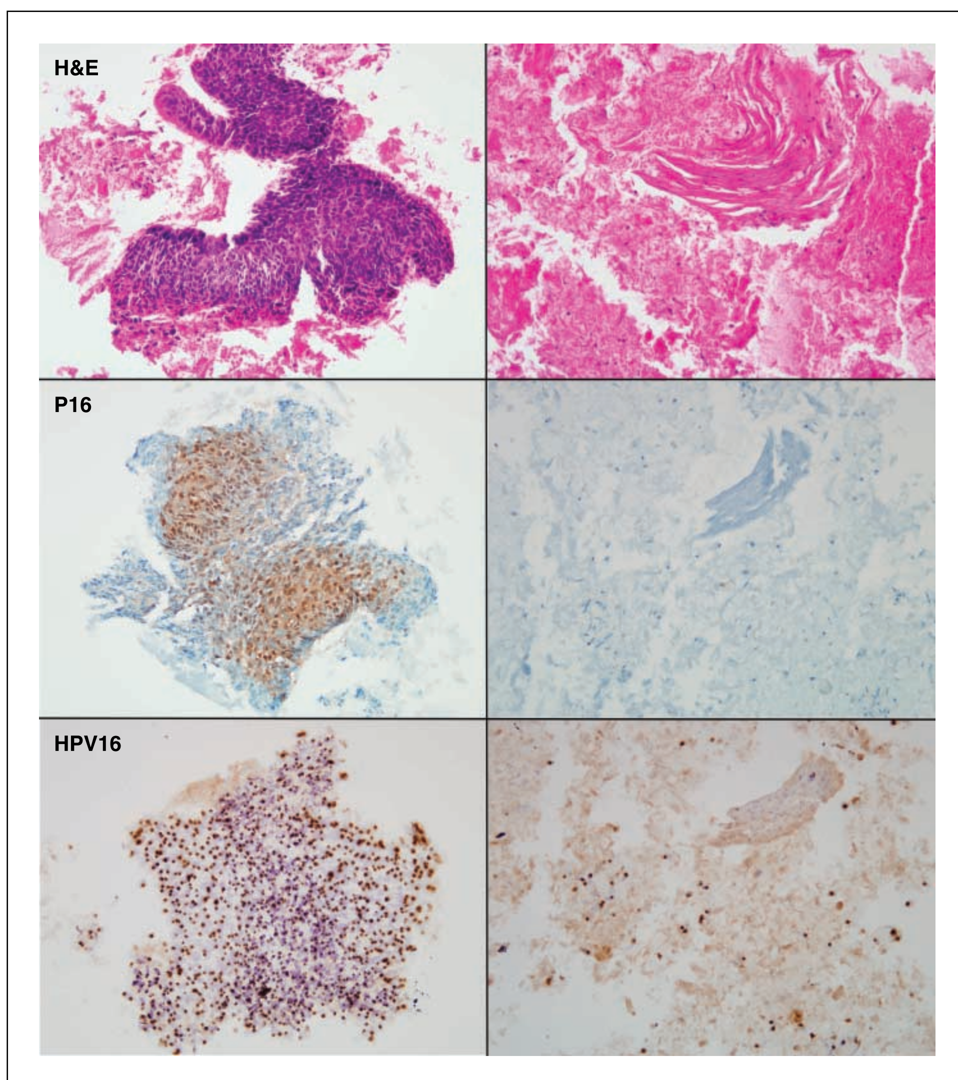

Fig. 1. Fine-needle aspirate of a metastatic squamous cell carcinoma evaluated by routine H&E staining (*row 1*), p16 immunostaining (*row 2*), and HPV *in situ* hybridization (*row 3*). A fragment of viable tumor (*left column*) is strongly p16 positive and HPV16 positive. In areas of cellular degeneration (*right column*), the tumor cells lose their p16 immunoreactivity but retain their HPV16 hybridization signals.

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Table 1. HPV16 *in situ* hybridization and p16 immunohistochemistry of fine-needle aspirates of cervical lymph node metastases by site of primary tumor origin

Anatomic subsite	HPV16 ISH (%)	P16 IHC (%)
Oropharynx	10/19 (53)	13/19 (68)
Non-oropharyngeal	0/46 (0)	1/46 (2)
Larynx	0/17 (0)	0/17 (0)
Oral cavity	0/16 (0)	1/16 (6)
Hypopharynx	0/3 (0)	0/3 (0)
Nasopharynx	0/2 (0)	0/2 (0)
Facial skin	0/3 (0)	0/3 (0)
Lung	0/3 (0)	0/3 (0)
Salivary gland	0/1 (0)	0/1 (0)
Thyroid	0/1 (0)	0/1 (0)
Unknown	3/10 (30)	3/10 (30)

In other words, p16 staining is either absent (no staining) or present (strong and diffuse nuclear and cytoplasmic staining). Accordingly, p16 expression was scored as positive if any staining was observed in the aspirated squamous cells. Immunohistochemical interpretation was done without knowledge of HPV status or tumor origin.

Extended HPV analysis. HPV *in situ* hybridization was extended to other high-risk HPV types in selected cases. In those cases where there was discordance between p16 immunohistochemical staining and HPV16 status (i.e., p16 positive and HPV16 negative) consistent with a non-HPV16 high-risk type, a more extended *in situ* hybridization analysis was done that included probes for HPV subtypes 6, 11, 18, 31, 33, 35, 45, 51, and 52. In those cases where a primary site could not be identified even by panendoscopy with systematic biopsies of the upper respiratory tract, the biopsy specimens from patients with HPV-positive metastases were evaluated in an effort to detect the presence of HPV-infected cells not initially recognized by histologic review. To evaluate the possibility of false-negative results due to the limited number of cells in the fine-needle aspirates, HPV16 *in situ* hybridization and p16 staining were also done on the surgically excised oropharyngeal carcinomas in those cases where the fine-needle aspirates were HPV16 negative.

Statistical evaluation. Primary tumor location was categorized as a dichotomous variable (oropharynx and non-oropharynx). Factors associated with oropharyngeal tumor location were evaluated by cross-tabulations and analyzed by the use of Fisher's exact test. *P* values are two sided unless otherwise specified. Statistical analysis was conducted using STATA software, version 7 (STATA, College Station, TX).

Results

Three hundred seventy-one patients were diagnosed with metastatic squamous cell carcinoma involving a cervical lymph node based on cytopathologic evaluation of a fine-needle aspirate. Of the 371 fine-needle aspirate specimens, 119 (32%) included preparation of a cell block. On microscopic review of the cell block, 77 of the 119 (65%) cell blocks contained at least some squamous cells. These 77 cases formed the study group for subsequent HPV16 and p16 analysis.

The quantity and quality of the squamous cells in the cell blocks were highly variable. In 37 (48%) of the study cases, squamous cells were present as cohesive nests of viable cells in sufficient numbers to easily permit a diagnosis of squamous cell carcinoma. In the other 40 cases, the samples were composed of squamous cells that were scant in number (8 cases, 10%), degenerated (15 cases, 19%), or both scant and degenerated (17 cases, 22%).

Of the 77 fine-needle aspirates diagnosed as metastatic squamous cell carcinoma, the primary sites of tumor origin included the oropharynx (n = 19, 25%), larynx (n = 17, 22%), oral cavity (n = 16, 21%), hypopharynx (n = 3, 4%), facial skin (n = 3, 4%), lung (n = 3, 4%), nasopharynx (n = 2, 3%), submandibular gland (n = 1, 1%), and thyroid (i.e., squamoid variant of anaplastic carcinoma; n = 1, 1%). In 10 (13%) cases, the primary site of origin was never established. Seven of these patients underwent neck dissection, and the fine-needle aspiration diagnosis of metastatic squamous carcinoma was confirmed histopathologically. The other three patients underwent radiation therapy without a neck dissection, such that the fine-needle aspiration diagnosis was not confirmed histopathologically. In 2 (3%) other cases where a primary squamous cell carcinoma was not detected, final histopathology of the resected neck mass showed a branchial cleft cyst.

Overall, HPV16 was detected in 13 of the 77 (17%) fine-needle aspirates. By anatomic subsite (Table 1), HPV16 was detected in 10 of 19 (53%) metastases from the oropharynx but in none of the metastases from non-oropharyngeal sites ($P < 0.0001$, Fisher's exact test). HPV16 was detected in 3 of 10 (30%) cases where the site of tumor origin was unknown. HPV16 was not present in the two branchial cleft cysts. In all HPV16-positive cases, hybridization was visualized as punctate signals within the nuclei. The signal varied from one or two inconspicuous dots to large number of confluent dots. Of note, the intensity of the hybridization signals did not seem to be diminished in degenerated samples. In 7 of the 13 HPV16-positive cases, the hybridization signals were intense and abundant, although the squamous cells were markedly degenerated (Fig. 1).

High expression of p16 has been advocated as a reliably surrogate marker for the presence of HPV16. Frequently inactivated in non-HPV–related HNSCC, the *p16(INK4A)* tumor suppressor gene is up-regulated in the presence of HPV16, and its protein product can readily be detected immunohistochemically in these HPV16-positive cancers (8, 12, 17). By immunohistochemistry, p16 overexpression was noted in 13 of 19 (68%) metastases from the oropharynx but in only 1 of 48 metastases from non-oropharyngeal sites ($P < 0.0001$, Fisher's exact test). In the positive cases, staining intensity was strong in the viable tumor cells but was diminished to absent in the degraded tumor cells (Fig. 1). P16 staining was present in 3 of 10 (30%) cases where the site of origin was unknown. Weak staining was noted in the two branchial cleft cysts and one metastasis from the oral cavity.

The presence of p16 expression by immunohistochemistry was strongly associated with the presence of HPV16 by *in situ* hybridization: 12 of 13 HPV16-positive tumors exhibited p16 overexpression, whereas 4 of 64 HPV16-negative tumors were p16 positive (92% versus 6%; $P < 0.0001$, Fisher's exact test). In the single case of a HPV16-positive tumor that did not overexpress p16, the aspirated material was very degenerated. However, immunohistochemical analysis of the surgically excised neck mass showed strong p16 staining in the viable tumor. Of the four HPV16-negative and p16-positive tumors that were evaluated with an extended panel of *in situ* hybridization probes, HPV18 was detected in one (25%) case (Table 2).

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Table 2. HPV *in situ* hybridization and p16 immunohistochemical staining of oropharyngeal cancers

Case	FNAs of lymph node metastases		Resections of primary tumors		
	HPV16 ISH	P16 IHC	HPV16 ISH	P16 IHC	Additional HPV ISH probes*
1	–	–	–	–	ND
2	–	–	–	–	ND
3	–	–	–	–	ND
4	–	–	–	–	ND
5	–	–	+	+	ND
6	–	+	–	+	HPV18+
7	–	+	–	+	–
8	–	+	–	+	–
9	–	+	–	+	–
10	+	–	+	+	ND
11	+	+	ND	ND	ND
12	+	+	ND	ND	ND
13	+	+	ND	ND	ND
14	+	+	ND	ND	ND
15	+	+	ND	ND	ND
16	+	+	ND	ND	ND
17	+	+	ND	ND	ND
18	+	+	ND	ND	ND
19	+	+	ND	ND	ND

Abbreviations: ISH, *in situ* hybridization; IHC, immunohistochemistry; ND, not done.
*Additional *in situ* hybridization probes for HPV subtypes 6, 11, 18, 31, 33, 35, 45, 51, and 52.

One potential limitation of HPV16 analysis of fine-needle aspirates is the underestimation of HPV16 frequency as a result of limited tumor sampling. To evaluate the effect of sampling error in HPV-negative cases, we analyzed the primary oropharyngeal carcinomas for HPV16 status and p16 expression and then compared HPV16 profiles between the resected primary tumors and the corresponding aspirated lymph node metastases (Table 2). In all but one case (case 5: a fine-needle aspirate with scant and degenerated squamous cells), the HPV profiles were concordant.

The 10 patients with metastatic squamous cell carcinoma of unknown primary site had undergone panendoscopy with biopsy of various sites of the upper respiratory tract. Thirty percent of metastases from unknown primary sites were positive for HPV16, strongly suggesting origin from the oropharynx (Table 3). In one of these cases, a small cluster of atypical epithelial cells were microscopically identified deep within one of the crypts, and these cells were found to be strongly p16 positive by immunohistochemistry and HPV16 positive by *in situ* hybridization (Fig. 2). HPV16 was not identified in any non-oropharyngeal site in the patients with HPV16-positive metastases of unknown origin (Table 3).

Discussion

The ability to adapt a tissue-targeted approach to aspirated cells represents a critical transition in the clinical implementation of a highly relevant biomarker. Use of aspirated cells as a substrate for HPV16 assessment could facilitate the diagnosis of a HNSCC, direct the search for its site of origin, predict clinical outcome, and

Table 3. HPV16 status of fine-needle aspirations from patients with primary cancers of unknown origin

Case	LN	Tonsils				BOT		NP	Pyriform sinus	
	FNA	Tonsillectomy		Biopsy		Ipsilateral	Contralateral		Ipsilateral	Contralateral
		Ipsilateral	Contralateral	Ipsilateral	Contralateral					
1	$X^{+/+}$			$X^{-/-}$	$X^{-/-}$			$X^{-/-}$	$X^{-/-}$	
2	$X^{+/+}$	$X^{+/+}$	$X^{-/-}$			$X^{-/-}$	$X^{-/-}$	$X^{-/-}$	$X^{-/-}$	$X^{-/-}$
3	$X^{+/+}$	$X^{-/-}$	$X^{-/-}$			$X^{-/-}$	$X^{-/-}$		$X^{-/-}$	$X^{-/-}$
4	$X^{-/-}$	X	X			X		X	X	X
5	$X^{-/-}$							X		
6	$X^{-/-}$			X	X	X	X	X	X	X
7	$X^{-/-}$			X	X	X	X	X	X	X
8	$X^{-/-}$	X	X			X	X	X	X	X
9	$X^{-/-}$			X		X		X	X	X
10	$X^{-/-}$			X		X		X	X	

NOTE: Analysis was also done on the corresponding biopsies of the upper respiratory tract in those patients with HPV16-positive metastases.
Abbreviations: X, site of tissue sampling; $^{-/-}$, p16 negative and HPV16 negative; $^{+/+}$, p16 positive and HPV16 positive; LN, lymph node; BOT, base of tongue; NP, nasopharynx.

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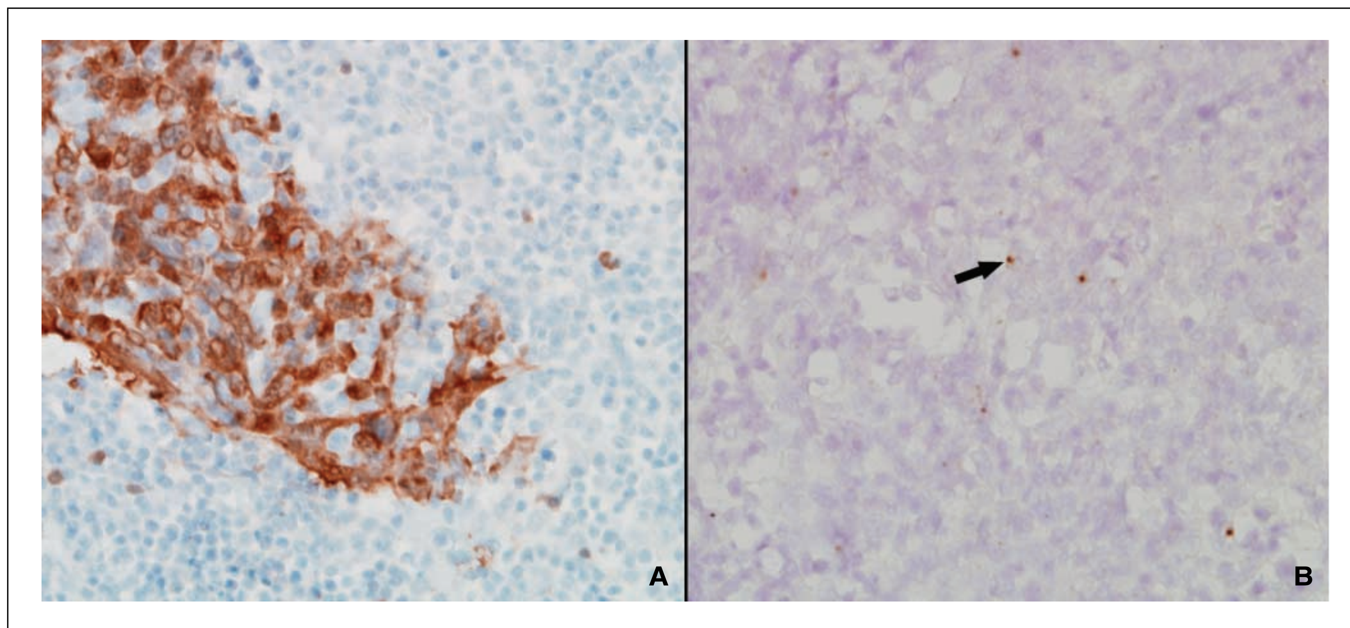

Fig. 2. HPV16 analysis of a resected tonsil from a patient with a HPV16-positive metastasis and an occult primary tumor. Although carcinoma was not appreciated by initial histopathologic evaluation, HPV analysis reveals collections of atypical cells deep within the tonsillar crypts that are positive by p16 immunohistochemistry (*A*) and HPV16 hybridization analysis (*B*). Arrow, focal nuclear hybridization signals.

potentially select patients most likely to benefit from vaccine-based therapeutic trials, all while abrogating the need for tissue acquisition via a more aggressive surgical procedure. The feasibility of an *in situ* hybridization approach for HPV detection in cervical lymph node aspirates has been confirmed in a limited number of studies (20, 21). The clinical application of these previous studies, however, has been constrained by small case numbers and an inability to link viral detection with relevant clinical variables.

In the present study, we focused on HPV16 detection as a means to establish site of tumor origin for patients presenting with metastatic HNSCC. This is not the first localization strategy to take advantage of the fact that certain oncogenic viruses target specific regions of the upper aerodigestive tract. Detection of Epstein-Barr virus in a neck metastasis has been shown to reliably point to the nasopharynx as the site of tumor origin (22–26). The role of viral probes as a tool to localize tumor origin has been expanded recently by the following observations: (*a*) the tonsil and base of tongue are now recognized as the most likely head and neck site to harbor a small and silent tumor in patients presenting with lymph node metastases (27); (*b*) 40% to 60% of squamous cell carcinomas arising from the oropharynx are associated with HPV16 (1, 3, 9, 28–30); and (*c*) HPV16 persists in these tumors even after they have metastasized to other sites (12, 31). Using an *in situ* hybridization approach on aspirated cells, we detected HPV16 in 53% of metastatic oropharyngeal carcinomas but in none of those metastatic carcinomas arising from non-oropharyngeal sites. Detection of HPV16 in fine-needle aspirates of neck masses emphatically points to the oropharynx as the site of tumor origin.

The ability to locate the site of tumor origin in fine-needle aspirates could simplify and fine tune subsequent clinical management. For patients requiring endoscopic evaluation, detection of HPV16 should direct selective biopsies to the oropharynx and may eliminate the need for non-selective biopsies of non-oropharyngeal sites altogether. As HPV16 seems to infect and transform the specialized reticulated epithelium of the deep tonsillar crypts, tonsillectomy is preferable to a superficial biopsy of the tonsil surface for cancer detection. When a cancer is not detected in these selective biopsies by standard light microscopy, additional HPV analysis can be useful in identifying HPV160-infected cells (Fig. 2). For HPV-positive HNSCCs, the common practice of radiating Waldheyer's ring in its entirety, inclusive of the nasopharynx, would seem excessive in light of the remarkable site specificity of HPV16 infection.

HPV analysis of fine-needle aspirates may also be useful as a diagnostic tool, particularly for cystic squamous lesions of the lateral neck. Metastatic oropharyngeal carcinomas involving cervical lymph nodes frequently undergo cystic degeneration causing clinical confusion with benign cystic squamous lesions. Even fine-needle aspiration cytopathology is not always helpful in separating benign lymphoepithelial cysts with reactive squamous atypia from cystic squamous cell carcinomas (32, 33). This diagnostic quandary emerged in a subset of our study cases. Although the absence of HPV16 may not be helpful in confirming the benign nature of a branchial cleft cyst, the presence of HPV16 would seemingly provide compelling evidence of the malignant nature of a metastatic oropharyngeal carcinoma.

As HPV analysis becomes more routinely applied in clinical practice, strategies are needed to optimize accurate determination of HPV status in fine-needle aspirates. In the present study, we used an *in situ* hybridization catalyzed signal amplification method. This method may be preferable to others, including PCR-based strategies: (*a*) it is relatively inexpensive and readily feasible for routine use by many diagnostic laboratories; (*b*) it is very sensitive, permitting visualization of single copies of HPV16 in infected cells (19); and (*c*) it permits direct visualization of viral tissue distribution, thus substantiating HPV as a causal agent. We did HPV16 *in situ* hybridization in

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parallel with p16 immunohistochemical staining. The finding that p16 overexpression is strongly associated with the presence of HPV16 further supports the role of p16 expression as a surrogate marker of HPV infection. P16 immunohistochemistry and HPV16 *in situ* hybridization are complementary, not redundant, in HPV analysis of fine-needle aspirates. The intensity of p16 staining in fine-needle aspirates seems to be inversely correlated with the integrity of the HPV-positive tumor cells: the more degraded the specimen, the weaker the staining. In contrast, the HPV16 hybridization signal persists even in markedly degraded samples. HPV16 *in situ* hybridization alone would not detect the 5% to 10% of oropharyngeal carcinomas that are associated with high-risk subtypes of HPV other than HPV161,9. P16 overexpression in a HPV16-negative tumor points to the presence of one of these other subtypes and provides a rational for an expanded set of hybridization probes in these selected cases. In the present study, HPV18 was detected in 25% of the p16-positive tumors that were HPV16 negative. In some cases, selection of viral probes should be expanded to include other oncoviruses. In particular, the microscopic presence of "lymphoepithelial" features (e.g., syncytial cytoplasm, prominent nucleoli, and absence of keratinization) and/or the failure to clinically document the primary tumor site should prompt consideration of Epstein-Barr virus analysis in addition to HPV analysis.

Over the past three decades, the objective of fine-needle aspiration of neck masses has remained simple and unchanged: collect cells from a mass so that a diagnosis can be rendered based on their morphologic appearance. Application of an expanding armament of potentially useful biomarkers will undoubtedly strain access to this valuable resource and limit implementation unless current practices are modified to accommodate new applications. The clinical need to establish the status of HPV, p16, and other promising biomarkers in fine-needle aspirates of neck masses places a broader expectation on the fine-needle aspirate and argues for the routine preparation and retention of materials (e.g., cytospins and cell blocks) for these less traditional studies.

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