

Role of Cytology in the Diagnosis and Management of HPV-Associated Head and Neck Carcinoma

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Key Words

Human papillomavirus · Oropharynx · Squamous cell carcinoma · Fine-needle aspiration

Abstract

Human papillomavirus (HPV)-associated head and neck squamous cell carcinoma (HNSCC) accounts for up to 25% of all HNSCCs. These tumors largely arise from the oropharynx, particularly the tonsil and base of tongue. The first manifestation of HPV-associated HNSCC is frequently as metastasis to cervical lymph nodes that can be documented by fine-needle aspiration. These metastases are often cystic with a predominantly non-keratinizing, basaloid morphology. Knowledge of the HPV status of metastatic HNSCC has significant treatment and prognostic implications as HPV-associated tumors have a more favorable prognosis than conventional HNSCC. Accordingly, HPV testing should be performed on any squamous cell carcinoma of unknown primary identified in neck lymph nodes. HPV detection may be performed using a variety of methods including p16 as a surrogate immunohistochemical marker, in situ hybridization and/or polymerase chain reaction detection of viral DNA or RNA. Further investigation is needed to determine the optimal method for HPV detection in fine-needle aspiration specimens. Cytology screening for HPV-associated HNSCC does not appear to be effective. Greater understanding of the natural history of oral HPV infections is needed before knowing if oral HPV testing may be useful as a screening test.

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Introduction

The overall incidence of head and neck squamous cell carcinoma (HNSCC) in the United States has decreased along with the decline in cigarette smoking seen since the 1970s [1, 2]. Surprisingly, while the incidence of HNSCC has declined at most anatomic sites, oropharyngeal HNSCC has been increasing [1, 2] and now accounts for 20–25% of all HNSCCs [3, 4]. Similar trends have been noted in European nations as well [5]. These oropharyngeal carcinomas are now recognized as representing a clinically distinct variant of HNSCC resulting from infection with human papillomavirus (HPV) [1–4, 6].

Like conventional HNSCC, HPV-associated HNSCC occurs in men three times as frequently as women, but the HPV-associated cancers differ in a number of other important respects [3, 5, 7–9]. HPV-associated HNSCC arises predominantly in the oropharynx (especially the tonsil and base of tongue). Patients are slightly younger than those with smoking-related HNSCC (<60 vs. >60 years of age). HPV-associated HNSCC is most frequently associated with HPV type 16 in 85–90% of cases. Viral transmission is through sexual contact with sexual behavior-associated risk factors, such as the number of sexual partners, correlating with disease [8, 10]. The precise sexual behaviors posing the greatest risk for viral transmission are not known. The male predominance of disease correlates with increased prevalence of oral infection by HPV type 16 in males [11] which may be due to the ease of female to male viral transmission via oral sex [12],

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hormonal factors [13] or immunologic factors [14, 15]. Cigarette smoking and marijuana use have also been implicated as risk factors [8, 11]. At the cellular level, HPV-associated HNSCCs overexpress E6 and E7 viral oncoproteins. E6 expression promotes the loss of p53 protein while E7 inactivates the Rb protein product, which in turn leads to p16 overexpression [16, 17].

Of greatest clinical significance, HPV-associated HNSCC is associated with an improved prognosis relative to conventional HNSCC [18–27]. Since HPV-associated and conventional HNSCC are now recognized as clinically distinct entities, there is recognition that treatment should be optimized according to whether the tumor is HPV-associated or not. Newly formed clinical trials for HPV-associated HNSCC are largely focused on the concept of deintensified therapy [28]. Because of the responsiveness of HPV-associated HNSCC to conventional therapy, trials are being explored to use less intensive chemotherapy and/or radiotherapy to optimize clinical outcomes while simultaneously decreasing treatment-associated morbidity [28].

Pathology

The most common morphologic variant of HPV-associated HNSCC is that of a non-keratinizing, basaloid squamous cell carcinoma (SCC) [3, 29]. This basaloid morphology should be distinguished from that seen in the clinically aggressive basaloid variant of HNSCC [30] that is non-HPV-related [31, 32]. Other histologic patterns have now been described in association with HPV infection including papillary SCC [33] and lymphoepithelial carcinoma-like tumors [34, 35]; however, these variants share the same favorable prognosis. Recently, small cell carcinoma of the oropharynx has been described as a rare morphologic variant of HPV-associated malignancy that is associated with poor clinical outcome, much like small cell carcinoma arising at other sites [36, 37].

Oropharyngeal HNSCCs, most of which are HPV-associated, commonly present with low T-stage disease with small or even clinically occult primary tumors. Simultaneously, patients frequently have advanced N-stage disease with cervical lymphadenopathy often being the presenting clinical finding [38]. Cervical lymph node metastases from HPV-associated HNSCC are often large in size and cystic in nature [39]. The cystic quality is sufficiently characteristic of these tumors that an HPV-associated oropharyngeal HNSCC should always be suspected in a patient with a cystic metastatic SCC in a cervical lymph node.

Cytologic Diagnosis of HPV-Associated HNSCC

Screening for HPV-Associated HNSCC

The concept of an oral Papanicolaou (Pap) test to screen for pre-invasive HPV-associated oropharyngeal HNSCC is appealing as a counterpart to the tremendous success of the Pap test as a screening tool for carcinoma of the uterine cervix. Unfortunately, both oral rinses and oral brushing specimens have failed at detecting squamous precursor lesions [40, 41]. Although oral brushings may yield diagnostic material in patients with a tonsillar mass, oral rinses are not sensitive for malignancy even in the presence of a known malignancy [41]. There are several obstacles to an oropharyngeal Pap test equivalent. Unlike with cervical neoplasia, lesser degrees of dysplasia than carcinoma in situ have not been convincingly demonstrated for HPV-associated HNSCC. Anatomical limitations are likely to be the single most important factor preventing the successful development of an oral Pap test [42]. The base of tongue is inaccessible to sampling by oral brushing. Although the surface of the tonsil can be sampled by brush, HPV-associated disease largely develops in the tonsillar crypts. These extensive invaginations of the tonsillar epithelium are also not readily sampled by standard brushing techniques putting the sites where HPV-associated disease arises out of reach of available sampling methods.

Fine-Needle Aspiration Diagnosis of HPV-Associated HNSCC

As indicated above, direct brushings of clinically apparent tonsillar masses can yield a diagnosis but are not used frequently in clinical practice [40, 41]. Fine-needle aspiration (FNA) also does not play a significant role in the diagnosis of HPV-associated HNSCC at the primary site due to the relative inaccessibility of the oropharynx and the often small size of the primary tumors.

Nevertheless, there is a significant role for cytology as the initial diagnostic modality for HPV-associated HNSCC. Since a majority of patients have cervical nodal metastatic disease as the initial manifestation of HPV-associated HNSCC, FNA is commonly the diagnostic method of choice for initial evaluation of such patients.

For any newly diagnosed metastatic SCC to a neck lymph node, determination of HPV status should now be a standard part of the cytologic work-up. Detection of HPV status in metastatic SCC is valuable for several reasons:

(1) *Identification of Primary Site.* Since the primary tumor in HPV-associated HNSCC is frequently occult,

FNAs from the involved neck nodes of these patients are evaluated as a malignancy of unknown primary site. Identification of HPV in the metastatic deposit is generally indicative of an oropharyngeal primary [43]. This knowledge can facilitate a targeted endoscopic examination to identify a potentially subtle abnormality facilitating biopsy confirmation of the primary tumor. If the primary tumor is still not identified, tonsillectomy with complete histologic sampling may be appropriate to try to identify the primary.

(2) *Guiding Directed Clinical Treatment.* In 3–9% of metastatic SCCs to the neck, a primary site cannot be identified despite extensive clinical, radiologic and pathologic evaluation [44]. In the absence of a known primary, all upper respiratory mucosa theoretically harboring an occult primary may be subjected to radiation therapy with the associated complications, including mucositis and xerostomia. In patients with no identifiable primary, presumptive localization of the primary site to the oropharynx based on positive HPV status allows directed radiation that spares the nasopharynx and significantly reduces patient morbidity.

(3) *Prognosis and Clinical Trial Selection.* As stated above, there is a favorable prognosis for HPV-associated HNSCC relative to conventional HNSCC [19–27]. Accordingly, clinical trials are being tailored to deintensified therapy for patients with HPV-associated HNSCC [28]. Novel therapies targeting the virus such as therapeutic HPV vaccines are also being investigated [45]. Conversely, for HPV-negative SCC, more intensive treatment protocols may be warranted.

(4) *Distinction of New Primary SCC versus Metastatic Disease.* Because SCCs arising from different sites exhibit marked morphologic and immunophenotypic overlap with one another, establishing the primary site based purely on cytologic appearance is generally not possible. It is worthwhile to compare the morphology of the current and previous tumor when the latter is available. Nevertheless, in most instances the morphological variability and overlap will be such that it will not be possible to offer more than an educated opinion as to the relationship of the two tumors. Ultimately, clinicopathologic correlation is needed relying on such factors as the initial pathologic stage of the previous tumor, site of the original tumor and new mass, and time interval between tumor presentations. In a patient with a known history of HPV-positive HNSCC, the HPV status can be valuable in resolving the uncertainty of whether a new presentation of SCC represents metastasis versus a new primary. For example, in patients with SCC in the lung, the HPV status of the tu-

mor has been shown to be valuable as a means of distinguishing metastatic oropharyngeal HPV-associated HNSCC (including some metastases occurring as late as 8 years after the initial primary presentation) from primary lung SCC [46].

Cytomorphology

Although any metastatic SCC to the neck with no known primary should be tested for HPV, there are patterns of SCC that are more likely to represent HPV-associated disease. Conventional smoking-related, non-HPV-associated HNSCC is often extensively keratinizing. These cancers are readily recognized by FNA, especially with Pap-stained, alcohol-fixed smears. In contrast, HPV-associated HNSCCs typically present with more challenging aspirates with prominent basaloid, undifferentiated, or cystic features, each of which is considered separately below. These patterns are not mutually exclusive so that the cytologic findings for a given case of HPV-associated HNSCC may show overlapping features including the presence of occasional keratinized tumor cells.

Basaloid Pattern

The term basaloid refers to cells having a phenotype resembling the basal reserve cell population of stratified epithelia. The scant cytoplasm of these immature cells offers few clues regarding cell lineage. Classification of basaloid neoplasms is dependent on the identification of a component of the tumor that exhibits a greater degree of differentiation characteristic of a specific entity. Thus, a basaloid carcinoma is only recognizable as SCC if either intercellular bridges or keratinization are identifiable. Such findings may be quite focal and demand thorough screening of the aspirate for diagnostic clues. In the absence of these characteristic features, ancillary studies (most commonly immunocytochemistry) are needed to distinguish definitively amongst the various considerations.

Basaloid HPV-associated HNSCC yields cellular aspirates with high-grade cytologic features. Cohesive clusters of basaloid cells as well as loose aggregates of similar appearing cells are present (fig. 1a). Individual cells have scant cytoplasm with intermediate sized hyperchromatic nuclei without prominent nucleoli. The presence of frequent mitoses, single cell necrosis, and nuclear molding may exquisitely mimic small cell carcinoma (fig. 1b). Focal keratinization helps establish the diagnosis of SCC (fig. 1c). Other helpful distinguishing features from small cell carcinoma are the presence of cohesive cellular groups and adenoid cystic-like areas in SCC (fig. 1a, d) [47–50].

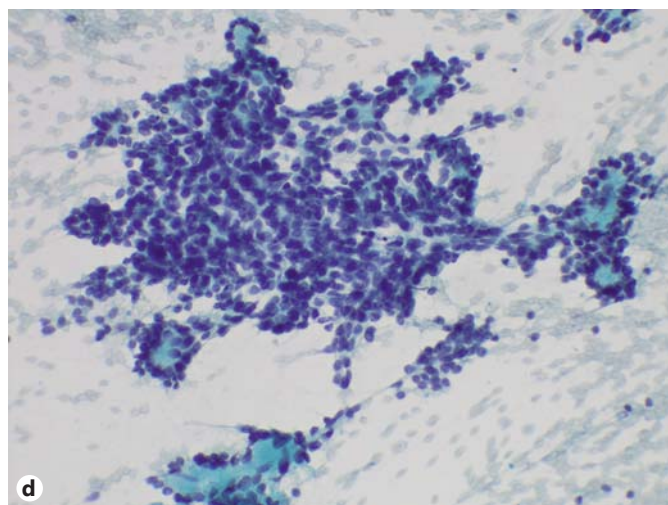
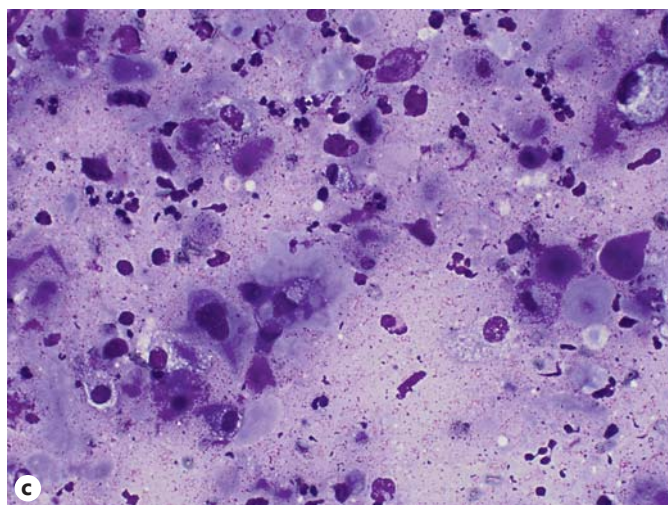
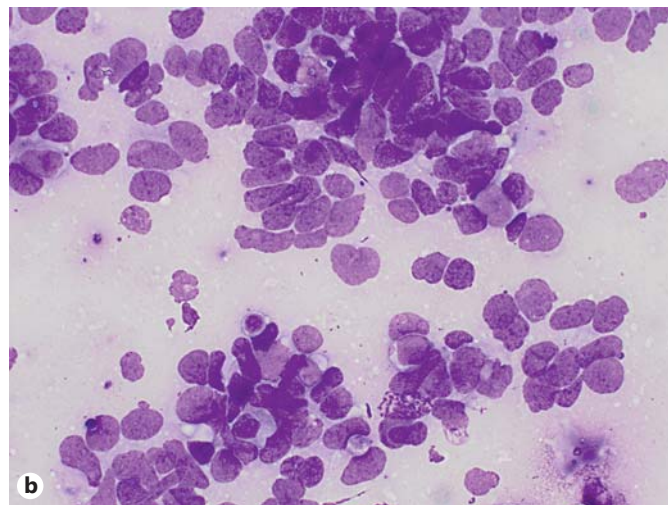
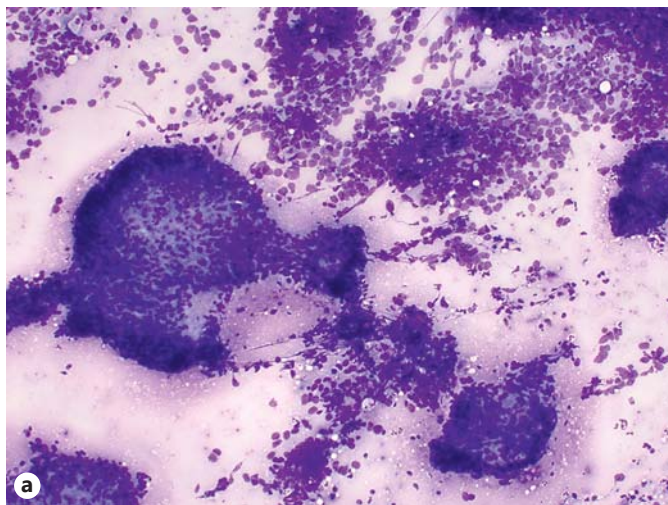


Fig. 1. FNA of HPV-associated HNSCC with a basaloid pattern. **a** The aspirate is markedly cellular with a mixture of cohesive clusters of cells and loose aggregates of basaloid cells (Romanowsky stain, $\times 100$). **b** The basaloid cells can exquisitely mimic small cell carcinoma with nuclear molding, mitoses, and single cell necrosis

(Romanowsky stain, $\times 400$). **c** A cystic component may be present with a mixed population of inflammatory cells, keratinized cells, and keratin debris (Romanowsky stain, $\times 400$). **d** Adenoid cystic carcinoma-like areas may be present with acellular stromal spheres present that are surrounded by basaloid cells (Pap stain, $\times 200$).

The differential diagnosis of HPV-associated HNSCC with basaloid features inevitably includes a non-HPV-related basaloid HNSCC. This distinction depends on HPV testing and is crucial clinically as non-HPV-related basaloid HNSCC has a worse prognosis than conventional HNSCC rather than the favorable prognosis of HPV-associated HNSCC [31, 32].

Immunohistochemistry is helpful in distinguishing amongst these basaloid neoplasms [51, 52]. SCCs express p63 and CK5/6, while neuroendocrine markers such as synaptophysin and chromogranin identify neuroendocrine carcinomas. p16 expression is sensitive for

detection of HPV-associated HNSCC, but is also positive in up to 26% of aggressive basaloid HNSCCs [31]. Of note, p16 is also not a specific surrogate marker for HPV infection in high-grade neuroendocrine carcinomas since activation of the p16 pathway may occur in non-HPV-associated small cell carcinoma [36, 53]. HPV in situ hybridization (ISH) is needed to distinguish HPV-associated HNSCC definitively from these mimics [31, 32].

Basaloid salivary gland neoplasms are also a consideration, primarily the clinically aggressive solid variant of adenoid cystic carcinoma. HPV-associated HNSCC may

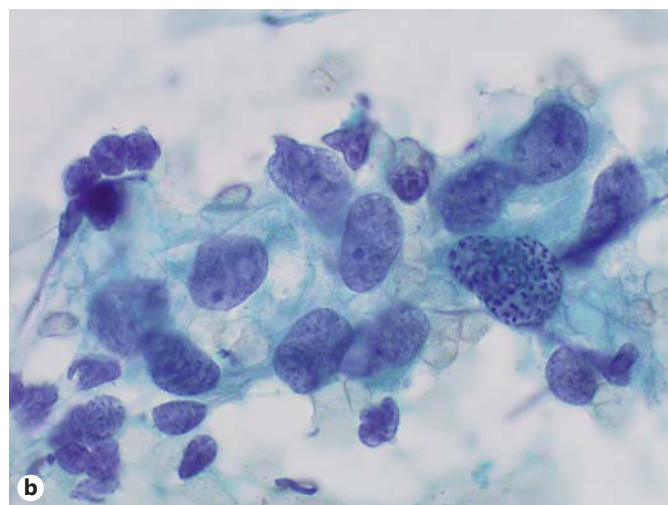
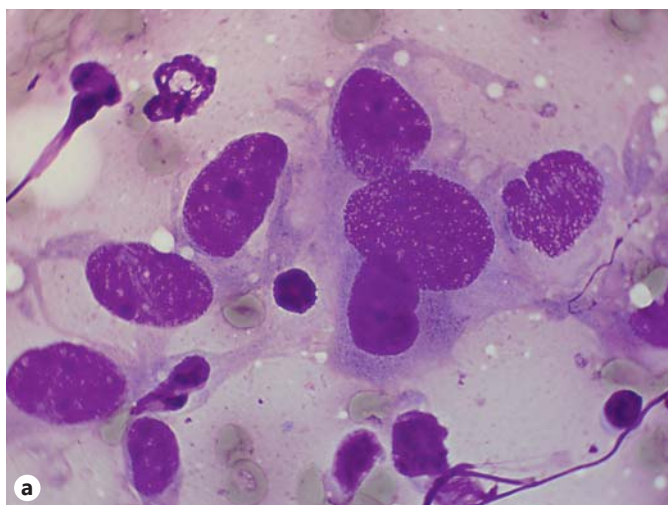


Fig. 2. FNA of HPV-associated HNSCC with an undifferentiated pattern. A syncytial cluster of malignant cells with vesicular chromatin and a single prominent nucleolus is present. Only a few lymphoid cells are present in these images, but may obscure the diagnosis particularly when the malignant cells are dispersed (**a** Romanowsky stain, $\times 1,000$; **b** Pap stain, $\times 1,000$).

exhibit finger-like projections or spherical aggregates of acellular basement membrane material surrounded by basaloid epithelial cells closely mimicking the stromal matrix characteristic of adenoid cystic carcinoma (fig. 1d). If present, squamous differentiation is a valuable distinguishing feature as this finding is absent in adenoid cystic carcinomas [48, 54]. Clinically, presentation of adenoid cystic carcinoma as a neck mass is typically limited to the upper neck with a primary mass in the tail of parotid or submandibular gland. Adenoid cystic carcinoma uncommonly metastasizes to the lower level neck lymph nodes that are most frequently involved by metastatic oropharyngeal HNSCC.

Undifferentiated Pattern

The undifferentiated pattern of HPV-associated HNSCC is morphologically identical to nasopharyngeal carcinoma (NPC) of the non-keratinizing undifferentiated type [34, 35]. Both differentiated and undifferentiated non-keratinizing NPCs are also highly associated with an oncogenic virus, Epstein-Barr virus (EBV). Also like HPV-associated HNSCC, NPC frequently presents clinically as lymph node metastasis in the neck; however, the involved lymph nodes are more frequently those in the posterior triangle. The differentiated subtype of NPC has the appearance of a non-keratinizing SCC, while the undifferentiated type is the variant classically described as lymphoepithelial carcinoma. Undifferentiated NPC

exhibits an intimate admixture of malignant cells with lymphocytes [55–60]. The malignant cells have scant cytoplasm with large vesicular nuclei having a single prominent nucleolus (fig. 2). The tumor is generally readily recognized as carcinoma when the cells form syncytial aggregates, but when present singly, distinction from lymphoma, particularly Hodgkin's disease or large cell lymphoma, is challenging. Keratin immunostains and the detection of EBV-encoded early mRNAs by ISH support a nasopharyngeal primary [61, 62]. Similar appearing undifferentiated carcinomas (with variable EBV association) have been described at other sites (including salivary gland), but with the classic presentation of cervical adenopathy a nasopharyngeal primary is most probable. As mentioned above, HPV-associated HNSCC may look identical to undifferentiated NPC (fig. 2) so that initial evaluation of an undifferentiated carcinoma in the neck should include testing for both EBV and HPV. In the United States, the undifferentiated pattern is now more likely to represent an HPV-associated oropharyngeal primary than an EBV-associated NPC, but NPC would be more probable in endemic areas such as Eastern Asia.

Cystic Pattern

As mentioned earlier, cystic metastatic SCC in a cervical lymph node is sufficiently characteristic that an HPV-associated oropharyngeal primary should be suspected in a patient with this finding [39].

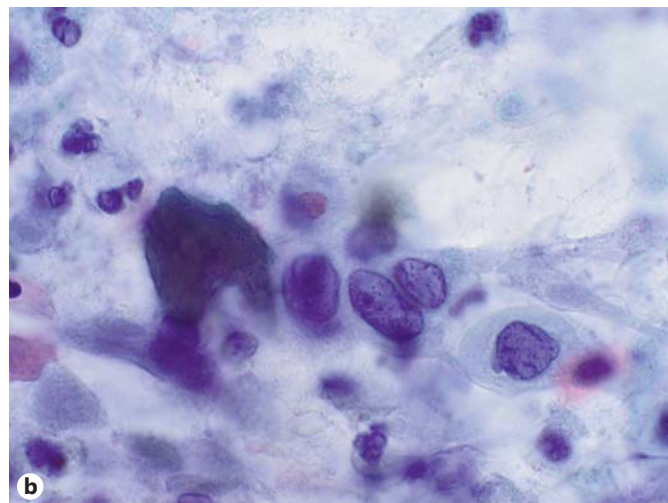
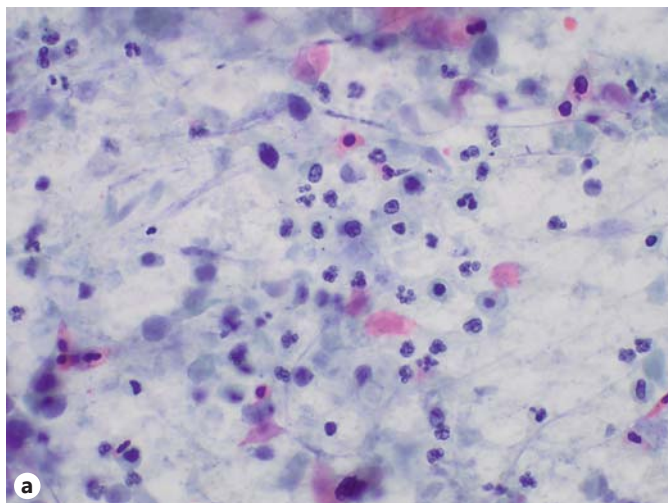


Fig. 3. FNA of HPV-associated HNSCC with a cystic pattern. **a** Only mildly atypical squamous cells with pyknotic nuclei may be evident in an inflammatory background (Pap stain, $\times 400$). **b** Close examination may be needed to appreciate the presence of a population of pleomorphic squamous cells (Pap stain, $\times 1,000$).

Cystic metastases of SCC are treacherous for both the practicing cytopathologist [63] and surgical pathologist and are a significant source of medicolegal claims in the head and neck region due to misdiagnosis as a branchial cleft cyst [64, 65]. The appearance of such aspirates may be deceptively bland and may in fact lack definitive evidence of malignancy.

As with cysts elsewhere, the presence of numerous macrophages identifies the cystic nature of the lesion. Nucleate and anucleate squames, inflammatory cells, and keratin debris may be seen (fig. 1c, 3a). These are non-specific findings that may be seen along with a benign lymphoid component in a variety of squamous epithelial-lined cysts including developmental cysts (branchial cleft cysts, thyroglossal duct cysts), sporadic lymphoepithelial cysts in the parotid, and in parotid gland benign lymphoepithelial lesions with HIV disease.

It is vital in all such aspirates that the specimen is thoroughly examined for any evidence of squamous atypia in order to not miss the focal presence of cells indicative of SCC (fig. 3b). Even in the absence of such cells, the aspirate should be interpreted with caution, maintaining skepticism toward the diagnosis of a benign developmental cyst in older adults, and having a low threshold for recommending excision for definitive diagnosis. HPV testing of such specimens is also useful in resolving diagnostic uncertainty [66, 67].

HPV Detection in Cytologic Material

In many patients, oropharyngeal HNSCC is treated non-surgically. Therefore, FNA may provide the only opportunity to sample tumor for assessing HPV status. For this reason and for all those stated above, accurate methods for determining HPV status are essential.

The presence of HPV infection can be assessed by a number of different methods in both surgical pathology material and cytologic specimens. HPV testing in surgical pathology material has been extensively studied. The simplest option is the use of immunohistochemistry for p16, a surrogate marker for HPV infection (fig. 4a). p16 is highly sensitive (approaching 100%) for the presence of HPV infection but with specificity of approximately 80% [68–70]. Overexpression of p16 occurs in a subset of non-HPV-associated SCCs as well as other tumors potentially in the differential diagnosis including small cell carcinoma [36, 53] and sinonasal undifferentiated carcinoma [71]. Also problematic is that p16 may be expressed in normal tonsillar crypt epithelium [72] as well as in the benign epithelium within a branchial cleft cyst [66, 67] and that there is no universally accepted threshold for p16 positivity.

Direct methods for identifying HPV infection include HPV DNA or RNA detection via polymerase chain reaction (PCR) analysis or ISH. PCR analysis has the advantage of high sensitivity, but also may theoretically detect clinically insignificant infections and is more technically

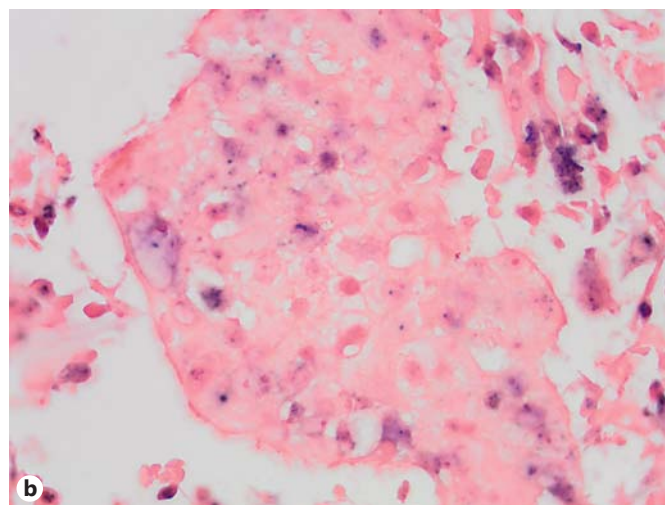
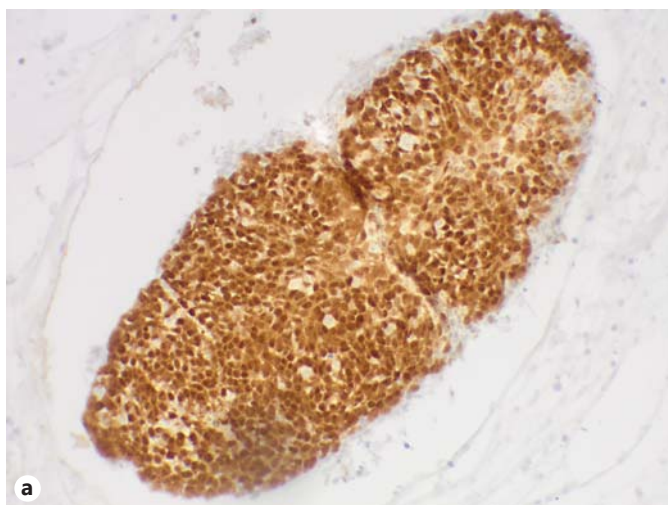


Fig. 4. Special stains for HPV-associated HNSCC. **a** Immunocytochemistry on cell block material often shows strong nuclear and cytoplasmic staining in all the malignant cells (p16 immunostain, $\times 200$). **b** High-risk HPV DNA ISH signal is often patchy and weak, potentially posing difficulty when limited material is present (HPV ISH, $\times 600$).

challenging to perform in many laboratories. ISH has slightly lower sensitivity but greater specificity providing the ability to directly visualize the presence of signal in the targeted population of malignant cells. Unfortunately, there is currently no consensus regarding the best method of HPV testing in surgical pathology material. Leading authorities advocate a combination of testing with the sensitivity of p16 and the specificity of high-risk HPV DNA ISH [69]. In our laboratory, we perform these tests in tandem reserving HPV DNA PCR testing for cases that are p16-positive and HPV DNA ISH-negative. The significance of such cases is unclear as there are conflicting data regarding if p16 positivity alone confers a favorable prognosis regardless of whether HPV DNA is detectable [18, 68, 73, 74]. A recent study using HPV RNA ISH indicates that many cases in which HPV DNA cannot be detected by ISH still represent HPV infection and are, therefore, indicative of diminished sensitivity of HPV DNA ISH in a subset of cases [75].

The optimal testing method for cytologic specimens is even less established. In a recent study, p16 immunocytochemistry on alcohol-fixed Pap-stained smears correctly localized HNSCC to the oropharynx in 74% of positive cases, but detected less than 50% of oropharyngeal primaries [76]. Another study comparing p16 staining in lymph node metastases to matched histologic primaries found 88% (2 false positives and 1 false negative) concordance in p16 staining for 25 cases of HNSCC [77]. These authors

considered a positive p16 result to be staining in 5% of cells, but noted that their 2 false-positive results would have been negative with a 10% threshold. In FNA cell blocks, both p16 immunocytochemistry and HPV DNA ISH have been successful in determining site of tumor origin in SCC cervical lymph node metastases [61, 78]. With adequate cell block material, testing may be performed in the same manner as on surgical pathology material (fig. 4). However, the sparse quantity of material often encountered in cell block preparations is potentially challenging both for interpretation of p16 immunocytochemistry and HPV ISH. The lack of a uniformly accepted threshold for defining a p16-positive result is problematic. In most instances, HPV infection is reflected by diffuse nuclear and cytoplasmic p16 immunoreactivity (fig. 4a). In surgical pathology material, thresholds ranging from 5 to 70% of positive cells have been advocated as constituting a p16-positive result [69, 77]. Conversely, in cytology series, any p16 staining has been interpreted as a positive result [69, 78]. Since p16 immunostaining may occur in the benign epithelium of a branchial cleft cyst [66, 67], a low threshold risks falsely positive p16 results. As for HPV DNA ISH (fig. 4b), detection of HPV DNA is commonly patchy in histologic material [72] increasing the likelihood of a falsely negative result in limited cell block material.

DNA ISH on alcohol-fixed smears has also been used successfully for HPV detection in HNSCC [79, 80] as has

PCR [81]. Recently, detection methods previously validated for HPV cervical Pap testing including proprietary commercial methods such as Hybrid Capture II [82] and Cervista [83] have successfully detected HPV DNA in HNSCC using archival air-dried, alcohol-fixed, and liquid-based preparations. The most rigorous testing to date has been a feasibility test using Hybrid Capture II on cytologic material obtained from surgically resected HPV-associated HNSCC [82]. In this study, Hybrid Capture II proved to have high sensitivity and specificity with rapid turnaround of testing, easy acquisition of adequate material for testing, easy interpretation of the testing result, and low cost.

With the current absence of consensus guidelines for HPV detection in cytologic material, each laboratory should select and optimize testing according to available resources and clinical demands. It should be emphasized that critical decision-making for treatment, clinical trial enrollment, and prognosis may rest solely on the determination of the HPV status of the patient's tumor. Accordingly, every effort should be made to validate whatever testing method is used within an individual laboratory to provide the highest possible level of quality assurance.

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