Chapter 9
Human Papillomavirus (HPV) Testing of Head and Neck Cancers

Eleanor Russell-Goldman and Jeffrey F. Krane

Abbreviations

AJCC	American Joint Committee on Cancer
CAP	College of American Pathologists
DNA	Deoxyribonucleic acid
EBER	EBV-encoded RNA
EBV	Epstein-Barr virus
FFPE	Formalin fixed paraffin embedded
FNA	Fine-needle aspiration
HPV	Human papillomavirus
hrHPV	High-risk HPV
IHC	Immunohistochemistry
ISH	*In situ* hybridization

E. Russell-Goldman · J. F. Krane (✉)
Department of Pathology, Harvard Medical School, Brigham and Women's Hospital, Boston, MA, USA
e-mail: erussell-goldman@bwh.harvard.edu; jkrane@bwh.harvard.edu

S. Roy-Chowdhuri et al. (eds.), *Molecular Diagnostics in Cytopathology*, https://doi.org/10.1007/978-3-319-97397-5_9

N	Nodes (extent of lymph node involvement by metastatic disease in TNM staging)
OPSQCC	Oropharyngeal squamous cell carcinoma
PCR	Polymerase chain reaction
Rb	Retinoblastoma
RNA	Ribonucleic acid
SQCC	Squamous cell carcinoma
T	Tumor (primary tumor description in TNM staging)

Key Terminology

IHC	A laboratory technique that allows the visualization of specific antigens in tissue by conjugating them to complementary antibodies with a reporter molecule. Common reporters are enzymes such as horseradish peroxidase. Enzymatic activation of the reporter leads to the production of a colored product that can be visualized with light microscopy
ISH	A DNA or RNA detection method that uses complementary probes to bind and identify specific DNA or RNA sequences. The probes can be conjugated to fluorescent or chromogenic reporter molecules, which can be detected using fluorescence or light microscopy, respectively
PCR	Molecular method that exponentially amplifies DNA sequences targeted by specific primers using a heat-stable DNA polymerase. This allows identification of targeted DNA sequences and their subsequent sequencing or other manipulations, if required

Key Points

- HPV-positive OPSQCC have a better prognosis and outcome than conventional head and neck SQCC
- HPV 16 is the most common HPV type associated with OPSQCC
- p16 IHC is a commonly used surrogate marker for hrHPV detection and is highly sensitive and moderately specific in surgical pathology specimens; however criteria for detecting hrHPV using p16 in cytology specimens is not well defined
- In surgical pathology specimens, p16 IHC is recommended as the first-line test for HPV and may be supplemented with other testing modalities if needed
- hrHPV can be detected using DNA and RNA isolation techniques that are highly specific and have variable sensitivity
- Current CAP guidelines recommend HPV testing in all cytologic material in cases of known or suspected OPSQCC when the HPV status is unknown or in cases of metastatic SQCC of unknown primary
- There is currently no preferred first-line test for cytology specimens
- While a positive p16 IHC result is considered to be at least 70% nuclear and cytoplasmic staining of at least moderate intensity in surgical pathology specimens, in cytologic material, the threshold for a positive p16 result needs to be individually validated in each laboratory
- Equivocal p16 IHC can be followed up by HPV PCR or ISH testing
- hrHPV testing performed on cytology specimens should be rigorously validated and interpreted with caution
- Negative HPV testing results in cytologic material should be repeated if a subsequent surgical pathology specimen becomes available
- In cases of metastatic SQCC of unknown primary, EBER testing should be performed either concurrently with HPV testing or in cases that prove to be HPV negative

Head and neck cancers encompass a variety of tumor types of the upper aerodigestive tract and salivary glands. Work over recent decades has shown significant molecular and clinical differences among head and neck tumor types. One of the most notable advances has been the recognition of human papillomavirus (HPV)-associated oropharyngeal squamous cell carcinoma (OPSQCC) as a distinct epidemiologic, morphologic, and molecular variant from non-HPV-associated head and neck squamous cell carcinoma [1]. High-risk HPV (hrHPV) has been detected in 70% of oropharyngeal carcinomas and is often associated with a nonkeratinizing basaloid morphology and a distinct clinical course with a more favorable outcome [2–5]. In particular, HPV type 16 has been shown to be most commonly associated with OPSQCC, being found in approximately 90% of cases, with HPV 18 being present in the majority of the remainder of cases [1, 6]. Although HPV has been detected in other head and neck carcinomas, the role and significance of HPV in these tumor types is less well defined [7]; therefore, HPV testing is currently only recommended for known or suspected cases of primary OPSQCC [8].

The HPV status of OPSQCC has been shown to be an independent prognostic factor for survival [5], and patients are now considered for deintensified therapy regimes in clinical trials based on tumor HPV status [4]. The recently published American Joint Committee on Cancer (AJCC) 8th Edition Cancer Staging System now considers high-risk HPV-associated (p16-positive) OPSQCC in its own chapter and incorporates different staging based on whether an OPSQCC is p16 positive or p16 negative [9]. These important insights have been gained from studies evaluating p16/HPV status on surgical pathology specimens.

There are two main scenarios in which the role of fine-needle aspiration (FNA) is increasingly important in the diagnosis and management of these HPV-associated OPSQCC patients. Firstly, many patients have metastatic nodal disease at initial presentation, as HPV-associated OPSQCC commonly presents with low tumor (T) but high

nodal (N) stage disease. FNA of involved lymph nodes, rather than a biopsy of the known primary site, is often the diagnostic method of choice. The second scenario is in patients presenting with metastatic disease of unknown primary site. This is the mode of presentation in 34% of patients with HPV-associated OPSQCC [10], making cytologic material often the only substrate available for both diagnosis and molecular work-up. In patients with metastatic SQCC of unknown primary site, Epstein-Barr virus (EBV)-associated nasopharyngeal carcinoma is also an important consideration. *In situ* hybridization for EBV-encoded RNAs (EBER) should be performed in HPV-negative specimens or tested concurrently with HPV.

The use of FNA material for HPV detection poses its own unique advantages and challenges. Metastatic OPSQCC can often present as necrotic and cystic nodal deposits with scant viable material for testing and a necrotic background that may interfere with testing [11]. Nevertheless, FNA remains an effective, low-cost, and minimally invasive method to rapidly evaluate a patient presenting with lymphadenopathy of unknown primary. Molecular testing of FNA material is also of particular value in patients presenting with SQCC following a history of a prior HPV-positive OPSQCC. In the lung, p16 testing can be used on FNA specimens to distinguish between metastases from an OPSQCC and a primary lung basaloid SQCC, which may be difficult to distinguish on morphological or clinical grounds alone. In this scenario, a positive p16 result is not entirely specific for HPV but suggests a metastasis in patients with a known history of HPV OPSQCC [12]. The recently published College of American Pathologists (CAP) guidelines for HPV testing in head and neck carcinoma now recommend hrHPV testing in all FNA specimens of SQCC in patients with known but previously untested OPSQCC, in patients with a suspected OPSQCC, or in a patient with metastatic SQCC of unknown primary [8]. Currently there is no specific recommendation for a preferred testing methodology in cytology specimens, but regardless of which testing method is used, internal laboratory

validation must be performed. If a hrHPV test is negative in a cytology specimen, follow-up hrHPV testing should be performed on subsequent surgical pathology specimens, if and when they become available. The discussion below addresses the main types of testing methodologies available and how they relate to HPV testing in cytologic material, predominantly referring to cell block preparations with additional discussion of newer liquid-based testing modalities (summarized in Table 9.1).

p16 Immunohistochemistry

The most frequently used HPV testing modality in surgical pathology material is p16 immunohistochemistry (IHC). This method detects the presence of the p16INK4a protein, a cell cycle regulator, which is upregulated during HPV infection and has been shown to be a reliable surrogate marker for hrHPV infection [13]. p16 IHC is the preferred first-line surrogate marker for the detection of hrHPV in surgical pathology specimens as it is highly sensitive, widely available, and relatively inexpensive to perform. The most commonly used p16 antibody is the E6H4 clone (Ventana Medical Systems, Inc., Oro Valley, AZ), which has recently been found to be the most specific and reliable p16 antibody in OPSQCC specimens, even at dilute concentrations [14, 15]; however, CAP guidelines do not specifically endorse any specific antibody or technique for p16 IHC [8]. In histologic tissue specimens, p16 is a highly sensitive marker (up to 100%) for the detection of HPV but has a specificity of approximately 80% [16–18]. CAP guidelines recommend at least 70% moderate to strong nuclear and cytoplasmic p16 positivity in tumor cells in order to report a positive result [8]. Currently, these guidelines refer mainly to surgical pathology specimens, with all testing carried out on formalin-fixed, paraffin-embedded (FFPE) tissue; there is no clear consensus on the interpretation of p16 IHC on cytologic material. Recent studies have attempted to answer this question and found p16 testing on

Table 9.1 HPV testing methods in cytology

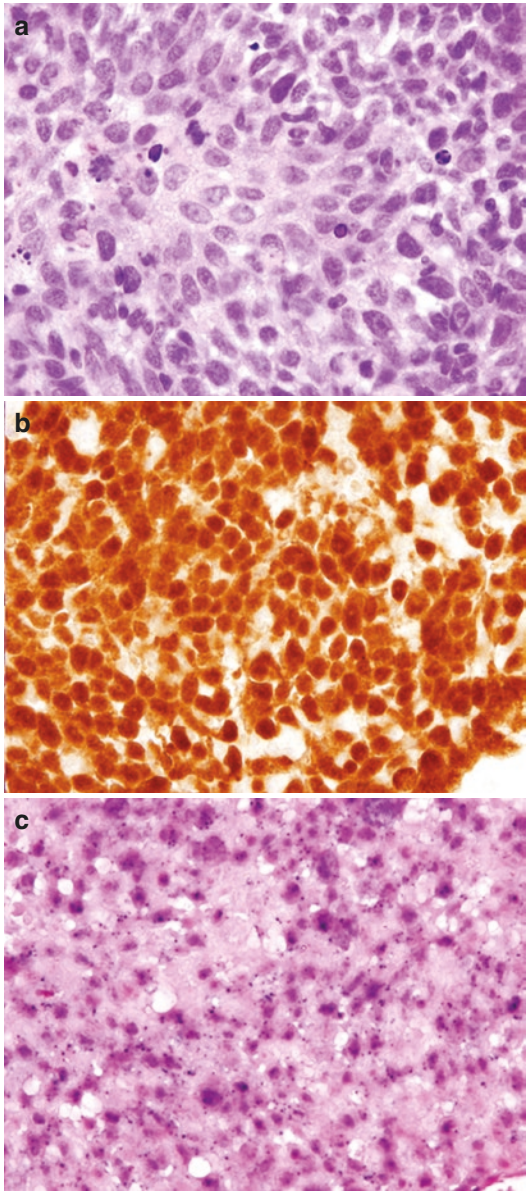
Method	Substrate	Widely available	HPV types tested	Correlates with in vivo viral activity	Sensitivity	Specificity	Positivity threshold in cytologic material
p16 IHC	Cell block	Yes	hrHPV	Yes	High	Moderate (varies with clinical context)	Not established
PCR	Cell block or smear material	Yes	LR and HR	No	High	Low	Not established
RNA ISH	Cell block	No	LR and HR[a]	Yes	High	High	Not established
DNA ISH	Cell block	No	LR and HR[a]	No	Low	High	Not established
Liquid-phase detection	Liquid-based cytology or smear material	Yes	HR	No	Likely high	Likely high	Not established

Abbreviations: *HR* high risk, *LR* low risk

[a]Although both LR and hrHPV probes are commercially available, LR testing is not routinely recommended

cytologic material resulted in variable sensitivities and specificities with suggested cutoffs for positivity ranging from 10% to 90% [11, 19, 20]. It is, therefore, prudent to interpret limited p16 IHC staining on cytologic material with caution, as specimen processing, low cellularity, and the presence of necrosis may all affect staining. CAP guidelines indicate that the threshold for determining a p16-positive result in cytologic material needs to be individually validated in each laboratory. We favor reporting p16 positivity on a cytology specimen when there is strong nuclear and cytoplasmic staining in the majority of cells in an at least moderately cellular specimen (Fig. 9.1). In cases with an equivocal result, subsequent HPV detection methods can be performed, as discussed below. A potential pitfall in the interpretation of p16 immunostaining is that p16 positivity has been reported in a number of non-HPV-related SQCC, in addition to other tumor types, including oropharyngeal small cell carcinoma, sinonasal undifferentiated carcinoma, clear cell carcinoma of the salivary glands, and HPV-related multiphenotypic sinonasal carcinoma [21–24]. Indeed, p16 positivity has also been reported in benign lymphoepithelial cysts [25]. Therefore, as p16 is a surrogate marker for HPV infection, a positive p16 result should always be interpreted in the context of the cytomorphology and the given clinical and radiological information. Despite these potential confounders, CAP guidelines consider p16 positivity alone adequate evidence of an HPV-associated OPSQCC when there is a known oropharyngeal primary or when there is a metastatic SQCC of unknown primary in upper or middle jugular lymph nodes with typical nonkeratinizing morphology [8].

Figure 9.1 FNA of HPV-associated OPSQCC. (**a**) The cell block demonstrates characteristic basaloid cells with mitotic activity and apoptotic debris (×1000). (**b**) Positive p16 immunocytochemistry on cell block material demonstrates strong and diffuse nuclear and cytoplasmic staining in the majority of cells (×1000). (**c**) Concurrent positive HR DNA ISH is seen as punctate dot-like hybridization signals in tumor nuclei (×1000)


a
b
c

RNA *In Situ* Hybridization (ISH)

The demonstration of transcriptionally active hrHPV is considered a gold standard for the diagnosis of a HPV-related malignancy. Viral oncoproteins E6 and E7 play an essential role in pathogenesis as they bind and disrupt cell cycle regulators TP53 and retinoblastoma (Rb) proteins, respectively [26]. The detection of E6/E7 mRNA by *in situ* hybridization (ISH) allows the direct visualization of hrHPV transcripts in FFPE tissue using chromogenic probes and light field microscopy. RNA ISH has been shown to be a sensitive marker for the presence of hrHPV and may be more sensitive than DNA ISH due to amplification of mRNA in the sample. RNA ISH has been shown to have strong agreement with p16 immunostaining, with a concordance rate of 96.4% [27]. RNA ISH may be able to detect cases of HPV infection when low copy numbers result in a negative DNA ISH result [28]. Until recently, RNA ISH has not been widely available as the hybridization and staining process were not automated. Recent work has demonstrated the utility of an automated hrHPV E6/E7 RNA ISH method which can detect the 18 most common types of HPV in a single assay, with sensitivity approaching 100% in OPSQCC specimens [29]. RNA ISH can be performed on cytology cell block material (FFPE), although there are no specific studies examining any potential differences between HPV RNA ISH in cytology cell blocks and surgically obtained FFPE tissue.

DNA *In Situ* Hybridization (ISH)

DNA ISH uses DNA probes, which are complementary to HPV viral DNA sequences, to detect HPV DNA in FFPE. Chromogenic probes allow direct visualization of HPV DNA in tumor cells using light microscopy, whereas fluorescent probes can also be used with fluorescence microscopy. DNA ISH is a highly specific method for HPV identification, with only a 1% false-positive rate reported [30]. It has been recommended that

a combination of sensitive p16 IHC and specific DNA ISH testing be employed for HPV detection [17]. A recent study evaluated the interpretation of DNA ISH on cytology cell blocks and found that approximately 30% of the cases were scored as difficult or moderately difficult to interpret [31]. The authors cited weak or non-specific staining as a barrier to interpretation and noted that there is often background debris or necrosis which may hinder interpretation. In cases where the result of DNA ISH appears negative or equivocal in a p16-positive cytology case, further hrHPV polymerase chain reaction (PCR)-based testing could be performed as a confirmatory test.

HPV Polymerase Chain Reaction (PCR)

HPV PCR can be performed on FFPE or non-formalin-fixed cytology specimens (smears, liquid-based cytology) and is one of the most commonly used methods for HPV detection. Studies have shown that PCR for HPV DNA is highly sensitive when performed on OPSQCC biopsies [32]. Commonly used primers, GP5+ and GP6+, detect both low- and high-risk HPV DNA [33]. A drawback of the PCR method is that it cannot distinguish between episomal and integrated DNA and therefore may detect the presence of HPV that is not transcriptionally active and therefore not clinically relevant. Specificity can be improved by utilizing multiplex assays such as PCR mass array and targeting the L1 gene of HPV [34]. One study examined PCR testing of scraped FNA smear material and demonstrated that this method had approximately 95% sensitivity and 100% specificity when compared to p16 testing of paired surgical specimens [35].

Liquid-Phase Assays

In addition to immunohistochemical and molecular testing on FFPE tissue, evaluation of HPV status can be performed using liquid-based assays. There are currently five FDA-approved assays that are widely used for the detection of hrHPV in

cervical cytology brushings and could be readily applied to head and neck OPSQCC FNA material. One such method is the Hybrid Capture 2 assay (Digene Corporation, Gaithersburg, MD), which is a liquid-phase hybridization assay using RNA probes to detect up to 13 hrHPV types. Pilot studies have demonstrated that the Hybrid Capture 2 assay is comparable to p16 immunohistochemistry and ISH for hrHPV detection in FNA material [36]. Another FDA-approved liquid-phase test for hrHPV types 16 and 18 is the Cervista assay (Hologic Inc., Bedford, MA), which uses a proprietary signal amplification method for the detection of specific DNA sequences. The Cervista assay has been found to have >90% agreement with concurrent p16 or ISH testing for hrHPV in FNA specimens of head and neck OPSQCC [37]. The Cobas 4800 test (Roche Molecular Systems, Pleasanton, CA) is an automated platform that performs real-time PCR on extracted DNA to detect hrHPV 16/18 and 12 additional high-risk strains. It has been demonstrated that the Cobas 4800 test has >90% agreement with concurrent p16 or DNA ISH testing for hrHPV in FNA specimens of head and neck OPSQCC and has a sensitivity of 100% and specificity of 86% [38]. The overall benefits of liquid-phase testing include the fact that special specimen processing requirements are minimal and the process is automated, resulting in greater reproducibility and short turnaround times. The high sensitivity and quick turnaround make liquid-based testing especially attractive as a screening test for patients with suspected hrHPV-associated OPSQCC.

Summary

The implementation of HPV testing in head and neck carcinomas, namely, OPSQCC, has revolutionized the management and prognosis for patients with hrHPV-positive OPSQCC. These patients may be spared extensive chemotherapy and radiotherapy regimes and overall have better outcomes than patients with conventional OPSQCC. There are a number of testing modalities available for the detection of hrHPV including evaluation of the surrogate marker p16

by IHC and direct detection of the virus by RNA and DNA isolation methods. Both the AJCC and CAP now recommend hrHPV testing as part of the work-up of OPSQCC. A positive p16 is reported for surgical specimens when at least 70% moderate to strong nuclear and cytoplasmic staining is present in tumor cells. The clinical relevance of HPV testing of head and neck carcinomas outside the oropharynx has not been established, and therefore routine HPV testing of these tumors is not currently recommended. The reporting of the HPV status of an OPSQCC should be included in the top-line diagnosis. Tumor grade or differentiation status is not reported as this does not impact prognosis in HPV-positive tumors. Preferred terminology is HPV-positive or p16-positive SQCC with a description of the testing method used and result included in the report.

For cytology specimens, CAP guidelines recommend hrHPV testing on all FNA specimens of known or suspected OPSQCC when hrHPV status has not previously been established and for metastatic SQCC of unknown primary. However, the choice of method of hrHPV testing in cytology specimens is left up to the pathologist or laboratory performing the test.

There is no single perfect test for HPV detection, and as discussed above, each test type has its own limitations and advantages (Table 9.1). Rigorous laboratory validation is essential for all HPV testing methods. The adequacy of the cytology specimen is paramount to reliable HPV testing, and evaluation of adequacy at the time of FNA is recommended to ensure optimal sampling. Until widely validated criteria for HPV testing in cytologic material are established, it is incumbent upon practicing pathologists to judiciously report HPV status and ensure that testing performed at the individual institution is thoroughly validated in order to provide optimal patient care.

References

1. Kreimer AR, Clifford GM, Boyle P, Kreimer AR, Clifford GM, Boyle P, et al. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomark Prev. 2005;14(2):467–75.

2. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst. 2008;100(4):261–9.
3. Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Paula Curado M, Ferlay J, Franceschi S, et al. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. J Clin Oncol. 2013;31(36):4550–9.
4. Krane JF. Role of cytology in the diagnosis and management of HPV-associated head and neck carcinoma. Acta Cytol. 2013;57(2):117–26.
5. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân DF, et al. Human papillomavirus and survival of patients with oropharyngeal Cancer. N Engl J Med. 2010;363(1):24–35.
6. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst. 2000;92(9):709–20.
7. Isayeva T, Li Y, Maswahu D, Brandwein-Gensler M. Human papillomavirus in non-oropharyngeal head and neck cancers: a systematic literature review. Head Neck Pathol. 2012;6(Suppl 1):104–20.
8. Lewis JS Jr, Beadle B, Bishop JA, Chernock RD, Colasacco C, Lacchetti C, et al. Human papillomavirus testing in head and neck carcinomas. Arch Pathol Lab Med. 2018;142(5):559–97.
9. O'Sullivan B, Lydiatt WM, Haughey BH, Brandwein-Gensler M, Glastonbury CM, Shah J. HPV-mediated (p16+) oropharyngeal cancer. In: Amin MB, Edge S, Greene F, Byrd DR, Brookland RK, Washington MK, et al., editors. AJCC cancer staging manual. 8th ed. New York: Springer International Publishing; 2017. p. 113–22.
10. Chernock RD, Lewis JS. Approach to metastatic carcinoma of unknown primary in the head and neck: squamous cell carcinoma and beyond. Head Neck Pathol. 2015;9(1):6–15.
11. Xu B, Ghossein R, Lane J, Lin O, Katabi N. The utility of p16 immunostaining in fine needle aspiration in p16-positive head and neck squamous cell carcinoma. Hum Pathol. 2016;54:193–200.
12. Bishop JA, Ogawa T, Chang X, Illei PB, Gabrielson E, Pai SI, et al. HPV analysis in distinguishing second primary tumors from lung metastases in patients with head and neck squamous cell carcinoma. Am J Surg Pathol. 2012;36(1):142–8.

13. Weinberger PM, Yu Z, Haffty BG, Kowalski D, Harigopal M, Brandsma J, et al. Molecular classification identifies a subset of human papillomavirus- associated oropharyngeal cancers with favorable prognosis. J Clin Oncol. 2006;24(5):736–47.
14. Shelton J, Purgina BM, Cipriani NA, Dupont WD, Plummer D, Lewis JS. P16 immunohistochemistry in oropharyngeal squamous cell carcinoma: a comparison of antibody clones using patient outcomes and high-risk human papillomavirus RNA status. Mod Pathol. 2017;30(9):1194–203.
15. Lewis JS, Shelton J, Kuhs KL, Smith D K. p16 immunohistochemistry in oropharyngeal squamous cell carcinoma using the E6H4 antibody clone: a technical method study for optimal dilution. Head Neck Pathol. 2017;0(0):1–8.
16. Smeets SJ, Hesselink AT, Speel E-JM, Haesevoets A, Snijders PJF, Pawlita M, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int J Cancer. 2007;121(11):2465–72.
17. Singhi AD, Westra WH. Comparison of human papillomavirus in situ hybridization and p16 immunohistochemistry in the detection of human papillomavirus-associated head and neck cancer based on a prospective clinical experience. Cancer. 2010;116(9):2166–73.
18. Schache AG, Liloglou T, Risk JM, Filia A, Jones M, Sheard J, et al. Evaluation of human papillomavirus diagnostic testing in oropharyngeal squamous cell carcinoma: sensitivity, specificity and prognostic discrimination. Clin Cancer Res. 2012;17(19):6262–71.
19. Jalaly JB, Lewis JS, Collins BT, Wu X, Ma XJ, Luo Y, et al. Correlation of p16 immunohistochemistry in FNA biopsies with corresponding tissue specimens in HPV-related squamous cell carcinomas of the oropharynx. Cancer Cytopathol. 2015;123(12):723–31.
20. Grimes R, Garcia-Buitrago MT, Jorda M, Ganjei-Azar P, Ferrell A, Gomez-Fernandez C. P16INKa immunocytochemistry in fine-needle aspiration cytology smears of metastatic head and neck squamous cell carcinoma. Acta Cytol. 2013;57(1):33–7.
21. Bishop JA, Westra WH. Human papillomavirus-related small cell carcinoma of the oropharynx. Am J Surg Pathol. 2011;35(11):1679–84.
22. Wadsworth B, Bumpous JM, Martin AW, Nowacki MR, Jenson AB, Farghaly H. Expression of p16 in sinonasal undifferentiated carcinoma (SNUC) without associated human papillomavirus (HPV). Head Neck Pathol. 2011;5(4):349–54.

23. Bishop JA, Rooper LM, Chiosea SI, Westra WH. Clear cell carcinoma of salivary glands is frequently p16 positive: a pitfall in the interpretation of oropharyngeal biopsies. Am J Surg Pathol. 2018;42(3):367–71.
24. Bishop JA, Andreasen S, Hang JF, Bullock MJ, Chen TY, Franchi A, et al. HPV-related multiphenotypic sinonasal carcinoma: an expanded series of 49 cases of the tumor formerly known as HPV-related carcinoma with adenoid cystic carcinoma-like features. Am J Surg Pathol. 2017;41(12):1690–701.
25. Cao D, Begum S, Ali SZ, Westra WH. Expression of p16 in benign and malignant cystic squamous lesions of the neck. Hum Pathol. 2010;41(4):535–9.
26. Asiaf A, Ahmad ST, Mohammad SO, Zargar MA. Review of the current knowledge on the epidemiology, pathogenesis, and prevention of human papillomavirus infection. Eur J Cancer Prev. 2014;23(3):206–24.
27. Ukpo OC, Flanagan JJ, Ma XJ, Luo Y, Thorstad WL, Lewis JS. High-risk human papillomavirus E6/E7 mRNA detection by a novel in situ hybridization assay strongly correlates with p16 expression and patient outcomes in oropharyngeal squamous cell carcinoma. Am J Surg Pathol. 2011;35(9):1343–50.
28. Bishop J, Ma X-J, Wang H, Luo Y, Illei PB, Begum S, et al. Detection of transcriptionally active high risk HPV in patients with head and neck squamous cell carcinoma as visualized by a novel E6/E7 mRNA in situ hybridization method. Am J Surg Pathol. 2012;36(12):1874–82.
29. Mills AM, Dirks DC, Poulter MD, Mills SE, Stoler MH. HR-HPV E6/E7 mRNA in situ hybridization. Am J Surg Pathol. 2017;41(5):607–15.
30. Thavaraj S, Stokes A, Guerra E, Bible J, Halligan E, Long A, et al. Evaluation of human papillomavirus testing for squamous cell carcinoma of the tonsil in clinical practice. J Clin Pathol. 2011;64(4):308–12.
31. Miller JA, Allison DB, Maleki Z. Interpretation of HPV DNA in situ hybridization in HPV-related head and neck squamous cell carcinoma: an achievable task in cell block and small biopsy material. J Am Soc Cytopathol. 2017;6(3):89–95.
32. Agoston ES, Robinson SJ, Mehra KK, Birch C, Semmel D, Mirkovic J, et al. Polymerase chain reaction detection of HPV in squamous carcinoma of the oropharynx. Am J Clin Pathol. 2010;134(1):36–41.

33. De Roda Husman AM, Walboomers JMM, Van den Brule AJC, Meijer CJLM, Snijders PJF. The use of general primers GP5 and GP6 elongated at their 3′ ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol. 1995;76(4):1057–62.
34. Walline HM, Komarck C, JB MH, Byrd SA, Spector ME, Hauff SJ, et al. High-risk human papillomavirus detection in oropharyngeal, nasopharyngeal, and oral cavity cancers comparison of multiple methods. JAMA Otolaryngol Head Neck Surg. 2013;139(12):1320–7.
35. Channir HI, Grønhøj Larsen C, Ahlborn LB, van Overeem Hansen T, Gerds TA, Charabi BW, et al. Validation study of HPV DNA detection from stained FNA smears by polymerase chain reaction: improving the diagnostic workup of patients with a tumor on the neck. Cancer Cytopathol. 2016;124(11):820–7.
36. Bishop JA, Maleki Z, Valsamakis A, Ogawa T, Chang X, WestraPai SI, et al. Application of the hybrid capture 2 assay to squamous cell carcinomas of the head and neck: a convenient liquid-phase approach for the reliable determination of human papillomavirus status. Cancer Cytopathol. 2012;120(1):18–25.
37. Guo M, Khanna A, Dhillon J, Patel SJ, Feng J, Williams MD, et al. Cervista HPV assays for fine-needle aspiration specimens are a valid option for human papillomavirus testing in patients with oropharyngeal carcinoma. Cancer Cytopathol. 2014;122(2):96–103.
38. Kerr DA, Pitman MB, Sweeney B, Arpin RN, Wilbur DC, Faquin WC. Performance of the roche cobas 4800 high-risk human papillomavirus test in cytologic preparations of squamous cell carcinoma of the head and neck. Cancer Cytopathol. 2014;122(3):167–74.