Published in final edited form as:
Am J Surg Pathol. 2012 December ; 36(12): 1874–1882. doi:10.1097/PAS.0b013e318265fb2b.

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

JA Bishop[1], **X-J Ma**[2], **H Wang**[2], **Y Luo**[2], **PB Illei**[1], **S Begum**[1], **JM Taube**[1,3], **WM Koch**[4], and **WH Westra**[1,3,4,5]

[1]Department of Pathology, The Johns Hopkins Medical Institutions, Baltimore MD

[2]Advanced Cell Diagnosistics, Hayward, CA

[3]Department of Dermatology, The Johns Hopkins Medical Institutions, Baltimore MD

[4]Department of Otolaryngology/Head and Neck Surgery, The Johns Hopkins Medical Institutions, Baltimore MD

[5]Department of Oncology, The Johns Hopkins Medical Institutions, Baltimore MD

Abstract

Background—Evidence for transcriptional activation of the viral oncoproteins E6 and E7 is regarded as the gold standard for the presence of clinically relevant human papillomavirus (HPV), but detection of E6/E7 mRNA requires RNA extraction and PCR amplification - a challenging technique that is restricted to the research laboratory. The development of RNA in-situ hybridization (ISH) probes complementary to E6/E7 mRNA permits direct visualization of viral transcripts in routinely processed tissues and has opened the door for accurate HPV detection in the clinical care setting.

Methods—Tissue microarrays (TMAs) containing 282 head and neck squamous cell carcinomas (HNSCCs) from various anatomic subsites were tested for the presence of HPV using p16 immunohistochemistry, HPV DNA ISH, and a RNA ISH assay (RNAscope®) targeting high risk HPV E6/E7 mRNA transcripts. The E6/E7 mRNA assay was also used to test an additional 25 oropharyngeal carcinomas where HPV status as recorded in the surgical pathology reports was equivocal due to conflicting detection results (i.e. p16 positive, DNA ISH negative).

Results—By the E6/E7 mRNA method, HPV was detected in 49 of 282 (17%) HNSCCs including 43 of 77 (56%) carcinomas from the oropharynx, 2 of 3 (67%) metastatic HNSCCs of unknown primary site, 2 of 7 (29%) carcinomas from the sinonasal tract, and 2 of 195 (1%) carcinomas from other head and neck sites. P16 expression was strongly associated with the presence of HPV E6/E7 mRNA: 46 of 49 HPV positive tumors exhibited p16 expression, whereas only 22 of 233 HPV negative tumors were p16 positive (94% versus 9%, $p < .0001$). There was also a high rate of concordance (99%) between the E6/E7 mRNA method and HPV DNA ISH. For

Correspondence to: William H. Westra, M.D., The Johns Hopkins Medical Institutions, 401 N. Broadway, Weinberg 2242, Baltimore, Maryland 21231-2410, Phone: 410-955-2163; Fax: 410-955-0115, wwestra@jhmi.edu.

Conflicts of Interest

the selected group of discordant HNSCCs (p16+/HPV DNA-), the presence of E6/E7 transcripts was detected in 21 of 25 (84%) cases.

Conclusions—The E6/E7 mRNA method confirmed the presence of transcriptionally active HPV-related HNSCC that has a strong predilection for the oropharynx and is strongly associated with high levels of p16 expression. Testing for HPV E6/E7 transcripts by RNA ISH is ideal because it confirms the presence of integrated and transcriptionally active virus, permits visualization of viral transcripts in tissues, and is technically feasible for routine testing in the clinical laboratory.

Introduction

High risk human papillomavirus (HPV), particularly type 16, is now recognized as a causative agent in a subset of head and neck squamous cell carcinomas (HNSCCs).[14, 15] These HPV-related HNSCCs are distinct from HPV-unrelated HNSCCs in a number of important respects including response to therapy and patient survival. HPV-related HNSCCs are more sensitive to chemoradiation, and improved locoregional control consistently translates into significantly improved overall and disease free survival.[3, 12, 29] Accordingly, HPV detection in HNSCC is now regarded as a powerful biomarker indicating a more favorable clinical outcome such that routine HPV assessment is becoming part of the standard pathologic evaluation of all oropharyngeal carcinomas. Indeed, both the College of American Pathologists and the American Joint Committee on Cancer have recently recommended routine HPV testing as part of the standard pathologic evaluation of resected oropharyngeal squamous cell carcinomas for the purpose of molecular tumor staging.[30] As more is understood of the unique natural history of HPV-positive HNSCC - from viral infection to viral persistence to viral-induced malignant transformation – applications for HPV testing will undoubtedly continue to increase. Indeed, detection of HPV is emerging as a valid biomarker for discerning the presence and progress of disease encompassing all aspects of patient care from early cancer detection,[9, 32] to more accurate tumor staging,[5] to selection of patients most likely to benefit from specific treatments,[31] to post-treatment tumor surveillance.[1, 8]

There is currently no standard approach for HPV testing of clinical samples. Instead, methods of HPV testing across laboratories vary considerably reflecting the biases and tendencies of individual investigators, and the cost to benefit ratio of each technique.[21] Detection strategies differ not just in design, but in their detection targets. These targets have included HPV DNA, HPV RNA, viral oncoproteins, cellular proteins (e.g. p16 protein) and HPV-specific serum antibodies. For widespread implementation in the clinical arena, detection methods must be accurate, technically feasible and cost effective. For example, immunohistochemical staining for viral proteins is relatively undemanding and inexpensive, but the performance of this approach has been too inconsistent to be used as a reliable detection method. At the other extreme, detection of mRNA E6/E7 transcripts is generally regarded as the gold standard for HPV detection, but the demand for expertise and complex tissue processing (e.g. microdissection of fresh frozen tissue) limits its application as a routine diagnostic tool.

The recent development of RNA in-situ hybridization (ISH) probes complementary to E6/E7 mRNA now permits direct visualization of viral transcripts in routinely processed tissues.[19, 27, 28] The purpose of this study was to test the effectiveness of this novel strategy against other commonly used HPV detection methods, and to validate certain distinguishing characteristics of HPV-related HNSCC including its preferential targeting of the oropharynx.

Methods

Tumor samples

Study approval was obtained from the Johns Hopkins Medical Institutions Internal Review Board. Tumor blocks were collected from 282 patients who had undergone biopsies or resections of HNSCCs at the Johns Hopkins Hospital and other centers between the years of 1996 and 2005. Although this case diversity allowed us to test the E6/E7 mRNA method across a wide cross section of patients with HNSCC, including patients from many different treating institutions, it did not facilitate evaluation of patient follow up. The precise sites of tumor origin were determined from a review of the medical records. The anatomic sites included the oropharynx (n = 77), oral cavity (n=109), larynx (n=64), hypopharynx (n=20), sinonasal tract (n=7), nasopharynx (n=2). Three of the samples were of lymph node metastases from patients with unknown primary sites. Slides from each case were reviewed, and then one or more formalin-fixed and paraffin-embedded tissue blocks were selected for tumor tissue transfer. The core cylinders measured 0.7 mm in diameter, and a minimum of three cores were transferred from the donor blocks to the recipient tissue microarray (TMA) blocks to minimize the effect of tumor heterogeneity. TMA sections were evaluated by p16 immunohistochemistry, HPV DNA ISH, and HPV RNA ISH.

At the Johns Hopkins Hospital, all biopsied and resected oropharyngeal squamous cell carcinomas are routinely evaluated for HPV using both p16 immunohistochemistry and HPV DNA ISH.[23] We performed a diagnostic search of the surgical pathology files of the Johns Hopkins Hospital in an attempt to identify cases showing discordant results (i.e. p16 positive, HPV DNA ISH negative). These cases were then selected for HPV analysis using the E6/E7 mRNA method to determine the value of this method in resolving these conflicting findings. A tumor block was obtained for E6/E7 mRNA analysis, and the results were compared to the p16 immunohistochemistry and HPV DNA ISH findings as reported in the medical records.

P16 immunohistochemistry

Five-micron sections from the tissue microarrays were evaluated by immunohistochemistry for expression of a biomarker of HPV E7 oncoprotein activity, the CDK-inhibitor p16. Sections of formalin-fixed and paraffin-embedded tissues were deparaffinized and subjected to antigen retrieval using 10 mM citrate buffer (92° C for 30 minutes). P16 expression was evaluated by use of a mouse monoclonal antibody against p16 (MTM Laboratories, Heidelberg, Germany) visualized using the Ultra view polymer detection kit (Ventana Medical Systems, Inc. Tucson, AZ) on a Ventana BenchmarkXT autostainer (Ventana). P16 expression was scored using a 4-tiered system: 0 = completely negative staining; 1 = focal staining (less than 20% of tumor cells); 2 = patchy staining (20 – 50% of tumor cells); 3 = diffuse staining (greater than 50% of tumor cells). As a surrogate marker of HPV infection, only staining that was diffuse (3) was regarded as positive for p16 overexpression.

DNA in situ hybridization

Five-micron sections from the tissue microarrays were evaluated for the presence of HPV DNA by ISH. Two different detection assays were used. A type 16 specific assay was performed using the ISH catalyzed signal amplification method for biotinylated probes (DAKO GenPoint, Carpinteria, CA). Briefly, 5-μm 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, Carpintera, CA). Signal amplification was performed by consecutive application of streptavidin-HRP complex and biotinyl tyramide. Visualization of positive hybridization signals was performed by

incubation with the chromogenic substrate diaminobenzidine. For broader high risk HPV detection, we also used the Ventana Inform HPV III Family 16 Probe (B) kit (Ventana Medical Systems, Tucson, AZ). For this assay the slides were conditioned using Ventana cell conditioner #2 and ISH-protease 3. Hybridization was performed using the HPV III Family16 probe set that captures HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66. Signals were detected with the ISH iView Blue Plus Detection Kit, which is an indirect biotin-streptavidin system that detects fluorescein-labeled probes. The kit utilizes an alkaline phosphatase enzyme and NBT/BCIP substrate chromogen reaction that provides an intense blue, permanent color and a red counter stain. All reagents are provided pre-diluted and ready-to-use on BenchMark Series automated slide stainers (Ventana Medical Systems, Tucson, AZ).

For both detection assays, punctate hybridization signals localized to the tumor cell nuclei defined an HPV-positive tumor. HPV16-positive controls included an HPV16-positive oropharyngeal cancer and the SiHa and CaSki cell lines. The SiHa cell line is known to harbor 1 to 2 copies of integrated HPV16 per cell, and the CaSki cell line is known to harbor 60 to 600 copies of integrated HPV16 per cell.

RNA in situ hybridization

RNA ISH for high risk HPV E6/E7 mRNA was performed manually using the RNAscope® HPV kit (Advanced Cell Diagnostics, Inc., Hayward, CA) according to the manufacturer's instructions. Briefly, 4 μm formalin-fixed and paraffin-embedded tissue sections were pretreated with heat and protease prior to hybridization. In the tissue microarray (TMA) study, the TMA sections were hybridized separately with a target probe to the HPV 16 genotype and a cocktail of target probes to high-risk HPV genotypes 18, 31, 33, 35, 52, and 58. In the study of the 25 p16+/DNA ISH- cases, whole tissue sections were hybridized with a single cocktail of 18 high-risk HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82). The preamplifier, amplifier and HRP-labeled probes were then hybridized sequentially, followed by color development with DAB. Probes to the bacterial gene dapB and the endogenous UBC mRNA were used as negative and positive controls, respectively, for each case. Specific staining signals were identified as brown, punctate dots present in the cytoplasm and/or nucleus. All three stained slides of each case, HPV, UBC and dapB, were examined together to determine HPV status. HPV status was scored qualitatively as either positive or negative, using the dapB-stained slides as reference. HPV positive cases had definitive punctate brown staining present in at least a subset of tumor cells.

The immunohistochemical and ISH studies were independently scored by 2 different study pathologists (WHW, JAB). Any discrepant cases were resolved by consensus review.

Results

Analysis of tissue microarrays

The TMA contained carcinomas from 282 patients with squamous cell carcinomas from various anatomic sites of the head and neck including the oropharynx (n = 77), oral cavity (n=109), larynx (n=64), hypopharynx (n=20), sinonasal tract (n=7), and nasopharynx (n=2). Three of the samples were of lymph node metastases from patients with unknown primary sites. A summary of HPV status by anatomic site using the various detection strategies are shown in Table 1. By the E6/E7 mRNA method, HPV was detected in 49 of 282 (17%) HNSCCs. Stratified by site of origin, HPV was detected in 43 of 77 (56%) carcinomas from the oropharynx, 2 of 3 (67%) metastatic HNSCCs of unknown primary site, 2 of 7 (29%) carcinomas from the sinonasal tract, and in only 2 of 195 (1%) carcinomas from other head

and neck sites (oral cavity = 1, larynx = 1). Of the 49 HPV positive cases, 42 (86%) were due to HPV16 while one of the other types (i.e. 18, 31, 33, 35, 52, and 58) was detected in the other 7 positive cases. By anatomic subsite, an HPV positive tumor of the oropharynx was more likely to be related to the HPV 16 than an HPV positive tumor from a non-oropharyngeal site (88% vs. 50%, p = .01). For all of the HPV positive HNSCCs, the presence of HPV16 was mutually exclusive of the other high risk genotypes: there were no cases that were positive for both the HPV16 probe and the probe cocktail targeting HPV types 18, 31, 33, 35, 52, and 58.

There was a high rate of concordance (99%) between the E6/E7 mRNA method and HPV DNA ISH for determining HPV status. 231 of the 233 (99%) HPV negative tumors by the E6/E7 mRNA method were also negative by DNA ISH. The discrepant cases were from the sinonasal tract (n=1) and larynx (n=1). Conversely, 47 of the 49 (96%) HPV positive cases by the E6/E7 mRNA method tested positive for HPV using the combination of DNA probes (i.e. HPV16 and the wide spectrum cocktail). The 2 discrepant cases were from the oropharynx (n=1) and larynx (n=1). Correlation with DNA ISH was not as strong when the DNA probes were used individually. The DNA probe cocktail failed to detect HPV in 10 cases (20%) that were HPV positive using the E6/E7 mRNA method. These cases were positive using the HPV16 specific DNA probe, but the presence of relatively few hybridization signals per tumor cell suggested low viral copy numbers per host genome (Figure 2). The HPV16 specific DNA probe failed to detect HPV in 8 cases (16%) that were HPV positive by the E6/E7 mRNA method. Not unexpectedly, 7 of these false negative were due to the presence of a non-16 HPV type.

P16 expression was also strongly associated with the presence of HPV E6/E7 mRNA: 46 of 49 HPV positive tumors exhibited high p16 expression, whereas only 22 of 233 HPV negative tumors were p16 positive (94% versus 9%, p < .0001). These p16 positive and E6/E7 mRNA negative cases were from the oropharynx (6 of 77, 8%), larynx (8 of 64, 13%), oral cavity (8 of 109, 7%), hypopharynx (1 of 20, 5%) and sinonasal tract (1 of 5, 20%) (Figure 3). Using HPV E6/E7 mRNA expression to measure the prevalence of HPV-related HNSCC for a given anatomic site, the positive predictive value of p16 positivity as a surrogate marker of HPV was 0.88 (0.74 – 0.95, 95% confidence interval) for HNSCCs arising in the oropharynx. In sites not preferentially targeted by HPV (i.e. oral cavity, larynx and hypopharynx), however, the positive predictive value of p16 staining was only 0.13 (0.02 – 0.42, 95% confidence interval). The p16 negative and E6/E7 mRNA positive cases were from the oropharynx (1 of 32, 3%), larynx (1 of 56, 2%) and sinonasal tract (1 of 4, 25%). For all sites, the negative predictive value of p16 negativity as a marker for the absence of HPV was 0.99 (0.96 – 1.00, 95% confidence interval).

E6/E7 mRNA analysis of discordant (p16 positive/HPV DNA negative) clinical cases

The practice at the Johns Hopkins Hospital is to routinely perform HPV testing of all oropharyngeal squamous cell carcinomas using an algorithm that incorporates both p16 immunohistochemical staining and DNA ISH. Using this approach, we have previously reported a small but significant subset of oropharyngeal squamous cell carcinomas (18%) characterized by strong p16 immunohistochemical staining in the absence of detectable HPV by DNA ISH.[23] Targeting this selected subset of cases using the E6/E7 mRNA method with a cocktail of 18 high-risk HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82), we were able to detect E6/E7 mRNA transcripts in p16 positive cases where one (17 of 20, 85%) or both (4 of 5, 80%) of the DNA ISH methods were negative (Table 2, Figure 4).

Discussion

Using the novel E6/E7 mRNA method in a large and anatomically diverse group of HNSCCs, we confirmed the distinctiveness of HPV-related HNSCC. First, HPV is clearly an etiologic agent in a subset of HNSCC and not a mere passenger virus. Not only do tumor cells express mRNA E6/E7 transcripts - a decisive indicator of biological activity - but the tight coupling of viral transcription to HPV DNA integration and perturbations of the Rb tumor suppressor pathway (e.g. overexpression of the p16 tumor suppressor gene) point to the sustained and critical role for HPV in cellular transformation (Figure 5). In 255 (90%) of the 282 HNSCCs, there was complete concordance between DNA ISH, RNA ISH and p16 immunohistochemistry as independent measures for the presence and biological activity of HPV (Figure 1).

Second, high risks types of HPV show preferential targeting of the oropharynx.[4, 23] Although the human papillomavirus has long been detected in squamous cell carcinomas of the head and neck (HNSCC), calculations of its site specific prevalence have been impeded by variance in detection methodologies. As just one example, estimates of HPV prevalence in squamous cell carcinoma of the oral cavity range from 0 to 100% depending on the detection strategy of preference.[13, 18] Out of this fog, refinement of detection assays is now clarifying the true incidence, distribution, and relevance of HPV-related tumorigenesis of the head and neck: quantitative PCR methods now help distinguish clonal from non-clonal (e.g. contaminant or passenger virus) patterns of HPV infection; DNA in-situ hybridization permits direct visualization of HPV distribution in tissue specimens; and p16 immunohistochemistry facilitates recognition of HPV-induced disruption of the retinoblastoma (Rb) tumor suppressor pathway (Figure 5). Using the novel E6/E7 mRNA approach, we confirm that most oropharyngeal squamous cell carcinomas harbor transcriptionally active HPV, while most HNSCCs arising in non-oropharyngeal sites do not. Transcriptionally active HPV was detected in 43 of 77 (56%) squamous cell carcinomas of the oropharynx, but in only 1 of 107 (0.9%) oral squamous cell carcinomas and 1 of 64 (1.6%) laryngeal squamous cell carcinomas. The low frequency of transcriptionally active HPV corroborates the findings of Lewis et al[19] who, using the E6/E7 mRNA method, detected transcriptionally active HPV in only a small subset (2.7%) of HNSCCs arising in the larynx, hypopharynx and oral cavity. The more frequent detection of mRNA E6/E7 transcripts in cervical lymph node metastases from unknown primary sites, and in squamous cell carcinomas of the sinonasal tract is not unexpected. Many lymph node metastases of unknown primary sites presumably originate from the tonsils[5, 6], and up to 20% of sinonasal HNSCCs have recently been noted to harbor high risk HPV.[7, 10] The application of the E6/E7 mRNA method to a large number of routinely processed tissues will further help pinpoint the anatomic distribution of HPV-related tumorigenesis in the head and neck and even non-head and neck sites such as the lung where variance in methodologies has obscured the prevalence and importance of HPV in a site specific manner.[25]

Third, HPV-16 is the dominant type of high risk HPV that is detected in oropharyngeal HNSCC. Using two different probes that separately target HPV16 and non-16 high risk types (18, 31, 33, 35, 52 and 58), we detected HPV16 in 88% of those oropharyngeal HNSCCs expressing mRNA E6/E7 transcripts. In these cases, HPV16 was exclusively present. In contrast to cervical squamous cell carcinoma where coinfection with multiple HPV types is common[2], we did not detect any case where an HPV16-positive HNSCC co-expressed mRNA E6/E7 transcripts from another high risk HPV type. The absence of redundancy for other high risk HPV types would seemingly point to the sufficiency of HPV16 mRNA E6/E7 transcription as a transforming agent, acting independently of other high risk HPV types. A non-16 type was detected in 2 of the 4 positive HNSCCs arising in non-oropharyngeal sites. The biology of HPV-related tumorigenesis may be different across

the various subsites of the head and neck; and the evaluation of HPV-related tumorigenesis may require a different and more comprehensive panel of HPV-specific probes outside of the oropharynx.

The widespread use of HPV DNA ISH in the clinical setting has recently been propelled by automation.[17] By diminishing the impact of technical inconsistencies, high throughput automation promises to decrease turnaround time for large case volumes, enhance standardization across diagnostic laboratories and improve reproducibility. Using the Ventana Inform HPV III Family 16 Probe kit (Ventana Medical Systems, Tucson, AZ), we found that this automated approach underestimated the number of HPV-positive HNSCCs: HPV DNA was not detected in 20% of those HNSCCs expressing mRNA E6/E7 transcripts. These apparent false negatives may reflect limitations of this assay to detect HPV at low viral copy numbers since hybridization signals - albeit at low numbers - were visualized using a more laborious manual HPV16 specific DNA ISH. The transcription of viral mRNA provides a natural target amplification step that innately enhances viral detection by RNA ISH, even in those HNSCCs where virus is present at low copy numbers (Figure 2).

Immunostaining for p16 protein has recently been regarded as a practical alternative for HPV testing based on a high correlation between HPV detection and p16 overexpression in recent studies.[11, 16, 20, 22, 23] The absence of a direct and exclusive mechanistic link between HPV DNA integration and p16 expression, however, warns against a casual application of p16 testing alone, particularly for HNSCCs arising outside of the oropharynx. In sites that have not been confirmed as being preferentially targeted by HPV such as the oral cavity, larynx and hypopharynx, the likelihood that p16 over-expression truly reflected the presence of transcriptionally active HPV (i.e. positive predict value) was very low. For these non-oropharyngeal cancers, and even for a subset of oropharyngeal HNSCCs, the possibility of encountering elevated p16 expression by non-viral related mechanisms must be considered. Admittedly, a false positive rate for HPV detection based solely on p16 overexpression may be acceptable when it comes to prognostication: p16 overexpression has been associated with improved patient outcomes for patients with oropharyngeal HNSCCs quite independent of HPV status.[3, 20] Any false positive results, however, may be unacceptable when HPV status is used for the selecting of patients who may benefit from novel therapies such as therapeutic vaccines. In such a scenario, HPV testing should be stringent and highly specific.

To improve the accuracy of HPV detection, some have advocated detection algorithms that utilize the strengths of various detection assays used in combination.[23, 24, 26] Although these algorithms are able to determine the HPV status for most oropharyngeal carcinomas, there remains a subset of cancers that yield conflicting results. At the Johns Hopkins Hospital where all oropharyngeal squamous cell carcinomas are evaluated using a combination of detection assays, up to 18% of cases are p16 positive by immunohistochemistry but HPV negative by DNA ISH. Application of the E6/E7 mRNA method to the clinical care setting could dramatically reduce the number of these equivocal cases. We found that the E6/E7 mRNA assay confirmed the presence of transcriptionally active HPV in 84% of those p16 positive cases where one or more of the HPV DNA probes failed to detect HPV. The fact that even a broad spectrum cocktail of 18 high-risk HPV genotypes failed to demonstrate HPV in the remaining p16 positive cases (16%) argues that p16 may be induced by non-viral mechanisms in a subset of p16 positive oropharyngeal carcinomas.

Testing for HPV E6/E7 transcripts by RNA ISH is an ideal platform for HPV detection and may provide a standalone test for discerning HPV status of head and neck cancers. First, it confirms the presence of integrated and transcriptionally active virus by permitting the

visualization of viral transcripts directly in tissue sections. Second, it is technically feasible and easily transferrable into the diagnostic pathology laboratory. Indeed, the imminent availability of the E6/E7 mRNA method to a widely available automated staining platform promises to enhance standardization across diagnostic laboratories, decrease turnaround time for large case volumes, and improve reproducibility among clinical trials. Third, the transcription of viral mRNA provides a natural target amplification step that may dramatically improve viral detection in clinical samples and clarify the status of those perplexing tumors that are p16 positive by immunohistochemistry but HPV negative by DNA ISH. Forth, it is prognostically useful. In our evaluation of the E6/E7 mRNA assay, we found that the presence of E6/E7 mRNA transcripts was tightly coupled to the expression of other powerful prognostic markers (e.g. p16 expression); and others have more directly observed that HPV status as discerned by the E6/E7 mRNA method strongly correlates with patient outcomes.[27]

Acknowledgments

Sources of Funding: The RNA in situ hybridization assays in the study were funded by and performed by the coauthors from Advanced Cell Diagnostics, Inc. (H.W., X-J. M., and Y.L.), who have stocks in this company and stands to profit by any use of this testing through the company. Construction of the tissue microarrays for this study was funded by the National Institutes of Health (R01 DE013152-11).

We would like to thank Fay Wang and Allissa Nielsen for technical assistance in the RNAscope assay.

References

1. Agrawal Y, Koch WM, Xiao W, et al. Oral human papillomavirus infection before and after treatment for human papillomavirus 16-positive and human papillomavirus 16-negative head and neck squamous cell carcinoma. Clin Cancer Res. 2008; 14:7143–7150. [PubMed: 18981014]
2. Andersson E, Karrberg C, Radberg T, et al. Type-dependent E6/E7 mRNA expression of single and multiple high-risk human papillomavirus infections in cervical neoplasia. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology. 2012
3. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010; 363:24–35. [PubMed: 20530316]
4. Begum S, Cao D, Gillison M, et al. Tissue distribution of human papillomavirus 16 DNA integration in patients with tonsillar carcinoma. Clin Cancer Res. 2005; 11:5694–5699. [PubMed: 16115905]
5. Begum S, Gillison ML, Ansari-Lari MA, et al. Detection of human papillomavirus in cervical lymph nodes: a highly effective strategy for localizing site of tumor origin. Clin Cancer Res. 2003; 9:6469–6475. [PubMed: 14695150]
6. Begum S, Gillison ML, Nicol TL, et al. 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. Clin Cancer Res. 2007; 13:1186–1191. [PubMed: 17317828]
7. Bishop JA, Guo TW, Smith DS, et al. Human papillomavirus-related carcinomas of the sinonasal tract. Mod Pathol. 2012; 25:305A.
8. Chuang AY, Chuang TC, Chang S, et al. Presence of HPV DNA in convalescent salivary rinses is an adverse prognostic marker in head and neck squamous cell carcinoma. Oral Oncol. 2008; 44:915–919. [PubMed: 18329326]
9. Edge, SB.; Byrd, DR.; Compton, CC.; Fritz, AG.; Greene, FL.; Trotti, A, III. AJCC Cancer Staging Handbook. New York: 2010.
10. El-Mofty SK, Lu DW. Prevalence of high-risk human papillomavirus DNA in nonkeratinizing (cylindrical cell) carcinoma of the sinonasal tract: a distinct clinicopathologic and molecular disease entity. The American journal of surgical pathology. 2005; 29:1367–1372. [PubMed: 16160480]

11. El-Naggar AK, Westra WH. p16 expression as a surrogate marker for HPV-related oropharyngeal carcinoma: A guide for interpretative relevance and consistency. Head Neck. 2012; 34:459–461. [PubMed: 22180304]
12. Fakhry C, Westra WH, Li S, 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; 100:261–269. [PubMed: 18270337]
13. Franceschi S, Munoz N, Bosch XF, et al. Human papillomavirus and cancers of the upper aerodigestive tract: a review of epidemiological and experimental evidence. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 1996; 5:567–575.
14. Gillison ML. Human papillomavirus-associated head and neck cancer is a distinct epidemiologic, clinical, and molecular entity. Seminars in oncology. 2004; 31:744–754. [PubMed: 15599852]
15. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst. 2000; 92:709–720. [PubMed: 10793107]
16. Gorgoulis VG, Zacharatos P, Kotsinas A, et al. Alterations of the p16-pRb pathway and the chromosome locus 9p21-22 in non-small-cell lung carcinomas: relationship with p53 and MDM2 protein expression. The American journal of pathology. 1998; 153:1749–1765. [PubMed: 9846966]
17. Guo M, Gong Y, Deavers M, et al. Evaluation of a commercialized in situ hybridization assay for detecting human papillomavirus DNA in tissue specimens from patients with cervical intraepithelial neoplasia and cervical carcinoma. J Clin Microbiol. 2008; 46:274–280. [PubMed: 17977987]
18. Ha PK, Pai SI, Westra WH, et al. Real-time quantitative PCR demonstrates low prevalence of human papillomavirus type 16 in premalignant and malignant lesions of the oral cavity. Clin Cancer Res. 2002; 8:1203–1209. [PubMed: 12006539]
19. Lewis JJS, Ukpo OC, Ma XJ, et al. Transcriptionally-active high-risk human papillomavirus is rare in oral cavity and laryngeal/hypopharyngeal squamous cell carcinomas - a tissue microarray study utilizing E6/E7 mRNA in situ hybridization. Histopathology. 2012 Epub ahead of print.
20. Lewis JS Jr, Thorstad WL, Chernock RD, et al. p16 positive oropharyngeal squamous cell carcinoma:an entity with a favorable prognosis regardless of tumor HPV status. The American journal of surgical pathology. 2010; 34:1088–1096. [PubMed: 20588174]
21. Robinson M, Sloan P, Shaw R. Refining the diagnosis of oropharyngeal squamous cell carcinoma using human papillomavirus testing. Oral Oncol. 2010; 46:492–496. [PubMed: 20227331]
22. Shapiro GI, Edwards CD, Kobzik L, et al. Reciprocal Rb inactivation and p16INK4 expression in primary lung cancers and cell lines. Cancer Res. 1995; 55:505–509. [PubMed: 7834618]
23. 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; 116:2166–2173. [PubMed: 20186832]
24. Smeets SJ, Hesselink AT, Speel EJ, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int J Cancer. 2007; 121:2465–2472. [PubMed: 17680565]
25. Srinivasan M, Taioli E, Ragin CC. Human papillomavirus type 16 and 18 in primary lung cancers--a meta-analysis. Carcinogenesis. 2009; 30:1722–1728. [PubMed: 19620233]
26. Thavaraj S, Stokes A, Guerra E, et al. Evaluation of human papillomavirus testing for squamous cell carcinoma of the tonsil in clinical practice. J Clin Pathol. 2011; 64:308–312. [PubMed: 21345874]
27. Ukpo OC, Flanagan JJ, Ma XJ, et al. 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. The American journal of surgical pathology. 2011; 35:1343–1350. [PubMed: 21836494]
28. Wang F, Flanagan J, Su N, et al. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. The Journal of molecular diagnostics : JMD. 2012; 14:22–29. [PubMed: 22166544]

29. Weinberger PM, Yu Z, Haffty BG, et al. Molecular classification identifies a subset of human papillomavirus--associated oropharyngeal cancers with favorable prognosis. J Clin Oncol. 2006; 24:736–747. [PubMed: 16401683]

30. Wenig, BM.; Barnes, L.; Carlson, DL., et al. Protocol for the Examination of Specimens from Patients with Carcinomas of the Pharynx. Based on AJCC/UICCTNM 7th ed. College of American Pathologists; Available from URL: http://www.cap.org.

31. Worden FP, Kumar B, Lee JS, et al. Chemoselection as a strategy for organ preservation in advanced oropharynx cancer: response and survival positively associated with HPV16 copy number. J Clin Oncol. 2008; 26:3138–3146. [PubMed: 18474879]

32. Zhao M, Rosenbaum E, Carvalho AL, et al. Feasibility of quantitative PCR-based saliva rinse screening of HPV for head and neck cancer. Int J Cancer. 2005; 117:605–610. [PubMed: 15929076]

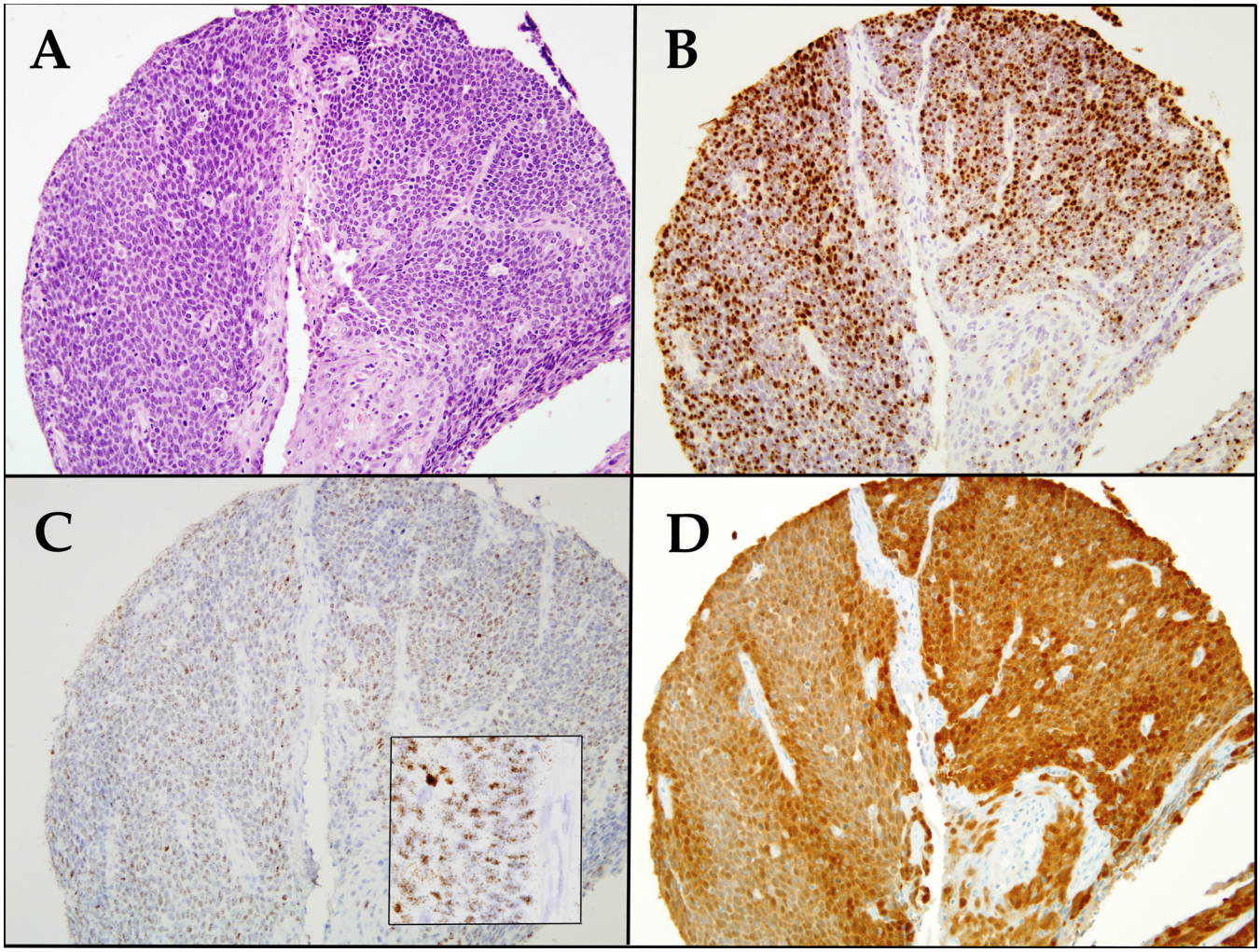

Figure 1.
In this non-keratinizing squamous cell carcinoma of the oropharynx (A, hemotoxylin and eosin stain), HPV DNA in situ hybridization establishes the presence of virus (B), HPV mRNA in situ hybridization confirms transcriptional activation of mRNA E6/E7 (C), and overexpression of the cyclin D kinase inhibitor p16 disruption indicates disruption of the retinoblastoma tumor suppressor pathway (D, inset at higher magnification highlights the granular nature of the hybridization signals).

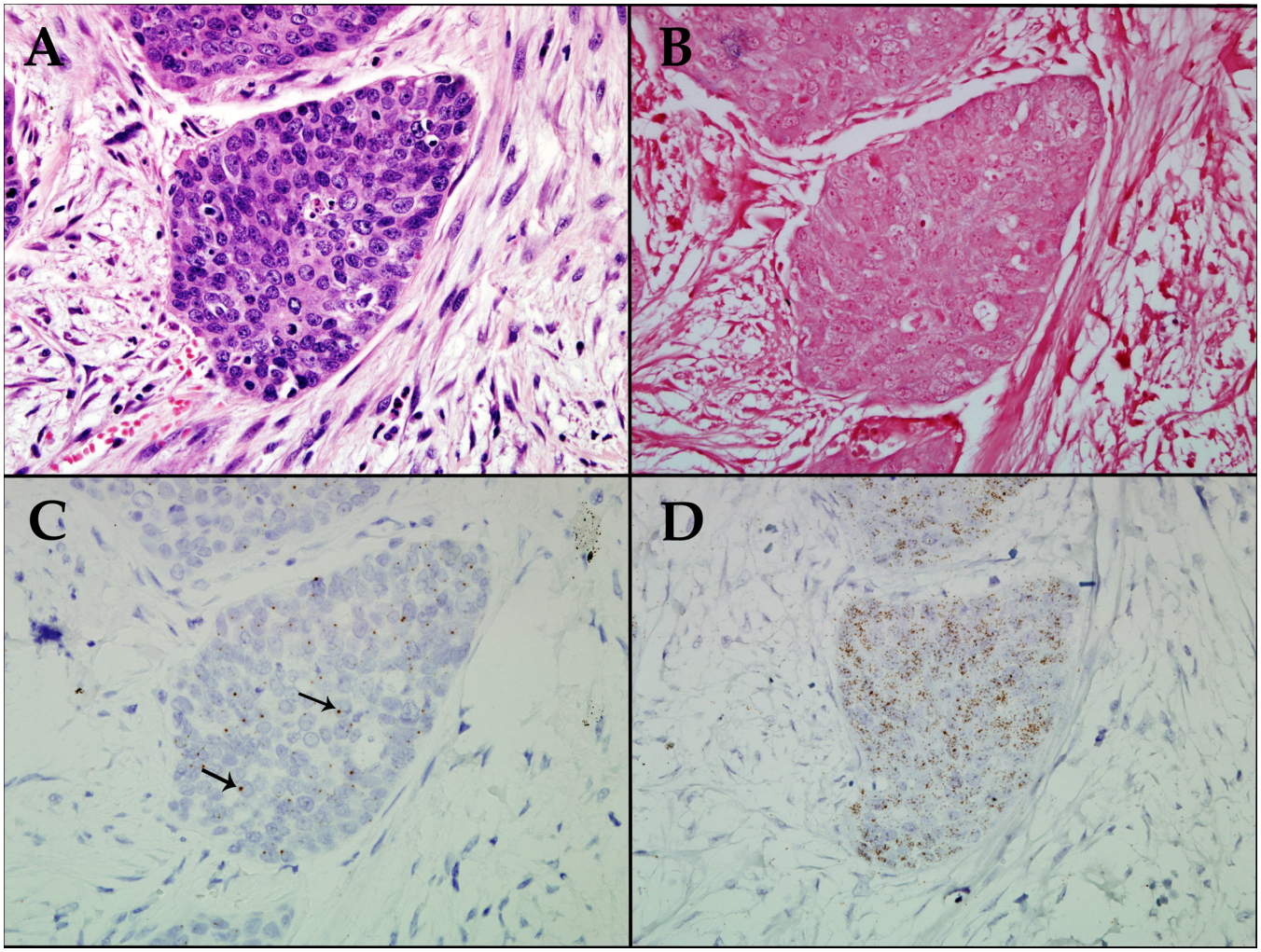

Figure 2.
In this HPV-related oropharyngeal squamous cell carcinoma (A, hemotoxylin and eosin stain), HPV was not detected using the Ventana Inform HPV III Family16 probe for HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66 (B), but the presence of HPV at low viral copy numbers was evident using the type 16 specific probe (DAKO GenPoint, Carpinteria, CA) (C, arrows point to small hybridization signals within nuclei of tumor cells). The mRNA transcripts are seen as numerous granular signals (D).

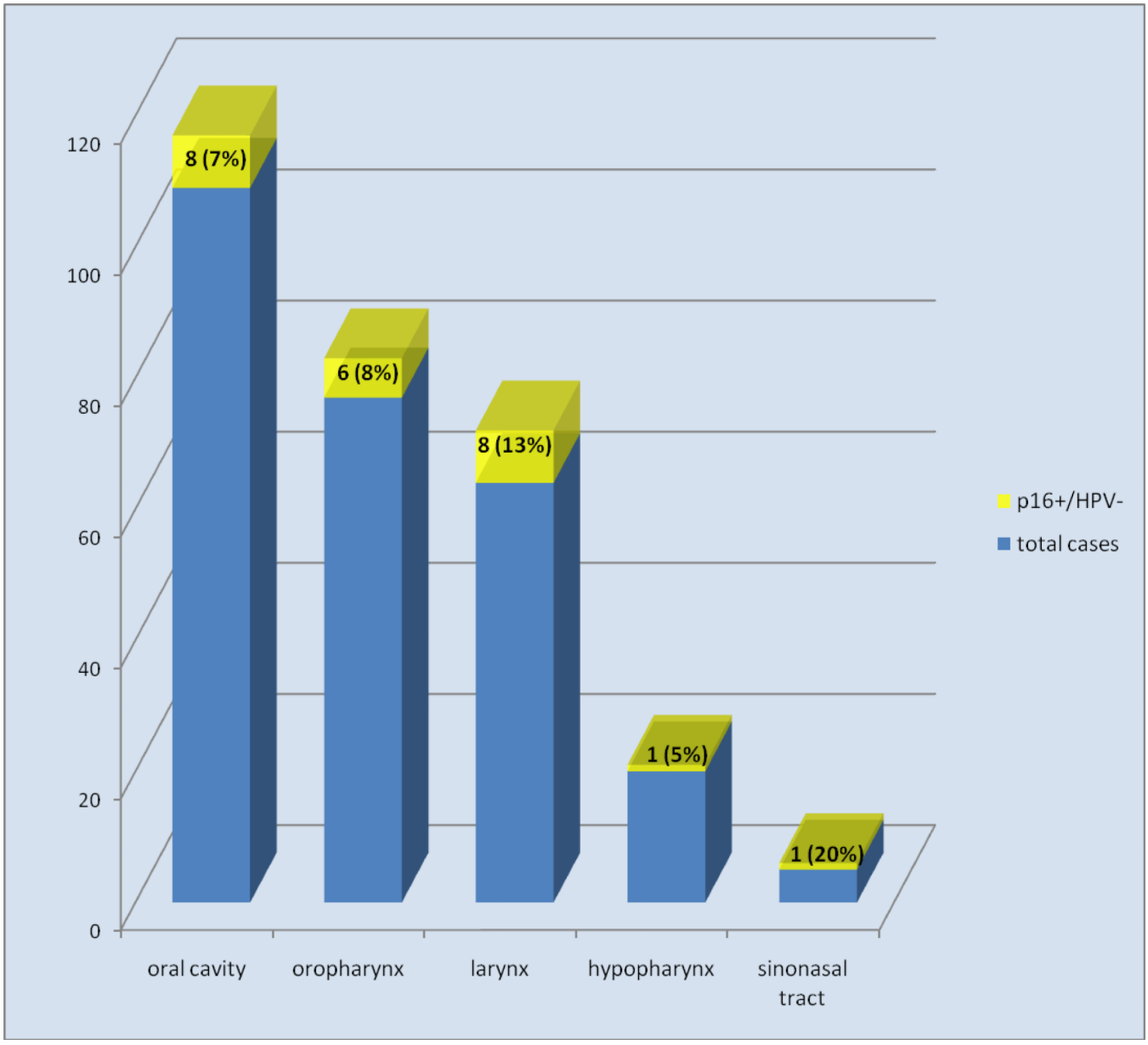

Figure 3.
The frequency of P16 positive and E6/E7 mRNA negative squamous cell carcinomas by anatomic subsites of the head and neck. The relative uniform frequency of these discrepant cases across all anatomic subsites including those that do not appear to be targeted by HPV suggests that non-viral mechanisms may play a role in p16 overexpression in some head and neck squamous cell carcinomas.

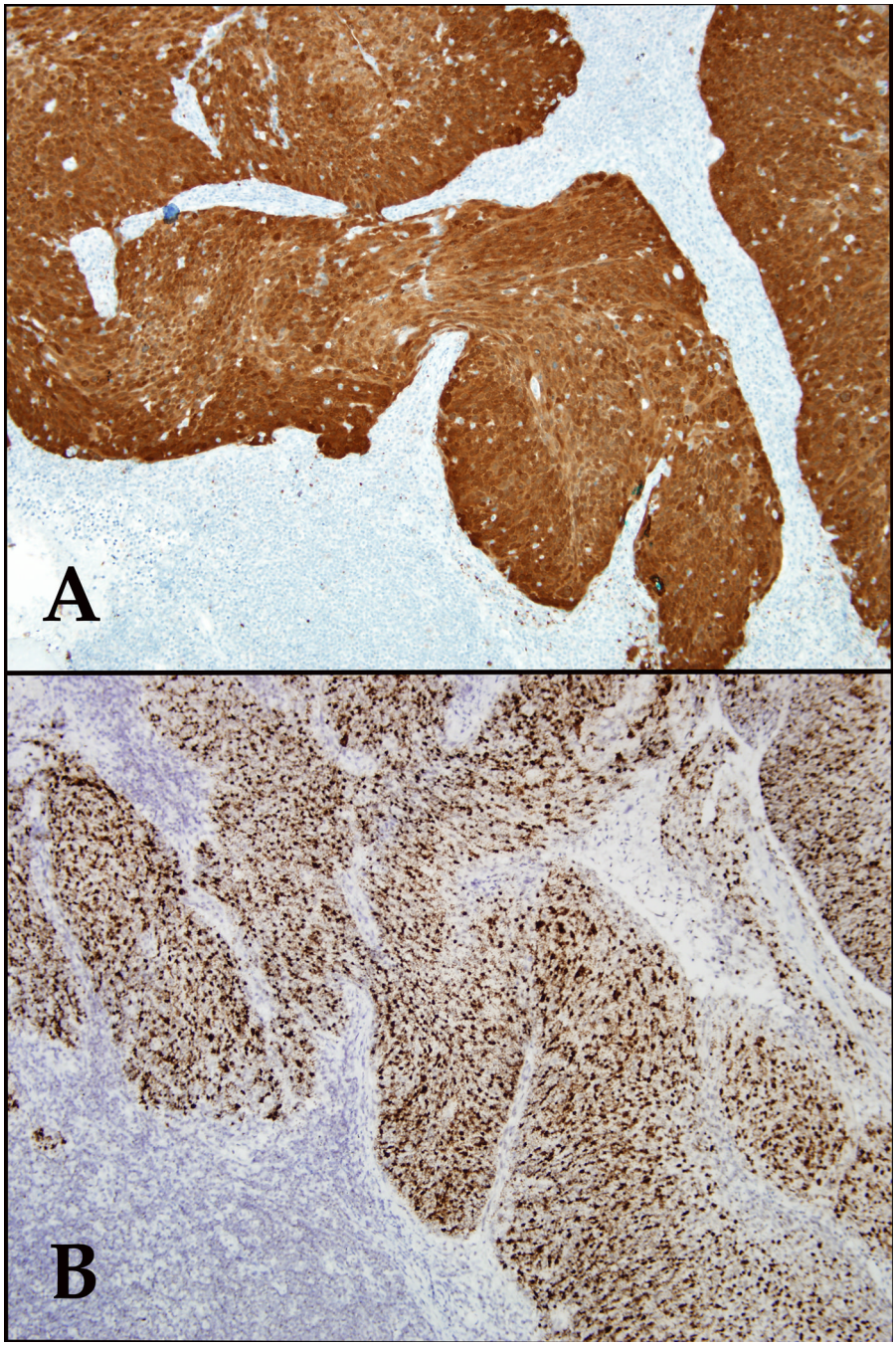

Figure 4.
The HPV status of the oropharyngeal carcinoma (case 13 of Table 2) was equivocal due to the presence of strong p16 staining (A) but the absence of hybridization signals by HPV DNA in situ hybridization (not shown). Despite the inability to detect HPV DNA, abundant E6/E7 mRNA transcripts are detected using the E6/E7 mRNA method (B).

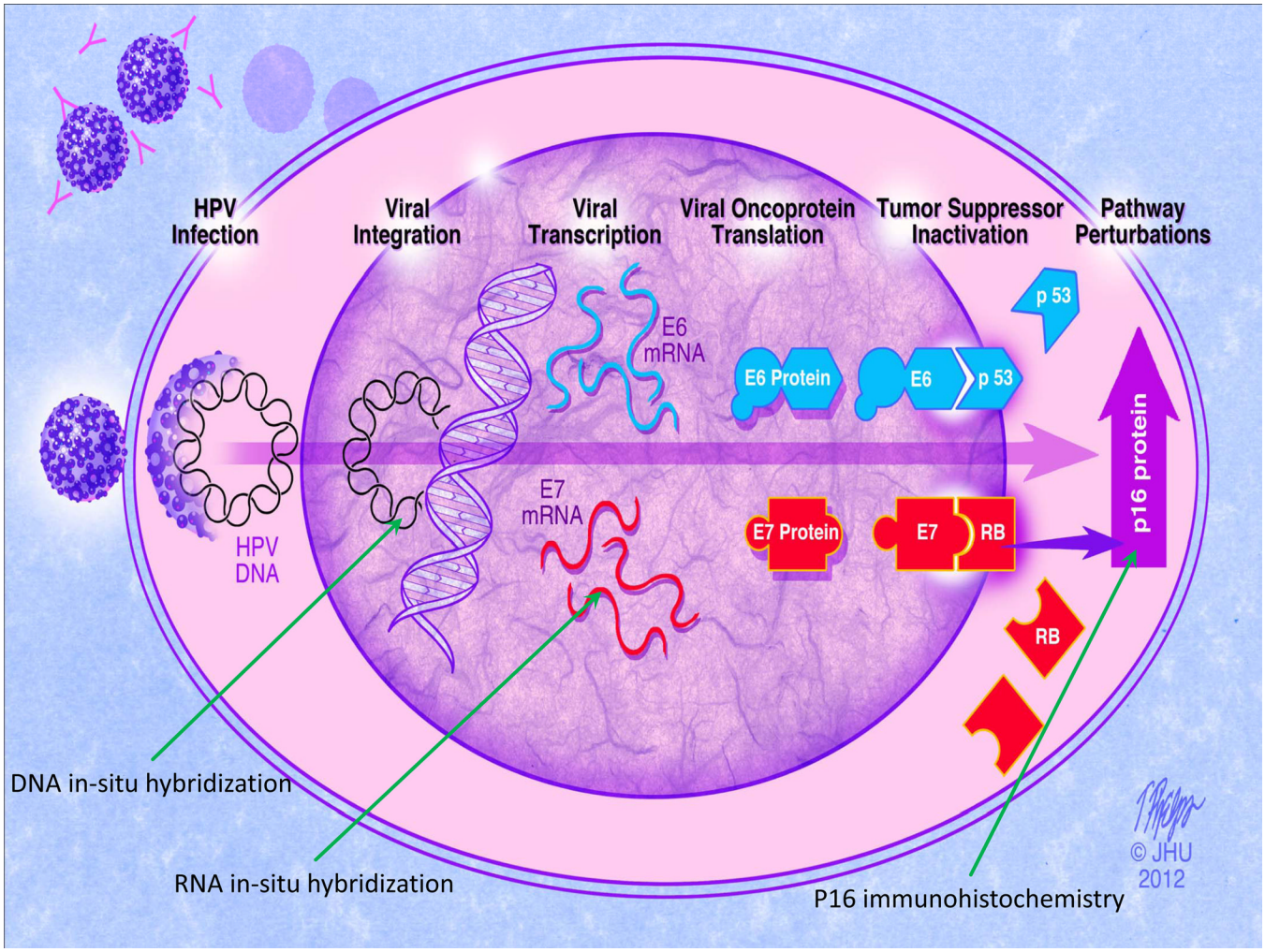

Figure 5.
The biology of HPV infection and malignant transformation provides several sequential points for HPV recognition. Concurrent evidence for HPV DNA, transcriptional activation, and disruption of key growth regulatory pathways (e.g. retinoblastoma pathway) confirms the presence of clinically and biologically relevant HPV.

Table 1

Summary of HPV detection in head and neck squamous cell carcinomas across a spectrum of anatomic subsites

	OP (%) n=77	Oral (%) n=109	Larynx (%) n=64	HP (%) n=20	UP (%) n=3	SNT (%) n=7	NP (%) n=2	Overall (%) n=282
HPV E6/E7 mRNA	38 (49)	1 (1)	0 (0)	0 (0)	2 (67)	1 (14)	0 (0)	42 (15)
type 16	5 (6)	0 (0)	1 (2)	0 (0)	0 (0)	1 (14)	0 (0)	7 (2)
non-16 types*	43 (56)	1 (1)	1 (2)	0 (0)	2 (67)	2 (29)	0 (0)	49 (17)
total								
HPV DNA	39 (51)	1 (1)	0 (0)	0 (0)	2 (66)	1 (14)	0 (0)	43 (15)
type 16	31(40)	1 (1)	1 (2)	0 (0)	2 (66)	2 (29)	0 (0)	37 (13)
broad spectrum**	42 (55)		1 (2)	0 (0)	2 (66)	2 (29)	0 (0)	47 (16)
total								
P16 protein	45 (58)	8 (7)	8 (13)	1 (5)	2 (66)	3 (43)	0 (0)	67 (24)

OP, oropharynx; HP, hypopharynx; UP, unknown primary; SNT, sinonasal tract; NP, nasopharynx; HPV18, 31, 33, 35, 52, and 58; HPV 16, 18, 33, 35, 45, 51, 52, 56 and 66.

Table 2

RNAscope findings in p16 positive oropharyngeal squamous cell carcinomas where HPV DNA was not detected by one or more in situ hybridization methods

Case	Site	P16 IHC	HPV ISH		
			DNA		RNA
			wide spectrum*	type-16**	RNAscope
1	tonsil	+	ND	−	−
2	lymph node	+	ND	−	+
3	tonsil	+	ND	−	+
4	tonsil	+	ND	−	+
5	BOT	+	ND	−	+
6	tonsil	+	−	ND	+
7	BOT	+	−	+	+
8	lymph node	+	−	ND	−
9	lymph node	+	−	−	+
10	tonsil	+	−	+	+
11	BOT	+	−	−	+
12	tonsil	+	−	+	+
13	tonsil	+	−	−	+
14	BOT	+	−	ND	−
15	BOT	+	−	+	+
16	BOT	+	−	ND	+
17	tonsil	+	−	+	+
18	BOT	+	−	+	+
19	sinonasal	+	−	+	+
20	BOT	+	−	+	+
21	BOT	+	−	−	−
22	tonsil	+	−	+	+
23	BOT	+	−	−	+
24	BOT	+	−	+	+

Case	Site	P16 IHC	HPV ISH		
			DNA		RNA
			wide spectrum*	type-16**	RNAscope
25	BOT	+	–	+	+

IHC, immunohistochemistry;

*, Ventana Inform HPV III Family 16;

**, DAKO type 16-specific HPV probe; BOT, base of tongue; ND, not done