p16 Immunohistochemistry in Oropharyngeal Squamous Cell Carcinoma Using the E6H4 Antibody Clone: A Technical Method Study for Optimal Dilution

James S. Lewis Jr.^{1,2} · Jeremy Shelton¹ · Krystle Lang Kuhs^{2,3} · Derek K. Smith^{4,5}

Received: 12 August 2017 / Accepted: 25 November 2017 / Published online: 30 November 2017
© Springer Science+Business Media, LLC, part of Springer Nature 2017

Abstract

Routine testing for p16 immunohistochemistry (with selective HPV-specific test use) has been recommended for clinical practice in oropharyngeal squamous cell carcinoma (OPSCC). Data suggests that the E6H4 clone performs best for this purpose, yet no studies have evaluated the optimal antibody concentration for OPSCC testing. We evaluated three concentrations (undiluted, 1:5, and 1:10) of the primary antibody solution for E6H4 using tissue microarrays from a cohort of 199 OPSCC patients with a > 70% staining cutoff for positivity. Concordance was evaluated using percent agreement and Cohen's kappa. The concentrations were evaluated for sensitivity and specificity using high risk HPV RNA in situ hybridization (RNA-ISH) and also correlated with Kaplan–Meier overall survival analysis. Inter-rater agreement was very high between p16 results at each concentration and also with RNA in situ hybridization ($p < 0.0001$ for all). Agreement between p16 undiluted and 1:5 dilution (agreement 98.2%; Kappa 0.943; $p < 0.0001$) was very high and between p16 undiluted and 1:10 dilution (agreement 79.2%; Kappa 0.512; $p < 0.0001$) much lower. Intensity of the staining did decrease with the 1:5 and 1:10 dilutions compared to undiluted, but not in a manner that obviously would change test interpretation or performance. Results suggest that the E6H4 antibody performs well at dilutions of up to 1:5 fold with a minor decrease in staining intensity, minimum loss of sensitivity, and no loss of specificity in OPSCC patients. This could result in reagent and cost savings.

Keywords Oropharyngeal · Squamous cell carcinoma · Immunohistochemistry · P16 · Antibody · Human papillomavirus · E6H4

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12105-017-0871-5>) contains supplementary material, which is available to authorized users.

✉ James S. Lewis Jr.
James.lewis@vanderbilt.edu

¹ Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, 3020D Vanderbilt University Hospital, Nashville, TN 37232-7415, USA

² Department of Otolaryngology, Vanderbilt University Medical Center, Nashville, TN, USA

³ Division of Epidemiology, Vanderbilt University School of Medicine, Nashville, TN, USA

⁴ Department of Biostatistics, Vanderbilt University Medical Center, Nashville, TN, USA

⁵ Department of Oral and Maxillofacial Surgery, Vanderbilt University Medical Center, Nashville, TN, USA

Introduction

It is now well established that high-risk human papillomavirus (HPV)-positive oropharyngeal squamous cell carcinoma (OPSCC) is a distinct entity amongst head and neck cancers with unique epidemiology, biology, and prognosis [1–6]. In contrast to conventional head and neck SCC patients, those with HPV-positive OPSCC tend to be younger and predominantly Caucasian and male, with higher average numbers of sexual partners and lower or absent tobacco use. Despite high rates of nodal metastasis at presentation, HPV-positive OPSCC patients have consistently been shown to have a more favorable prognosis than those who are HPV negative [3–5].

Due to the unusual signaling pathway disruptions caused by the high risk HPV E7 oncoprotein, p16 (which functions as a tumor suppressor protein in normal cells) is aberrantly overexpressed [7–9]. p16 immunohistochemistry has widespread availability, ease of interpretation, and

extensive high quality outcomes research in the literature showing it to be independently associated with improved survival. For these reasons, it has become the recommended first line (and usually standalone) prognostic test for patients with OPSCC. The recent publication of the College of American Pathologists evidence-based guidelines for HPV testing in head and neck carcinomas highlights the central role of p16 in the HPV work up of new oropharyngeal and cervical nodal metastatic SCC [10, 11].

What has not been addressed to date are the precise technical details of p16 immunohistochemical testing. This test has become a critical prognostic biomarker for routine clinical practice, and has the potential to become an actual therapeutic (or “theragnostic”) biomarker [2, 12]. For this reason, just as with receptor immunohistochemistry in breast cancer, there needs to be standardization in the technical methods and interpretation through method comparison studies and even the establishment of proficiency testing. Our group recently published a large oropharyngeal SCC antibody comparison study that showed the E6H4 antibody to perform best as a prognostic marker, in interpretation, in staining intensity, and in specificity using the antibody in its “ready to use” form [13]. However, the staining conditions recommended by the manufacturer are not necessarily optimized for oropharyngeal SCC testing, as the antibody is used for cervical biopsies and also for myriad other surgical pathology applications. It has been observed by many groups that the primary antibody can be diluted (potentially several fold) without apparent decline in staining performance [14, 15]. Based on these few published studies and upon much clinical practice experience, we performed a formal dilution study of E6H4 immunohistochemistry in a large oropharyngeal SCC tissue microarray cohort, evaluating results by their correlation with high risk HPV status by RNA in situ hybridization and with patient disease outcomes.

Materials and Methods

A tissue microarray of retrospectively identified OPSCC patients from 1998 to 2007 was created as previously described [15, 16]. According to the amount of available

biopsied or resected tumor tissue, duplicate 2 mm punches (or if inadequate tumor tissue present, then 0.6 mm punches) were taken from each case. Approximately 75% of cases on the array had the larger (2 mm) punches. 243 cases were evaluated in total, 44 of which were excluded due to missing tumor (“core loss”) or insufficient evaluable tumor, leaving a total of 199 cases. Patients were treated without regard to their p16 status.

p16 Immunohistochemistry

Immunohistochemistry was performed for p16 on formalin-fixed, paraffin-embedded tissue sections using the E6H4 antibody (Ventana Medical Systems, Inc.) on a Leica Bond automated instrument (Leica Biosystems, Inc.) with antigen retrieval consisting of 10 min in the ER1 proprietary antigen retrieval solution. Three sets of stains were performed, all in the same laboratory and with the same staining conditions, but the dilution was varied from undiluted (so called “ready to use”) to 1:5 and up to 1:10. Primary antibody solution was diluted using Leica’s BOND primary antibody diluent. The Bond Polymer Refine detection system was used for visualization. Slides were then dehydrated, cleared and coverslipped. Staining was interpreted by the study pathologist (J.S.L.). Cases were considered suitable/sufficient for interpretation if at least 10% of the cross-sectional area across the two cores consisted of tumor cells. Both nuclear and cytoplasmic staining was required for a cell to be considered “positive” and staining distribution was read in a binary manner using the criteria put forth in recent guidelines as negative = no staining or staining in less than 70% of tumor cells versus > 70% and positive = staining in > 70% of tumor cells regardless of intensity. Intensity was assessed as weak (1), moderate (2), or strong (3) for comparison between the dilutions. As a further check on the utility of the tissue microarray (TMA) punches for the evaluation of p16 immunohistochemical status, results of the undiluted TMA staining were compared to p16 immunohistochemical staining on whole tissue sections, which had previously been performed for all patients on the TMAs as part of a separate research project [14].

Table 1 Correlation table between the results of p16 immunohistochemistry at each concentration with HPV status by RNA in situ hybridization

	Sensitivity	Specificity	PPV	NPV
TMA Undiluted	0.97 (0.92, 0.99)	0.90 (0.74, 0.98)	0.98 (0.94, 1.00)	0.85 (0.68, 0.95)
TMA 1:5 Dilution	0.95 (0.90, 0.98)	0.96 (0.82, 1.00)	0.99 (0.96, 1.00)	0.82 (0.65, 0.93)
TMA 1:10 Dilution	0.75 (0.67, 0.82)	0.94 (0.81, 0.99)	0.98 (0.94, 1.00)	0.49 (0.36, 0.61)

HPV human papillomavirus, TMA tissue microarray, PPV positive predictive value, NPV negative predictive value

Fig. 1 Examples of discrepant p16 immunohistochemistry between dilutions. **a** Undiluted staining showing diffuse, strong, nuclear and cytoplasmic staining; **b** 1:5 dilution staining showing <70% nuclear and cytoplasmic staining of moderate intensity; **c** 1:10 dilution staining showing less than 25% of tumor cells and with only weak to modest nuclear and cytoplasmic staining. (All images 20× magnification)

RNA In Situ Hybridization (RNA-ISH) for High Risk HPV

In situ hybridization for high risk HPV E6/E7 mRNA had been performed and interpreted as previously described [15] by hand using the RNAscope HPV kit (Advanced Cell Diagnostics, Inc., Hayward, CA) according to the manufacturer's instructions. Probes covered HPV types 16, 18, 31, 33, 35, 52, and 58. Positive staining was identified as brown, punctate dots present in the nucleus and/or cytoplasm. Control probes for the bacterial gene DapB (negative control) and for the housekeeping gene ubiquitin C (positive control evidence of adequate RNA) were also included on each case. Cases in which there was <10% surface area consisting of tumor were excluded. The array (and corresponding control) slides and were classified in a binary manner as either positive or negative. Positive cases had to have granular cytoplasmic and/or nuclear brown staining that was higher than the signal on the DapB negative control slide.

Statistical Analysis

Each concentration was assessed in terms of its sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). These values were assessed using RNA-ISH as the standard for transcriptionally-active high risk HPV status. Percent agreement and Cohen's kappa were calculated to assess agreement between the undiluted microarray and the two diluted forms. Specimens missing for a given diagnostic test were presumed to be missing completely at random and omitted from analyses involving the missing observation.

We assessed differences in overall survival (OS) in patient groups defined by immunohistochemistry findings in tumor tissue obtained at the time of initial diagnosis and treatment. Patients were considered to have died of their disease if their cancer had recurred and they died with known disease in their bodies. Kaplan–Meier survival plots were used to estimate differences in survival between these groups. The significance of these differences was assessed with the log rank test.

Additionally, to determine which test was most prognostic, an accelerated failure time model was fit on the observations in which the tests made concordant predictions. A permutation test was then performed using the

total log-likelihood of the discordant observations as a test statistic. This process generated the distribution of the test statistic under the null hypothesis that neither test is more likely to generate predictions that are more consistent with observed overall survival. The observed log-likelihood was then compared to the simulated null distribution with significant results representing one test being more consistent with observed survival than the other.

Results

Results of all immunostaining are presented in Supplemental Table 1 by individual patient. Supplemental Table 2 shows correlation rates between p16 immunohistochemistry on the tissue microarrays with microarray high risk HPV RNA-ISH and also p16 immunohistochemical staining results on whole tissue sections (which was performed for prior studies and which used a 1:1 dilution [14]). Table 1 shows the performance of p16 immunohistochemistry at each concentration compared with HPV RNA-ISH. The 1:10 dilution demonstrated a marked decrease in both sensitivity and negative predictive value compared to undiluted, although it demonstrated comparable specificity and positive predictive value.

Only three discordant results were observed between undiluted and 1:5 dilution staining among the 170 patients

for which results of both tests were available. For each discordant case, the undiluted staining resulted in a positive result, while the 1:5 dilution was negative (Fig. 1). This resulted in a high percent agreement (98.24%) and significant kappa statistic ($p < 0.001$). There were 36 discordant results observed between undiluted and 1:10 dilution staining among the 173 patients for which results of both tests were available. The vast majority (34; 94.4%) of these were also cases for which the undiluted staining was positive and the 1:10 dilution was negative (Fig. 1). Compared with undiluted, percent agreement with 1:10 dilution (79.19%) was significantly lower than that with the 1:5 dilution (chi square test; $p < 0.001$). Table 2 shows the correlation matrices for agreement between p16 immunohistochemistry for the various concentrations.

Staining intensity varied somewhat between the dilutions as well (Fig. 2). For the positive cases, 24 of the 170 (14%) undiluted, 31 of the 142 (21.8%) 1:5 dilution, and 28 of the 148 (18.9%) 1:10 dilution patients had less than 3+ intensity staining. These differences were not statistically significantly different (p values > 0.1 for all). All of the non 3+ intensity cases for all three dilutions were graded as 2+ (moderate intensity) with the exception of a single case. This one case was 1+ intensity and 80% of tumor cells positive with the undiluted staining, 1+ intensity with only 60% of tumor

Table 2 Correlation tables between the results of p16 immunohistochemistry using the various dilutions

Undiluted and 1:5 dilution			
p16 TMA 1:5	p16 TMA undiluted		
	Positive	Negative	
Positive	136	0	
Negative	3	31	
	139	31	
Sensitivity	97.84% (95% CI 93.82–99.55%)		
Specificity	100.00% (95% CI 88.78–100.00%)		
Agreement	Expected agreement	Kappa	p value
98.24%	69.06%	0.943	<0.0001
Undiluted and 1:10 dilution			
p16 TMA 1:10	p16 TMA undiluted		
	Positive	Negative	
Positive	105	2	
Negative	34	32	
	139	34	
Sensitivity	75.54% (95% CI 67.53–82.43%)		
Specificity	94.12% (95% CI 80.32–99.28%)		
Agreement	Expected agreement	Kappa	p value
79.19%	57.19%	0.5139	<0.0001

cells positive with the 1:10 dilution staining, and, for the 1:5 staining was noninformative due to core loss on the sections.

Kaplan Meier survival analysis results are shown in Fig. 3. High risk HPV RNA-ISH and all p16 immunohistochemistry results, regardless of dilution, were strongly prognostic for overall survival. The results for HPV RNA-ISH and undiluted and 1:5 dilution p16 immunohistochemistry results were remarkably similar, with stratification of survival that was similar throughout the follow up period. For p16 immunohistochemistry at the 1:10 dilution, the separation was much lower. All of these findings were reflected by the hazard ratios (and 95% confidence intervals) for overall survival presented in Table 3, supporting that only the 1:10 dilution had inferior stratification of overall survival.

Using the accelerated failure time model as the permutation test for undiluted against the 1:10 dilution, the difference in overall survival was highly significant ($p < 0.001$), favoring the undiluted staining as the better prognostic indicator. A similar comparison was intended between undiluted and 1:5 dilution p16 immunohistochemistry, but the low

number of discordances between the undiluted and the 1:5 dilution testing made that comparison impractical.

Discussion

Convincing evidence has accumulated that HPV status in oropharyngeal SCC is strongly and independently prognostic [1, 6, 8, 12, 17]. As p16 immunohistochemistry alone (or as a first test at least, before HPV specific testing) is now being recommended broadly across head and neck oncology for routine clinical practice testing [5, 11], the technical methods and staining interpretation need to be standardized. The soon to be published College of American Pathologists recommendations for HPV testing in head and neck cancer [18] will help to establish *when* testing should be performed and by *what* test, along with interpretation criteria; it does not extensively discuss the technical methods of assay performance. The current study was performed to better define some of the technical methods regarding p16

Fig. 2 Intensity differences between the p16 immunohistochemistry dilutions. This tumor has concordant results for all three dilutions using the $> 70\%$ nuclear and cytoplasmic staining cutoff. **a** Hematoxylin and eosin image of an OPSCC with moderate amounts of eosinophilic cytoplasm and no obvious keratin formation; **b** undiluted stain-

ing showing diffuse, strong intensity (3+) nuclear and cytoplasmic staining; **c** 1:5 dilution staining showing diffuse, moderate intensity (2+) nuclear and cytoplasmic staining; **d** 1:10 dilution staining showing diffuse, weak to moderate intensity (1+ to 2+) nuclear and cytoplasmic staining. (All images 10 $\times$ magnification)

immunohistochemistry using the E6H4 antibody, which, from extensive data, including a recently published head to head comparison of different clinically available p16 antibody clones [13], appears to be very well suited for oropharyngeal SCC. It also appears to perform better than other antibody clones.

There is no reason to believe that the “prediluted” or “ready to use” formulation of the E6H4 antibody, as provided by the manufacturer, is necessarily optimized for testing in oropharyngeal SCC as a prognostic marker and surrogate of transcriptionally-active high risk HPV. As such, research with suitable patient numbers and calibration to high risk HPV specific test results and to patient outcomes is needed. The current study was designed specifically for this purpose.

We found that p16 immunohistochemistry with the E6H4 clone in oropharyngeal SCC was functionally similar with dilution of the primary antibody solution up to 1:5. There were only three cases that were different between them (negative with the 1:5 dilution, positive with undiluted). Two of these three patients were HPV RNA-ISH positive and one

Table 3 Kaplan Meier overall survival analysis for high risk HPV mRNA status and p16 immunohistochemistry using the various dilutions

	Hazard ratio for overall survival	95% Confidence intervals	p value
High Risk HPV mRNA	0.203	0.117–0.352	<0.001
p16, Undiluted	0.267	0.153–0.466	<0.001
p16, 1:5 Dilution	0.216	0.122–0.383	<0.001
p16, 1:10 Dilution	0.340	0.192–0.603	<0.001

died of disease with distant metastases. Statistically, however, there is no significant difference between these p16 immunohistochemical results. The single finding that does argue in favor of the undiluted method over the two dilutions is staining intensity. The p16 staining is noticeably stronger for the undiluted staining compared to the 1:5 and 1:10 dilutions (Fig. 3). Given that it is the presence of nuclear and cytoplasmic staining in more than 70% of tumor cells that is the cutoff, slightly less intense staining may not be much

Fig. 3 Kaplan–Meier curves for overall survival for high risk HPV RNA in situ hybridization and p16 immunohistochemistry at the various dilutions. HPV human papillomavirus, RNA ribonucleic acid

of a concern, however. We did not identify any nonspecific or background staining problems with the p16 immunohistochemistry at any of the concentrations. The 1:10 dilution clearly proved suboptimal. Performance declined with many more “false negative” p16 results, poorer correlation with high risk HPV status, and less discrimination of outcomes between patients with positive and negative results.

Technical method studies such as this are expected to help shape the practices of laboratories and to help establish reference testing methods that can be used for proficiency testing, similar to other biomarkers such as receptor immunohistochemistry in breast cancer. Laboratories should seek to perform p16 immunostaining when it is recommended in routine practice and with methods shown in the literature to perform well in outcome stratification and in correlation with high quality HPV tests such as RNA-ISH or RT-PCR. Individual laboratories should not be expected to show direct correlation with patient outcomes themselves.

Our results suggest that a much greater number of p16 immunohistochemistry tests can be performed per volume of primary antibody solution. Some dilution of the primary antibody solution for E6H4 (up to 1:5) appears to be reasonable, if not optimal. Of course, while there is no agreed upon standardization for p16 immunohistochemistry in oropharyngeal SCC, and as of yet no proficiency testing, it is up to individual pathologists and laboratories to decide. It is our hope that such decisions will be made on scientific data such as in this work and others, rather than just based on corporate recommendations for the products.

The potential cost impact of using less E6H4 antibody solution is not entirely clear. It would result in savings on reagent costs for laboratories but wouldn't necessarily be directly passed on to patients. All of this depends greatly on how laboratories are funded and how they are reimbursed for the tests performed, which are highly variable. Further, it depends greatly on the cost of the antibody solutions. E6H4, in particular, is currently one of the more expensive antibodies, and this likely will remain the case or increase, particularly as it may become an FDA-approved clinical test in the future.

In summary, this study on optimal concentration of the E6H4 antibody in oropharyngeal SCC finds, in a well powered and characterized study population, that dilution of the primary antibody solution up to 1:5 on a Leica Bond platform with standard antigen retrieval and incubation times, still provides robust performance as a prognostic marker and surrogate marker of high risk HPV. The results suggest non-inferiority with dilution of the primary antibody solution when using the recommended 70% nuclear and cytoplasmic staining cutoff.

Acknowledgements We would like to thank Donna M. Posey for her wonderful assistance with clerical support and spreadsheet data

management for the various aspects of this study. We also acknowledge the Translational Pathology Shared Resource (TPSR) supported by NCI/NIH Cancer Center Support Grant 2P30 CA068485-14 and the Vanderbilt Mouse Metabolic Phenotyping Center Grant 5U24DK059637-13.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed Consent Use of participant data was exempt from individual consent (study waiver) under institutional review board approval.

References

1. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *New Engl J Med*. 2010;363:24–35.
2. Gillison ML, Restighini C. Anticipation of the impact of human papillomavirus on clinical decision making for the head and neck cancer patient. *Hematol/Oncol Clin North Am*. 2015;29:1045–60.
3. Gillison ML, Chaturvedi AK, Anderson WF, Fakhry C. Epidemiology of human papillomavirus-positive head and neck squamous cell carcinoma. *J Clin Oncol*. 2015;33:3235–42.
4. Huang SH, Patel S, O'Sullivan B, et al. Longer survival in patients with human papillomavirus-related head and neck cancer after positive postradiation planned neck dissection. *Head neck*. 2015;37:946–52.
5. Rischin D, Young RJ, Fisher R, et al. Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. *J Clin Oncol*. 2010;28:4142–8.
6. Benson E, Li R, Eisele D, Fakhry C. The clinical impact of HPV tumor status upon head and neck squamous cell carcinomas. *Oral Oncol*. 2014;50:565–74.
7. Lewis JS Jr p16 Immunohistochemistry as a standalone test for risk stratification in oropharyngeal squamous cell carcinoma. *Head Neck Pathol*. 2012;6(Suppl 1):S75–S82.
8. Sedghizadeh PP, Billington WD, Paxton D, et al. Is p16-positive oropharyngeal squamous cell carcinoma associated with favorable prognosis? A systematic review and meta-analysis. *Oral Oncol*. 2016;54:15–27.
9. Westra WH. The changing face of head and neck cancer in the 21st century: the impact of HPV on the epidemiology and pathology of oral cancer. *Head Neck Pathol*. 2009;3:78–81.
10. O'Sullivan B, Huang SH, Su J, et al. Development and validation of a staging system for HPV-related oropharyngeal cancer by the International Collaboration on Oropharyngeal cancer Network for Staging (ICON-S): a multicentre cohort study. *Lancet Oncol*. 2016;17:440–51.
11. O'Sullivan B, Lydiatt WM, Haughey BH, et al. HPV-Mediated (p16+) Oropharyngeal Cancer, In: Amin MB, editor. *AJCC cancer staging manual*. 8th edn. Cham: Springer; 2016.

12. Yom SS, Gillison ML, Trotti AM. Dose de-escalation in human papillomavirus-associated oropharyngeal cancer: first tracks on powder. *Int J Radiat Oncol Biol Phys*. 2015;93:986–8.
13. Shelton J, Purgina BM, Cipriani NA, et al. 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;9:1194–203.
14. 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. *Am J Surg Pathol*. 2010;34:1088–96.
15. 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. *Am J Surg Pathol*. 2011;35:1343–50.
16. Lewis JS Jr, 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;60:982–91.
17. Marur S, D'Souza G, Westra WH, Forastiere AA. HPV-associated head and neck cancer: a virus-related cancer epidemic. *Lancet Oncol*. 2010;11:781–9.
18. Lewis JS Jr, Beadle B, Bishop JA, et al. (2017) Human papillomavirus testing in head and neck carcinomas—Guideline from the College of American Pathologists. *Archiv Pathol Lab Med*. **(e-pub ahead of print)**.