p16 Immunohistochemistry As a Standalone Test for Risk Stratification in Oropharyngeal Squamous Cell Carcinoma

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Abstract It is widely acknowledged that human papillomavirus (HPV)-related oropharyngeal carcinoma is a biologically unique form of head and neck cancer that should be singled out and treated differently. It is now incumbent to find a test (or combination of tests) that accurately identifies cancers with the associated favorable prognosis for proper patient counseling and management and for placing patients in the correct treatment arms in the emerging clinical trials that are attempting to establish unique treatment types and approaches. The test (or combination of tests) that are utilized must be widely available, reliable, easy to interpret, and well-validated. While HPV-specific testing seems completely logical to use as a single test or one of a combination of tests, it turns out to be quite complicated in practice. Because of the different forms of the virus, the differing types of HPV-specific tests can give different information. HPV DNA, RNA, and protein assays have varying sensitivities for virus detection and also varying availability from formalin-fixed, paraffin-embedded tissue. Since p16 protein over expression is very sensitive for the presence of transcriptionally-active HPV and since it correlates strongly with patient outcomes, is widely available, and easy to interpret, it appears to currently be the single test that combines all of the desired attributes in a risk stratification marker for widespread implementation in clinical and research study settings. This article will review the literature on p16 immunohistochemistry and its relation to

HPV-specific testing, discuss some practical issues related to its implementation, and present the case for why it should be the single test used for this purpose.

Keywords Human papillomavirus · p16 immunohistochemistry · Squamous cell carcinoma · Oropharynx · Survival · Head and neck

Introduction

In recent years, it has become clear that the identification of high-risk human papillomavirus (HPV) in oropharyngeal squamous cell carcinoma (SCC) is critical [1], because HPV-related tumors have a unique biology and also have improved treatment responses and patient outcomes [2, 3]. Not only does knowledge of HPV status provide important prognostic information but, in the future, it may guide specific treatment decisions as well. As the current treatments for head and neck SCC have substantial morbidity, there is a call for treatment deintensification, specific treatment regimens, and targeted agents for these HPV-related cancers. However, specific methods for risk stratifying patients and for determining tumor HPV status that can be universally applied remain elusive.

HPV is a DNA virus that is present in oropharyngeal SCC either in integrated and/or episomal form and has classically been detected by DNA-based PCR [4]. DNA is, in many ways though, just the “carcass” or the “body” of the virus. It turns out that HPV DNA is found very widely in head and neck squamous dysplasia, SCC, in other cancer types, in benign lesions such as papillomas, and even in some clinically and histologically normal tissues [4]. When one simply detects HPV DNA, it doesn’t allow for quantification of the virus present nor for assessment of the activity or biological relevance it may have.

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Fig. 1 In-situ hybridization assays for high-risk human papillomavirus. **a** DNA in situ hybridization utilizing the Ventana Inform HPV assay showing positive staining with strong, punctate, *blue* nuclear signals in the tumor cells ($\times 400$ magnification). **b** RNA in situ

hybridization using the Advanced Cell Diagnostics RNAscope assay showing positive staining with extensive, granular, *brown* cytoplasmic and nuclear signals in the tumor cells ($\times 100$ magnification)

More recently, it has become clear that it is the transcriptionally-active form of HPV that is critical for clinical relevance. There are many tests that reflect (or at least correlate with) biologically or transcriptionally-active HPV, including DNA in situ hybridization (ISH) [2, 5] (Fig. 1a), RT-PCR for E6 and E7 mRNA [6–8], and more recently RNA ISH [9] (Fig. 1b). In addition, since the HPV protein E7 degrades the retinoblastoma protein leading to aberrant overexpression of p16, p16 immunohistochemistry (IHC) has been shown to be a sensitive test for the presence of transcriptionally-active HPV in oropharyngeal SCC [2, 5, 9] (Fig. 2).

There are many potential reasons to test a patient's oropharyngeal SCC for HPV or HPV surrogate markers, including for risk stratification and patient counseling, for specific treatment regimens appropriate to their tumor's biology, to identify the presence of HPV and its molecules for virus-specific targeted therapies, and to identify epidemiological trends. Currently, the major needs are for risk stratification, patient counseling, and for patient stratification for clinical trials of tailored therapy (such as deintensification of radiation or omission of chemotherapy, for example).

Many advocate p16 IHC as a screening test which, when positive, is to be followed by an HPV specific test such as DNA ISH, PCR, or both [1, 7, 10, 11]. There is an emerging view in oropharyngeal SCC, however, that the high correlation with detectable HPV RNA [9] and the strong risk stratification for patient survival provided by p16 IHC make it a suitable single marker for defining a

Fig. 2 Immunohistochemistry for p16 showing a positive case with diffuse, strong, nuclear, and cytoplasmic staining in the tumor cells ($\times 100$ magnification)

patient's tumor as biologically and clinically favorable for treatment and counseling. There are many who disagree with this view, though, insisting on the need for HPV-specific testing. This article reviews the data comparing p16 to HPV-specific tests and to combinations of tests, examines p16 in risk stratification in oropharyngeal SCC, and tries to make the case for why p16 IHC alone may be the best approach for addressing these current needs.

p16 Immunohistochemistry, HPV-Specific Tests, and Test Combinations

There is an extremely large amount of data looking at p16 IHC and HPV-specific testing in oropharyngeal SCC. Most of the studies are retrospective, but several prospective studies have been performed and analyzed as well [2, 3, 5, 9, 12]. There is very high correlation between the HPV-specific tests (of all types) [7, 9] and also between p16 immunohistochemistry and HPV-specific testing, particularly with the newer HPV RNA tests (Table 1) [2, 3, 5, 8–11, 13–15]. In a pooled analysis by Robinson et al. [1] of 496 patients from several studies utilizing DNA-based HPV testing, the authors reported that only 5% of cases were p16 positive/HPV negative and that 8% were p16 negative/HPV positive. More recent studies have utilized HPV E6/E7 mRNA testing and have found [7–9] only 1–7% of p16 positive tumors to be HPV RNA negative, and only 2–7% of p16 negative tumors to be HPV RNA positive. From all of this data, it is widely acknowledged that p16 overexpression is a very sensitive marker for transcriptionally-active HPV. However, despite the low rate of discrepant cases, the major critique of p16 IHC for use in any of the purposes mentioned above, is that it lacks specificity for the presence of transcriptionally-active HPV. The argument being that “a significant minority of p16 positive oropharyngeal SCC cases lack the virus, with p16 being overexpressed by other mechanisms [16].”

It has been suggested that screening patients’ tumors for p16 expression be performed first, and if positive, followed by an HPV-specific test, such as DNA ISH or DNA PCR (or both), for practical purposes [1, 7, 11]. The rationale is that this provides direct evidence that HPV is in the tumor and that, based on p16 overexpression, the HPV is

transcriptionally-active. Further, this strategy obviates the need for esoteric HPV RNA specific testing, which is currently expensive, complex, and quite limited in availability. What this approach fails to do, however, is to address those patients with discrepant results. Whatever form of testing is utilized, we know that some small fraction of results will be incorrect, either falsely positive or falsely negative. What about those patients who have p16 positive, HPV DNA testing negative tumors? Since DNA ISH lacks perfect sensitivity, and DNA PCR can also be falsely negative out of formalin-fixed, paraffin-embedded tissue, perhaps many of these patients still have cancers related to HPV and should still be regarded as having a “favorable prognosis.”

The concept that a minority of patients whose tumors are p16 positive actually lack HPV is probably partially driven by speculation on tumor biology but also likely by findings for p16 and HPV-specific tests in other cancer types. For example, in sinonasal undifferentiated carcinomas, most of the tumors overexpress p16 but none have been found to harbor HPV [17]. Similarly, in several types of lung non-small cell carcinoma, p16 is overexpressed in as many as 60% of cases, unassociated with the presence of HPV [18]. However, different cancer types and organ systems are biologically unique, so extrapolation to other tumors and subsites is not necessarily helpful or useful.

How could one actually define the potential entity of a highly p16 expressing but HPV completely absent oropharyngeal SCC? The only way to know definitively if a tumor is p16 positive but lacks HPV is to do extremely sensitive and reliable HPV testing for which negative results assure that no virus is present. This has never been effectively done. Assuming one did definitively identify p16 positive, HPV truly absent oropharyngeal SCC cases,

Table 1 Correlation rates between p16 immunohistochemistry and HPV-specific tests

Study	No. of p16 positive patients ^a	HPV DNA PCR	HPV DNA ISH	HPV DNA ISH followed by DNA PCR	HPV RNA RT-PCR	HPV RNA ISH
Dahlstrand et al. [14]	16	15 (93.8%)	NP	NP	NP	NP
Weinberger et al. [13]	18	17 (94.4%)	NP	NP	NP	NP
Reimers et al. [3]	29	25 (86.2%)	NP	NP	NP	NP
Ang et al. [2]	206	NP	192 (93.2%)	NP	NP	NP
Lewis Jr. et al. [5]	187	NP	139 (74.3%)	161 (86.1%)	NP	NP
Ukpo et al. [9]	148	NP	119/157 (75.8%)	NP	NP	147/148 (99.3%)
Schlecht et al. [8]	11	NP	6/10 (60%)	NP	10 (90.9%)	NP
Thavaraj et al. [10]	90	NP	75 (83.3%)	88 (97.8%)	NP	NP
Doxtader et al. [15]	25	NP	24 (96.0%)	NP	NP	NP
Totals	730	57/63 (90.4%)	555/675 (82.2%)	249/277 (89.9%)	10/11 (90.9%)	147/148 (99.3%)

NP not performed

^a Reflects the number of p16 positive patients for whom HPV-specific testing was available, not the overall number of p16 positive patients in each study. p16 positivity is defined as >70 or 75% positivity wherever it can be discerned from the study. When it cannot positivity is defined just by the binary cutoff used in the respective study

their biology and clinical behavior need to be defined. Given their rarity, this has been, to date, a practical impossibility.

p16 Immunohistochemistry, HPV-Specific Tests, and Patient Survival

It is widely discussed that there should be a “gold standard” for testing, and that this gold standard should be the presence of high risk HPV E6/E7 mRNA [7, 10]. While this is an attractive and popular concept, when it comes to patient risk stratification, it is somewhat misleading. While the presence of high risk HPV E6/E7 mRNA in a tumor is the ultimate way of showing that it harbors transcriptionally-active HPV, this doesn’t guarantee a favorable outcome. The true “gold standard” is actually patient survival. Whatever test or test combinations correlate best (or equally best) with survival should be utilized, whether they directly assess for the presence of high-risk HPV mRNA or not. RT-PCR for HPV mRNA is not perfectly sensitive for HPV, either. Although almost never discussed, how often are such assays falsely negative out of formalin-fixed, paraffin-embedded tumor specimens, particularly on small biopsies? Since many different HPV mRNA tests are not available at this time for cross validation studies, there is currently no data that addresses this question.

As previously mentioned, there is a wealth of data evaluating all forms of HPV-specific testing and p16 IHC in oropharyngeal SCC, and all tests consistently show strong correlation with patient survival. Among these, HPV RNA by RT-PCR, RNA by ISH, and p16 IHC have shown the best risk stratification [2, 5–7, 9]. For p16 expression in oropharyngeal SCC, virtually every study has shown strong differences in overall, disease-free, recurrence-free, and disease-specific survival, depending on the respective outcome measures provided in the particular study [2, 3, 5–7, 9, 12, 19]. This has been independent of treatment modality, either primary chemoradiation, induction chemotherapy followed by definitive radiotherapy, or primary surgery with or without postoperative chemo radiotherapy [2, 5, 9, 12, 19, 20]. Hazard ratios for overall, progression-

free, disease-free, and disease-specific survival for p16 positive versus negative cases are consistently low (Table 2) [2, 3, 5, 6, 12, 21]. Also, all major studies that performed multivariate survival analysis to control for other variables have found that positive p16 IHC independently correlates with better survival [2, 5, 6, 9, 12]. In particular, it is also prognostically independent of the histologic features of the SCC [22], such as keratinizing versus nonkeratinizing types (unpublished data), and, for most, if not all, specifically-defined SCC variants arising in the oropharynx, such as basaloid SCC [23], undifferentiated carcinoma [24], and adenosquamous carcinoma [25].

Focusing more on the practical question posed by combining tests, does adding HPV-specific testing to p16 IHC improve risk stratification? If so, does it do so in a biologically (and statistically) significant manner? So far, this has not been the case in any major study that has analyzed the data in a manner that addresses this question [5, 7, 9]. If there is a p16 positive, HPV truly absent cancer type and if it has an unfavorable biology, it should drag down the p16 positive tumor cohort survival relative to the cohorts for which HPV has actually been demonstrated or otherwise it is rare enough, or not prognostically different enough, to change the overall results. In the few studies comparing p16 IHC alone to p16 plus an HPV-specific test (and analyzing and presenting the data in a manner in which it can be evaluated), adding the HPV test has not significantly improved correlation with patient survival [5, 7, 9] or there have been problems with the methods, study cohorts, or conclusions [16].

RNA-based studies, such as RT-PCR and ISH are increasingly available. These are better than DNA-based tests and more data is emerging (and is expected to emerge) on their use. They offer the ability to assess tumors for transcriptionally-active HPV, but the emerging examples have not clearly shown that using them in isolation or adding them to p16 IHC results in improved correlation with patient survival. In a particular example, a very well performed study by Schache et al. [7], the investigators compared various HPV-related tests and test combinations, including quantitative RT-PCR for HPV E6/E7 mRNA, with each other for prediction of patient survival in an oropharyngeal SCC cohort (n = 108). They suggested that p16 results alone

Table 2 Hazard ratios for death or disease recurrence in the largest studies examining p16 immunohistochemistry alone for risk stratification in oropharyngeal squamous cell carcinoma

p16 cutoffs were those utilized in the respective study

NR not reported

	No. of patients	Overall survival	Progression free survival	Disease free survival
Weinberger et al. [21]	123	0.42 (0.2–0.9)	NR	0.36 (0.2–0.7)
Reimers et al. [3]	106	0.24 (0.05–1.05)	NR	0.13 (0.02–0.82)
Shi et al. [6]	111	0.42 (0.17–1.09)	NR	0.32 (0.16–0.66)
Ang et al. [2]	316	0.29 (0.20–0.43)	0.33 (0.24–0.46)	NR
Lewis Jr. et al. [5]	239	0.21 (0.12–0.36)	NR	NR
Rischin et al. [12]	185	0.36 (0.17–0.74)	0.39 (0.20–0.74)	NR

(70% cutoff) did not stratify patient risk as well as the combination of p16 and HPV mRNA or DNA detection. They concluded that “with the exception of p16 IHC or high-risk HPV DNA ISH in isolation, most of the other assays available provide a reasonable prognostic guide.” However, in the Kaplan–Meier estimates, the p-values for correlation with survival were very low for all tests and test combinations, and the differences in patient survival (both overall and disease-specific) between the p16 alone and p16 plus an HPV-specific test cohorts were less than 2 months. While the p-values and survival times were technically slightly worse for the cohort of p16-positive patients than for the HPV mRNA positive patients and the p16 positive and HPV mRNA positive patients, the magnitudes of the differences were extremely small, there was no formal presentation of the hazard ratios, and they did not specifically report on or analyze the p16 positive, HPV RNA negative patient cohort. Comparing two p-values to each other, as statisticians will tell you, is suspect, particularly if they are close to each other in magnitude [26]. So a single digit difference between two statistically significant p-values does not clearly support the position that one is better than the other. So, despite showing minor differences in outcome prediction, the contention that the combination of p16 with HPV-specific testing (of any of the other types) is better than p16 alone is not substantiated by the data [5]. It does raise the point that with improved and cross-validated RT-PCR, and other detection methods for HPV mRNA from formalin-fixed, paraffin-embedded specimens, they could very well become the ideal form of testing alone or in combination with p16.

RNA ISH is an example of a newer, and more user-friendly, HPV mRNA detection method. In a recent study of 196 patients with oropharyngeal SCC utilizing high-risk HPV RNA ISH, with a 75% cutoff for p16 IHC, it was found that the RNA ISH was positive in 99.3% (147 of 148) of the p16 positive cases, and positive in only 4 of the 41 (9%) p16 negative cases. Overall and disease-free survival were almost identical for the two tests, but were actually slightly better for p16 IHC alone [9]. The bottom line is that this compared tests which have a very high correlation with each other and with improved patient outcomes in oropharyngeal SCC. As such, it is very hard to make the case that one is better than the other for risk stratification, and further, it is very difficult to define the biology of the small cohorts of discrepant cases.

Technical Aspects and Practical Issues for p16 Immunohistochemistry

For p16 IHC, several practical issues arise for implementation. The first is which antibody type should be used and if this has an impact on its effectiveness and use as a

prognostic marker in oropharyngeal SCC. There are a few different monoclonal antibody clones available for use. The most commonly utilized is clone E6H4 (MTM Laboratories; Heidelberg, Germany) [2, 5, 10, 11], followed by other less commonly utilized clones such as 16P04 (NeoMarkers; Fremont, CA) [3], and JC8 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) [27]. While there has been no formal study comparing their use, studies utilizing all of these different antibodies have shown strong predictive value for their staining results [2, 3, 5, 27]. However, it may be of benefit to standardize the staining conditions across institutions for this test moving forward, and potentially to establish an FDA-approved version of the test.

The second issue relates to test interpretation. p16 IHC is typically very strong since it is nuclear and cytoplasmic. Most cases are either diffusely positive or completely negative while only a small subset of oropharyngeal SCC have partial p16 expression. From a large patient database at Washington University in St. Louis, 300 patients with oropharyngeal SCC have been tested for p16, and only 16 (5.3% of whole group and 6.8% of the 237 p16 positive cases) have positive staining in less than 75% of the cells (unpublished data). Although one study has reported a high number of partial p16 positive oropharyngeal SCC cases [8], the literature on p16 otherwise also supports that partial staining is uncommon [3, 5, 28]. However, specific (and detailed) data is difficult to garner from most of the individual studies.

The common question is “what cutoff should be used for p16 staining that correlates with transcriptionally-active HPV and with improved outcomes?” In the literature, very little direct evidence exists to answer this question. What cutoff of staining to utilize to call a tumor positive for p16 has a lot of indirect evidence, though, and also some recent direct evidence. In the many studies over the years, investigators have used any level of expression as positive but more often have used cutoffs of 50, 70, or 75% in order to binarily divide tumors as p16 positive versus negative. This has been based largely on the idea that when HPV is transcriptionally-active, E7 protein expression very effectively causes degradation of Rb protein, loss of suppression of p16 transcription, and thus very, very high levels of its expression (Figs. 2, 3). The vast majority of studies on p16 IHC that have shown high-risk stratification results and strong correlation with patient outcomes have used these “extensive expression” cutoffs [2, 3, 6, 11, 12].

A few studies appear to directly validate cutoffs of 50% or 75% as indicative of an HPV-related cancer. Schlecht et al. [8] compared extent of p16 staining to RT-PCR for high-risk HPV E6/E7 on fresh tissue and found that all but one of 11 cases with >75% p16 staining harbored viral RNA and only 1 of 14 cases with partial p16 staining in

Fig. 3 Immunohistochemistry for p16 showing a case with partial staining. Approximately 50 to 75% of the tumor cells have nuclear and cytoplasmic staining ($\times 100$ magnification)

<75% of tumor cells harbored viral RNA. In an analysis (accepted for publication) of 16 patients with partial p16 expression for whom we performed RT-PCR and RNA ISH for high-risk HPV E6/E7 mRNA on formalin-fixed, paraffin-embedded tissue, only 1 of 9 cases with <50% p16 staining harbored viral RNA (RNA ISH negative but RT-PCR positive with very low expression) while 3 of 5 cases with 50–75% p16 staining harbored viral RNA by both tests. The 2 cases with partial, but greater than 75% p16 staining both harbored viral RNA by both tests. As mentioned, from the larger oropharyngeal SCC patient database, of the 148 patients with >75% p16 expression, 147 (99%) were RNA ISH positive [9]. Again, these studies help to validate that, in oropharyngeal SCC, a cutoff of 50%, or better yet 75%, of tumor cells staining for p16 is required to correlate with the presence of transcriptionally-active HPV and improved patient outcomes.

The third practical issue regarding p16 IHC is cost. In an era of skyrocketing healthcare expenditures and rampant budget deficits, cost containment is important. Table 3 shows a comparison of technical costs for an average clinical laboratory and pathology practice for p16 IHC and for the various HPV-specific tests. The technical costs for all of the HPV-specific tests are from 2 to 16 times more per slide than for p16 IHC. To test all new cases of oropharyngeal SCC over 1 year in the United States alone, where an estimated 10,000 new cases of oropharyngeal SCC are diagnosed per year [29], adding HPV-specific testing to the p16 IHC positive cases (which would be estimated to represent approximately 75% of these 10,000 cases) could result in between \$412,500 and \$3,000,000 of extra expense just for the technical charges alone. If one also includes slide preparation costs and professional interpretation fees (for the ISH assays) and also considers

Table 3 Technical costs for p16 immunohistochemistry and various HPV-specific tests

Test	Technical cost per slide
p16 immunohistochemistry	\$25.00 ^a
HPV DNA PCR	\$400.00 ^b
HPV DNA In Situ Hybridization	\$55.00 ^c
HPV RNA In Situ Hybridization	\$157.00 ^d
HPV RTPCR for E6/E7 (research)	\$150.00 ^e

The provided figures are only estimated costs for the clinical laboratory at Barnes Jewish Hospital and Washington University in St. Louis, including for send out reference testing. Actual costs will vary by location, vendor, and contract

HPV human papillomavirus, PCR polymerase chain reaction, RT-PCR reverse transcriptase polymerase chain reaction

^a Estimate utilizing clone E6H4 on a Ventana Benchmark automated stainer

^b Estimate based on referral lab DNA PCR with genotyping

^c Estimate based on Ventana high-risk HPV cocktail assay performed on a Ventana Benchmark automated stainer

^d Estimate for testing using a cocktail of probes to seven major high risk HPV types

^e Based on average costs to perform testing in a research laboratory at Washington University in St. Louis

that rates of oropharyngeal SCC are continuing to increase annually, the costs would be much higher.

Summary

Amidst the epidemic of HPV-related oropharyngeal SCC, tests are needed for several purposes: for discerning favorable from unfavorable tumor types, for patient counseling about disease and prognosis, for treatment regimens appropriate to their tumor's biology, to identify the actual presence of HPV for virus-specific treatments, and to identify epidemiological trends. The most pressing of these needs is risk stratification. A consensus method for risk stratifying patients with oropharyngeal SCC is becoming critical. p16 IHC is emerging as a suitable single test for this purpose. It has been thoroughly studied, is widely available, inexpensive, and is easy to interpret, with clear guidelines on what staining cutoff to use. In fact, the 2011 National Comprehensive Cancer Network guidelines for head and neck cancer recommend p16 IHC testing alone for the purpose of counseling patients with oropharyngeal SCC about their prognosis (<http://www.nccn.org>) [30], and several large clinical trials have recently opened (such as RTOG-1016 and RTOG-0920) that are utilizing p16 IHC alone as the basis for patient inclusion or exclusion or for patient stratification. The choice to use p16 alone for trial entry or exclusion is a very important one which should not

be generalized, but which is better considered carefully by the planners on the details of the study. For other needs, particularly in the future, direct identification of HPV in tumors will be required. As HPV-specific tests, particularly RNA-based ones, become more widely available, clinically validated, and cheaper, they may replace p16 for this purpose, or be used in conjunction with it.

Considering the patients as a larger whole and considering the current clinical, practical, and financial factors for this issue, p16 IHC appears to be an excellent option as a standalone test. Future studies should be directed toward refining HPV-specific tests and identifying markers, particularly genetic ones, which can further substratify outcomes within the ever growing pool of p16 positive/HPV-related oropharyngeal SCC patients.

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