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Assessing p16 status of oropharyngeal squamous cell carcinoma by combined assessment of the number of cells stained and the confluence of p16 staining: a validation by clinical outcomes

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Abstract

Human papilloma virus (HPV) related oropharyngeal squamous cell carcinoma (OPSCC) has favorable prognosis relative to other head and neck squamous cell carcinomas. Criteria for predicting HPV status based upon p16 staining, including difficult cases with partial staining patterns, have been developed; however, clinical validation of these criteria and the clinical significance of partial p16 staining have not been reported. 81 archival OPSCC cases were initially stained for p16 by immunohistochemistry with clone G175-405. The percentage of p16+ cells and percentage of confluence of p16+ cells were categorized as 25%, 26-75% or >75%. Of all cases, 16 (20%) had partial p16 expression, with 26-75% p16+ cells. Applying previously developed criteria of >75% p16+ cells or >50% positive cells with >25% confluence, 48 (59%) patients were categorized p16+ and demonstrated expected clinical characteristics and superior disease-free survival (DFS) and overall survival ($p < 0.001$) compared to p16- patients. By themselves, the partial staining patients had intermediate outcomes however, separating the partial staining cases by degree of confluence showed those with >75% confluence had superior DFS ($p = 0.042$). When the 16 original partial staining cases were re-stained with the alternative anti-p16 E6H4 clone, p16 status remained concordant for all cases, but only 3 of the 16 were interpreted as demonstrating partial staining. This report shows the prevalence of partial p16 staining varies with the antibody utilized and clinically validates the application of a graded evaluation of both the number as well as confluence of positive cells for risk-stratification of patients with OPSCC.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

Keywords

human papillomavirus; oropharyngeal squamous cell carcinoma; p16 immunohistochemistry

Introduction

In recent decades, a variant of squamous cell carcinoma of the head and neck (HNSCC) has become apparent which has distinct clinical ramifications and is associated with high risk human papillomavirus (HPV). These HPV related oropharyngeal squamous cell carcinomas (OPSCC) have been shown to have a highly favorable clinical outcome despite an association with higher stage regional metastatic disease.(1-4) Epidemiologically, the incidence of HPV related OPSCC has been increasing at a striking rate.(5, 6)

Physicians differ on the appropriate methodology to diagnose HPV related OPSCC. Some favor in situ hybridization for viral RNA or DNA, while others offer evidence that immunohistochemical methods can serve as an efficient surrogate for direct detection of HPV.(7, 8) Carcinomas driven by HPV demonstrate overexpression of the p16 tumor suppressor protein in response to oncogenic loss of control of the cell cycle.(9) Therefore, p16 staining of tumor tissue by immunohistochemistry (IHC) has been presented as a surrogate marker for HPV infection. Interestingly, p16 positivity has also been shown to identify good prognosis OPSCC independent of HPV status.(8)

Interpretation of IHC staining for p16 in OPSCC is usually unequivocal, but a small proportion of cases may have partial staining.(10, 11) In order to set IHC staining thresholds for determining p16 positivity, and relating this positivity to high risk human papillomavirus, Lewis et al. have proposed >75% p16 positive cells or presence of >50% p16 positive cells with >25% confluence (where confluence is defined as groups of 10 contiguous cells) as a criteria for predicting positive HPV status based on correlation of these cutoffs with detection of transcriptionally active HPV.(10) However, clinical validation of the partial staining criteria or the clinical significance of partial p16 staining has yet to be reported. Herein, we show OPSCC cases with partial p16 staining have, as a group, intermediate clinical outcomes, between p16 positive and p16 negative cases, and this group can be appropriately risk stratified by further assessment of the confluence of staining. Additionally, the proportion of partial staining cases appears to be dependent upon the anti-p16 antibody clone utilized.

Materials and Methods

After obtaining Institutional Review Board approval, the University of Wisconsin Head and Neck Cancer Database was searched for consecutive patients with OPSCC treated with curative intent from 1990-2010. The oropharynx is defined here as the base of tongue or tonsil, the soft palate (SP) or adjacent posterior pharyngeal wall. Of these patients, 81 with archived tumor samples available at the University of Wisconsin Department of Pathology for review and additional p16 staining were included.

All 81 cases were studied by immunohistochemical staining performed on an automated platform (Ventana Medical Systems, Tucson AZ, USA) utilizing an antibody to p16 protein (BD Pharmingen, Purified mouse anti-human p16, Clone G175-405, 1:10 dilution) on archived formalin-fixed paraffin-embedded tissue from surgical specimens. Antigen retrieval was accomplished by incubation with Cell Conditioning 1 reagent (Ventana Medical Systems) at 100°C for 52 minutes. Cases that were classified as partial staining were additionally stained with CINtec® p16 Histology (Ventana Medical Systems, mouse anti-human p16, Clone E6H4, pre-diluted to an antibody concentration of 1.0 μg/mL and antigen retrieval with Cell Conditioning 1 reagent at 95°C for 44 minutes). Positive staining was defined as visual detection of nuclear and cytoplasmic staining, with nuclear positivity defined as any appreciable shade of oxidized 3,3-diaminobenzidine (DAB) beyond the baseline hematoxylin counter stain in the nuclei of neighboring non-tumor cells. The percentage of p16 positive cells were semi-quantitatively categorized into quartiles of ≤25%, 26-50%, 51-75%, and >75% by visual estimation at 200x magnification. Evaluation of confluence of p16 staining was introduced by Lewis et al(10) as a secondary criteria to determine which cases with 51-75% p16 positive cells harbored transcriptionally active HPV. In accordance to Lewis et al., confluence in this study was defined as groups of 10 contiguous cells demonstrating staining and quantitated as the overall percentage of p16 positive cells that were in confluent groups assessed for their proportion of the overall amount of p16 positive cells and semi-quantitatively categorized as ≤25%, 26-75% or >75%. p16 positive cases were defined as those demonstrating >75% staining or >50% staining with >25% confluence.(10) Assignment of cases into quartiles based on the number of positive cells and confluence was performed by consensus (S.B. and D.T.Y.).

Results of studies for p16 status were correlated with baseline clinical characteristics of the patients. Kaplan-Meier estimates of overall survival (OS) and disease-free survival (DFS) were calculated. Death was considered an event for OS with patients censored at the time of last follow-up. Any recurrence was considered an event for DFS calculation with patients censored at the time of death or last follow-up. The t-test was used to compare means (two-tailed) while the Chi-Square test was performed for categorical variables (two-sided). Log-rank test was used for univariate analyses and Cox Regression was performed for multivariate analysis. All statistical analyses were performed using IBM SPSS (Statistical Package for Social Sciences, version 22).

Results

Anti-p16 Clone G175-405

Table 1 demonstrates the baseline clinical characteristics for the patients, categorized by p16 status as evaluated by the criteria described by Lewis et al.(10) Median follow up of the patients was 3.4 years (maximum; 18.4 years). There were statistically significant differences in the age distribution, smoking status, T- and N- classification between the two cohorts of patients. p16 positive cases were associated with younger age, less smoking history, lower T-stage and higher N-stage at the time of diagnosis.

The categorization of cases by the number of p16 stained cells stained and percentage of confluence is shown in **Table 2**. The proportion of specimens that demonstrated 26-50% or

51-75% p16 positive cells were 5% and 15% respectively (16 cases total). These cases were combined to represent the partial p16 staining group. Correspondingly, cases with >75% p16 positive cells (**Figure 1A and B**) were deemed clearly p16 positive and those with ≤25% p16 positive cells (**Figure 1C and D**) were deemed clearly p16 negative. In terms of confluence of staining, 63% of cases showed >75% confluence and 30% showed ≤25% confluence. 7% (6 cases) demonstrated 26-75% confluence. Examples of partial p16 staining with the G175-405 clone are presented in **Figures 2-5**, alongside staining of the same cases with the E6H4 clone that is further discussed below.

Five-year DFS for the case cohorts with ≤25%, 26-75% and >75% p16 positive cells were 41%, 58%, and 81%, respectively ($p < 0.001$), with the partial staining cohort showing clinical outcomes that sit squarely between the good outcomes of the >75% cohort and the poor outcomes of the ≤25% cohort (**Figure 6A**). Interestingly, 5-year DFS for cases based on p16 confluence was 37%, 26% and 77% for the ≤25%, 26-75% or >75% cohorts, respectively ($p < 0.001$), suggesting that cases with 26-75% confluence had a similarly poor prognosis as those with <25% confluence (**Figure 6B**). Since >75% confluence appears to be associated with good prognosis, we attempted to risk stratify the 16 cases with partial staining (between 26-75% p16 positive cells) and intermediate outcomes (**Figure 6A**) by dichotomizing this cohort by whether or not they demonstrated >75% confluence (**Figure 6C**). We show that the partial staining cases with >75% confluence had significantly better DFS than those with <75% confluence ($p = 0.042$).

By applying the Lewis et al. criteria to the scored cases and categorizing cases as p16 positive if they demonstrated >75% p16 positive cells or >50% positive cells with >25% confluence, 48 (59%) patients were categorized as p16 positive and 33 (41%) as p16 negative (**Table 3**). The 2- and 5-year DFS and OS for p16 positive patients was significantly better than those categorized as p16 negative, respectively ($p < 0.001$). Alternatively, we also applied the criteria of >75% p16 positive cells or 26-75% positive cells with >75% confluence for identifying p16 positive cases and show 51 (63%) patients being categorized as p16 positive and 30 (37%) as negative using these criteria (**Table 3**). The 2- and 5-year DFS and OS for p16 positive patients was also significantly better than those labeled as p16 negative, respectively ($p < 0.001$).

On multivariate analysis for DFS, adjusting p16 status by baseline clinical characteristics, p16 status per Lewis criteria retained statistical significance (hazard ratio [HR] for failure in p16 negative 3.6; 95% confidence interval [CI]: 1.1-12.0, $p = 0.037$) while T-stage classification retained borderline significance (HR for failure in T1or T2 0.4, 95% CI 0.1-1.1, $p = 0.067$).

Anti-p16 Clone E6H4

The proportion of partial staining cases in this study (20%) was similar to that described by Chen et al.(11) (35%) who also utilized the G175-405 clone in their study. However, Lewis et al.(10), utilized the E6H4 clone and the proportion of partial staining was less than 4%, suggesting the G175-405 clone may be more susceptible to partial reactivity. Indeed, when we re-stained the 16 partial staining cases with the E6H4 clone, only 3 cases were

categorized as partially stained (**Table 4**). Final determination of p16 status showed perfect concordance between the two clones. Case 1 and 3 are examples where partial staining was found with both clones, indicated by patches of p16 positive cells found in a background of p16 negative cells (**Figure 2 and 3**). Because >75% of the positive cells in these cases were confluent, the cases were categorized as p16 positive. Case 12 (**Figure 4**) is an example where staining with clone G175-405 is interpreted as partial because in some areas, many of the tumor cells lacked nuclear staining (**Figure 4A**), but in other areas, tumor cells showed nuclear and cytoplasmic staining and >50% were confluent (**Figure 4B**). This same case stained with clone E6H4 clearly shows nuclear staining in of all tumor cells, thereby negating the dilemma of partial staining (**Figure 4C, D and Table 4**). Case 15 (**Figure 5**) is an example where staining with clone G175-405 is partial, showing scattered tumor cells with cytoplasmic and variable nuclear staining that are not confluent (**Figure 5A and B**) hence, the case is classified as p16 negative. However, no partial staining is evident with clone E6H4 (**Figure 5C and D**) and the case can be directly classified as p16 negative (**Table 4**).

Discussion

Evaluation of p16 staining on formalin-fixed paraffin-embedded tumor specimens to categorize OPSCC cases as p16 positive or negative is prognostically relevant. Clinicians routinely discuss p16 results with head and neck squamous cell carcinoma patients as they review anticipated treatment outcomes. However, determining whether cases are p16 positive or negative can be difficult in the small proportion of cases that demonstrate a partial staining pattern. Lewis et al. have shown that for these cases, the presence of >50% p16 positive cells with >25% confluence is associated with transcriptionally active HPV and recommend this criteria as a cutoff for defining p16 positivity.(10) We clinically validated this criteria in a retrospective cohort of 81 patients with OPSCC, 20% of whom demonstrated partial p16 staining, with between 26-75% of tumor cells demonstrating p16 expression after staining with the G175-405 clone. After dichotomizing all cases as p16 positive or negative based on Lewis et al., at a median follow up of 3.4 years, both DFS and OS were superior in p16 positive patients ($p < 0.001$) (**Table 3**). The clinical characteristics of the dichotomized patients were as expected, with the p16 positive patients tending to by younger with less smoking history and presenting with smaller primary tumors but more advanced regional metastases (**Table 1**).

As anticipated, disease-free survival for patients with >75% p16 positive cells in their tumors was superior to those with ≤25% p16 positive cells, but interestingly, the DFS curve for the partial staining cases with 26-75% positive cells fell between the other two curves (**Figure 6A**). This suggests that cases with partial p16 staining have intermediate outcomes and are likely comprised of a mixture of p16 positive and p16 negative patients. In accordance to Lewis at al., we assessed confluence of p16 staining as an additional discriminator.(10) After scoring the confluence of p16 staining in all 81 cases as <25%, 25-75%, or >75%, we show that cases with >75% confluence had superior outcomes compared to those with ≤75% confluence (**Figure 6B**). Surprisingly, the six cases with between 25-75% confluence did not have an intermediate outcome; rather, they had a 5-year

DFS of 26%, similar to that of the ≤25% confluence group (37% 5-year DFS). Accordingly, the results suggest >75% confluence is associated with superior outcomes; hence, it may be reasonable to consider partial p16 staining cases with >75% confluence as p16 positive. Indeed when the 16 partial staining cases are dichotomized by confluence, the cases with >75% confluence have superior DFS compared to those that do not (**Figure 6C**).

Overall, the findings support the notion that assessment of confluence of p16 staining can be a useful and clinically relevant discriminator of p16 status in cases with partial p16 staining. Based on our findings, we investigated the application of a modified discriminating criteria, defining p16 positive cases as >75% p16 positivity or 25-75% positivity with >75% confluence. Applying these criteria to the study cohort resulted in 51 patients being classified as p16 positive and 30 as negative, compared to 48 and 33, respectively, based on Lewis et al. (**Table 3**). The low number of partial staining cases and the challenge of overfitting limited our ability to compare the performance of the two methodologies, which will require additional studies on an independent patient cohort. However, 2 year DFS and OS in the p16 positive cases determined by the Lewis criteria were slightly higher than the modified criteria (**Table 3**), suggesting the modified criteria may not improve accuracy.

We also found that the proportion of partial staining is likely dependent upon the antibody clone utilized. The G175-405 clone generally showed weaker staining, especially in the tumor cell nuclei, similar to Chen et al's previous report.(11) The E6H4 clone showed more robust nuclear and cytoplasmic co-staining, boosting the number of p16 positive tumor cells to >75% in many of the same cases that showed <75% positive tumor cells with the G175-405 clone (**Table 4**). Of the 16 cases that showed partial staining with G175-405, only 3 showed partial staining with E6H4 and all three were deemed p16 positive based on almost 100% confluence of positive cells (**Figures 2 and 3**). Interestingly, E6H4 also appears to be more specific than G175-405, where G175-405 partial staining cases deemed p16 negative due to a lack of confluence were unequivocally p16 negative with hardly a single p16 positive cell when stained with E6H4 (**Table 4 and Figure 5**). In terms of final determination of p16 status (positive or negative) for the partial staining cases, no discordance between the clones was found (**Table 4**).

Caution needs to be used when assessing patchy p16 positivity in isolated cells. P16 over expression in isolated cells is a non-specific finding and irregular, scanty p16 staining in squamous cell carcinomas of the head and neck which occur *outside* of the oropharynx may not correlate with either HPV or a good prognosis. However, in the cervix(12) and in the oropharynx(10), strong, block-like immunohistochemical staining for p16 with more than 75% of positive cells stained in contiguity correlates well with presence of HPV.

In recent years, systematic evaluation of factors that influence outcome in oropharynx cancer patients has revealed several interesting findings. For example, each additional pack-year of tobacco smoking increases the risk of death. This factor serves to lower the favorable outcome for HPV-positive OPSCC patients who are heavy smokers quite close to the level of HPV-negative patients.(1) Although some have postulated that the introduction of intensity modulated radiation therapy (IMRT) in the early 2000's may be responsible for increasing tumor control rates in OPSCC, detailed studies suggest that other factors may explain this

outcome improvement (including the increased prevalence of HPV-positive tumors) as opposed to the use of IMRT.(13) These type of findings underscore the importance of detailed clinical outcome validation studies that carefully examine the relationship between new biomarkers, treatment techniques and population outcomes for individual cancers.

In summary, p16 positivity in OPSCC is associated with longer disease-free survival and the improved outcomes are likely related to carcinogenesis driven by high risk human papillomavirus.(1, 2, 4, 9) Criteria used to evaluate p16 immunohistochemical staining should include the percentage of cells stained as well as the confluence of staining, as the combination these criteria described by Lewis et al.(10), >75% p16+ cells or >50% positive cells with >25% confluence, can discriminate those cases with transcriptionally active HPV infection. Until now, these findings and the proposed criteria had not been validated by patient outcomes. We show that application of the criteria proposed by Lewis et al., or a modified version of the criteria, >75% p16 positivity or 25-75% positivity with >75% confluence, can effectively risk stratify a cohort of 81 OPSCC cases into two prognostically relevant p16 positive and p16 negative groups. We also show the proportion of partial staining cases is likely dependent upon the antibody clone utilized with many of the partial staining cases identified by clone G175-405 showing clear positivity or negativity when re-stained with clone E6H4.

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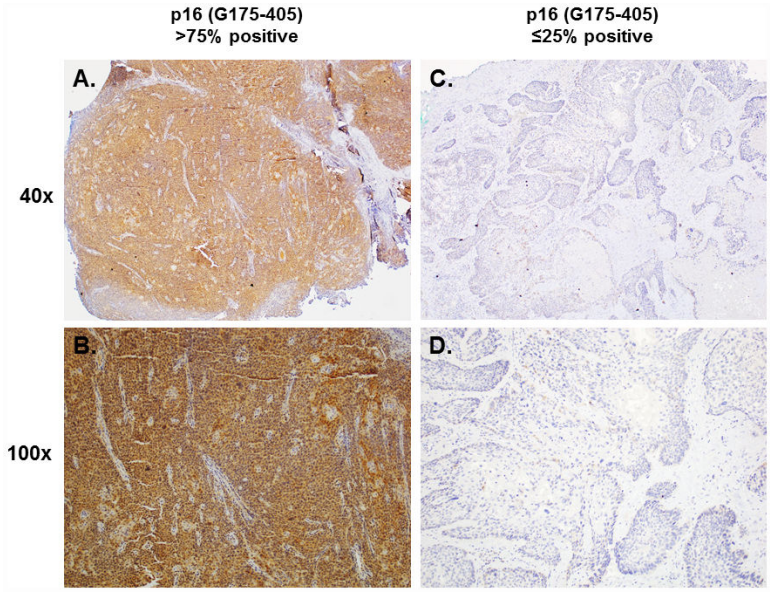

Figure 1.

Examples of cases that were not partially stained with anti-p16 clone G175-405. **A and B)** Example of a case categorized as p16 positive, demonstrating >75% p16 positive tumor cells with both nuclear and cytoplasmic p16 expression detected. **C and D)** Example of a case categorized as p16 negative, demonstrating ≤25% p16 positive tumor cells.

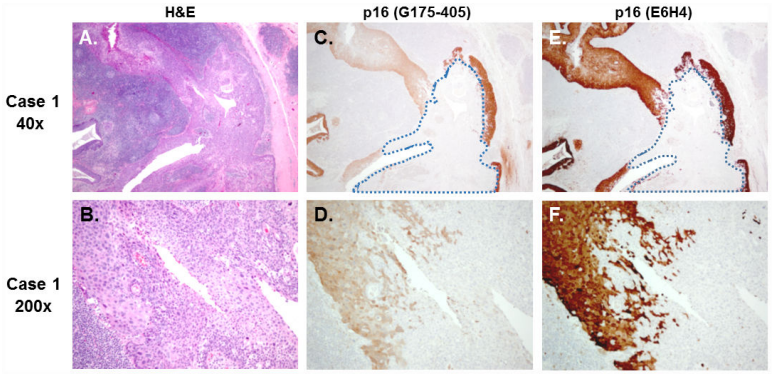

Figure 2.

Case 1, p16 positive. Partial p16 staining with both p16 clones and >75% confluence of positive cells. **A and B)** H&E stained sections at low and higher magnification showing extensive infiltration by squamous cell carcinoma. **C)** G175-405 clone showing patchy staining with 51-75% of tumor cells being positive (negative tumor outlined) and at high magnification, **D)** nearly 100% of the positive cells showing confluence. **E)** E6H4 clone showing relatively stronger staining, but a similarly patchy distribution with 51-75% of tumor cells being positive (negative tumor outlined) with 100% confluence at **F)** high magnification.

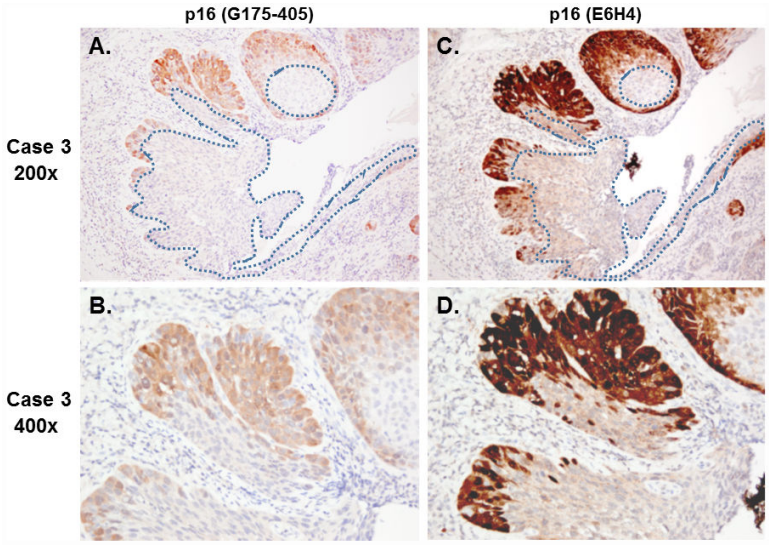

Figure 3.

Case 3, p16 positive. Partial p16 staining with both p16 clones and >75% confluence of positive cells. **A)** G175-405 clone showing 26-50% of tumor cells with nuclear and cytoplasmic p16 expression (negative tumor outlined) and **B)** >75% of the positive cells being confluent. **C)** E6H4 clone also showing 26-50% of tumor cells positive for p16 (negative tumor outlined) and **D)** >75% confluence of the positive cells.

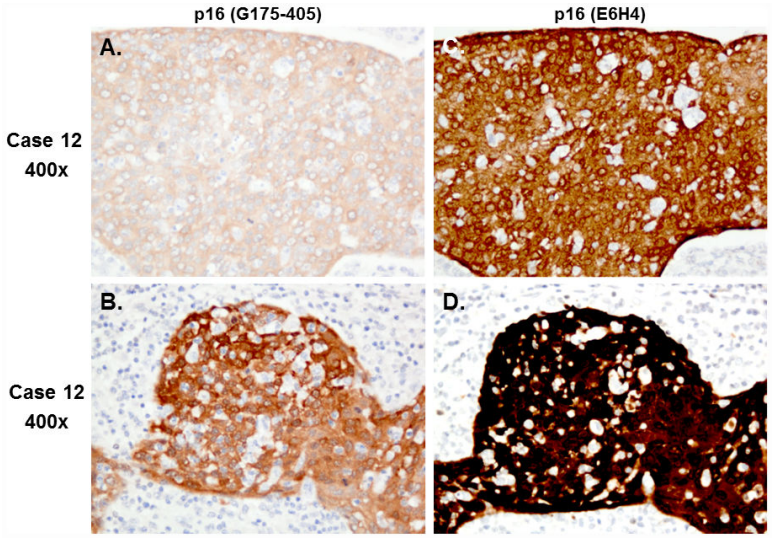

Figure 4.

Case 12, p16 positive. Partial staining with G175-405, but not E6H4. **A)** G175-405 clone showing patches of p16 negative tumor cells that demonstrate cytoplasmic but not nuclear staining, but also **B)** patches of p16 positive tumor cells with both cytoplasmic and nuclear staining with >75% confluence. **C and D)** E6H4 clone showing cytoplasmic and nuclear expression of p16 in >75% of tumor cells. Accordingly, the case is not considered partially stained, but simply p16 positive by E6H4.

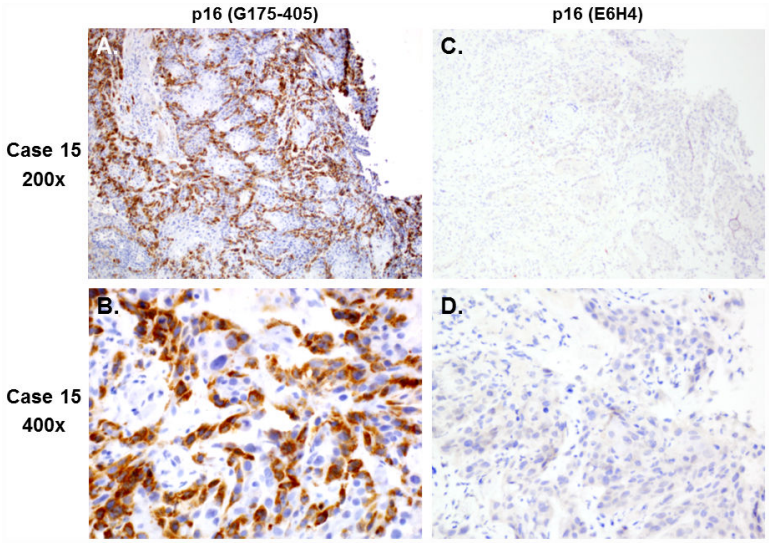

Figure 5.

Case 15, p16 negative. Partial staining with G175-405, but not E6H4. **A and B)** G175-405 clone showing 26-50% of tumor cells expressing cytoplasmic and variable nuclear p16, but the positive cells are scattered with <25% of the positive cells being confluent. Negative staining tumor is not outlined as the majority of the cells imaged are tumor cells**. C and D)** E6H4 clone showing no p16 expression in the tumor cells.

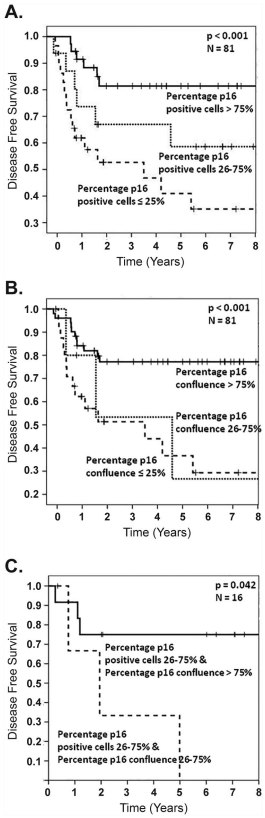

Figure 6.

Clinical outcomes associated with p16 interpretations. **A)** Disease free survival (DFS) by percentage of p16 positive cells. **B)** DFS by percentage of confluence of p16 positive cells. **C)** DFS of 16 cases with partial p16 staining (between 26-75% positive cells) dichotomized by degree of confluence.

Table 1

Baseline characteristics of the patients

Group (no.)	All (81)	p16 +*	p16 – *	p-value
Age (median; range)	58 years; 36-83 years	53.5 years; 36-83 years	62 years; 41-83 years	0.013
Gender (%)				
Male	57 (70)	35 (73%)	22 (67%)	
Female	24 (30)	13 (27%)	11 (33%)	0.5
Smoking (%)				
Non/Minimal smokers (< 10 PY)	13 (16%)	12 (25%)	1 (3%)	0.003
Moderate/ Heavy smokers	56 (69%)	26 (54%)	30 (91%)	(excluding not
Not known	12 (15%)	10 (21%)	2 (6%)	known)
Tumor-Site (%)				
Base-Tongue	30 (37%)	20 (42%)	10 (30%)	
Tonsil	42 (52%)	27 (56%)	15 (46%)	0.8
Soft-palate/pharyngeal wall	9 (11%)	1 (2%)	8 (24%)	(excluding SP/PPW)
Grade (%)				
Grade 1/2	33(41%)	19 (40%)	14 (43%)	
Grade 3	22 (27%)	14 (29%)	8 (24%)	
Not classified	26 (32%)	15 (31%)	11 (33%)	0.9
T-classification (%)				
T1/T2	61 (75%)	41 (85%)	20 (61%)	
T3/T4	20 (25%)	7 (15%)	13 (39%)	0.011
N-classification (%)				
N0/N1	29 (36%)	10 (21%)	19 (58%)	
N2/N3	52 (64%)	38 (79%)	14 (42%)	0.001
Treatment type (%)				
Definitive RT (± CT)	59 (73%)	37 (77%)	22 (67%)	
Post-operative RT (± CT)	22 (27%)	11 (23%)	11 (33%)	0.3

PY, pack-years; SP, soft-palate; PPW, posterior pharyngeal wall; RT, radiotherapy; CT, chemotherapy Grade 1 = well differentiated, Grade 2 = moderately well differentiated, Grade 3 = poorly differentiated

* p16 + or – by Lewis et al. criteria

Table 2

Categorization of cases by number of p16 positive cells and by percentage of confluence

	≤ 25%	26-50%	51-75%	> 75%
Categorization of cases by number of p16 positive cells	29 (36%)	4 (5%)	12 (15%)	36 (44%)
Categorization of cases by confluence of p16 positive cells	24 (30%)	5 (6%)	1 (1%)	51 (63%)

Table 3

Comparison of clinical outcomes by diagnostic criteria

	P16 Positive N (%)	P16 Negative N (%)	P16 Positive 2-/5- Year DFS, 2-/5- Year OS	P16 negative 2-/5- Year DFS, 2-/5- Year OS	Statistical significance (Log-Rank)
Lewis et al criteria: Positive when >75% or >50% positive AND > 25% confluence	48 (59%)	33 (41%)	85%/ 76%, 94%/ 80%	55%/ 39%, 66%/ 39%	p < 0.001 p < 0.001
Modified Criteria: Positive when >75% or 26-75% positive AND >75% confluence	51 (63%)	30 (37%)	82% 77%, 92%/ 76%	56%/ 34%, 66%/ 41%	p < 0.001 p < 0.001

DFS, disease free survival; OS, overall survival

Table 4

Comparison of p16 status and partial staining between two anti-p16 clones

	Case	Anti-pl6 G175-405	Anti-pl6 E6H4
pl6 positive	1	■	■
	2	■	■
	3	■	
	4	■	
	5	■	
	6	■	
	7	■	
	8	■	
	9	■	
	10	■	
	11	■	
	12	■	
pl6 negative	13	■	
	14	■	
	15	■	
	16	■	

■ partial staining