Published in final edited form as:

J Oral Pathol Med. 2016 July ; 45(6): 399–408. doi:10.1111/jop.12378.

A novel prediction model for human papillomavirus-associated oropharyngeal squamous cell carcinoma using p16 and subcellular β -catenin expression

Guoqing Qian¹, Zhongliang Hu^{1,5}, Hong Xu², Susan Müller³, Dongsheng Wang¹, Hongzheng Zhang¹, Sungjin Kim⁴, Zhengjia Chen⁴, Nabil F. Saba¹, Dong M. Shin¹, Andrew Y. Wang², and Zhuo Georgia Chen^{1,*}

¹ Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia, 30322, USA

² Ocean NanoTech, San Diego, California, 92126, USA

³ Department of Otolaryngology, Emory University School of Medicine, Atlanta, Georgia, 30322, USA

⁴ Department of Biostatistics and Bioinformatics, Emory University, Atlanta, Georgia, 30322, USA

⁵ Department of Pathology, Xiangya Hospital; Department of Pathology, Xiangya Medical School; Central South University, Changsha, Hunan, 410078, China

Abstract

BACKGROUND—p16 overexpression is a highly sensitive yet moderately specific biomarker for predicting human papillomavirus (HPV)-associated oropharyngeal squamous cell carcinoma (OPSCC). Nuclear β -catenin translocation has been linked to HPV positive OPSCC. However, whether the strategy of combining β -catenin with p16 can better predict HPV-associated OPSCC remains unknown.

METHODS—We evaluated the expression of p16 and β -catenin (nuclear and membrane) by immunohistochemistry staining in 101 OPSCC tissues and HPV status by HPV DNA *in situ* hybridization. Logistic regression models were used to evaluate single or multiple biomarkers for HPV prediction. The prediction power, sensitivity, and specificity were determined by receiver operating characteristic (ROC) analyses.

RESULTS—Our data showed that upon univariate analysis, p16 and nuclear β -catenin were positively correlated with HPV status while membrane β -catenin was inversely correlated with HPV status ($P < 0.01$). p16 showed the highest HPV predictive power, with area under the curve (AUC) of 0.9074 compared to 0.6762 for nuclear β -catenin and 0.7635 for membrane β -catenin,

*Correspondence: Zhuo G. Chen, PhD, Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia, 30322, USA. gzchen@emory.edu.

Disclosure/Conflict of Interest

No potential conflicts of interest were disclosed.

Author Contributions

Conceived and designed the experiments: ZGC AFW HX NFS DMS. Performed the experiments: GQ ZH HZ DW. Analyzed the data: ZH SM KS ZC. Wrote the paper: GQ.

respectively, indicating differential accuracies for HPV prediction. Multivariable analysis showed that p16 was significantly correlated with HPV while nuclear and membrane β -catenin showed marginal significance. The three-biomarker model was similarly sensitive (98.9% versus 100%) but more specific (88.9% versus 81%) than p16 alone, which also showed a good predictive value for overall ($P = 0.0002$) survival and disease-free ($P = 0.0158$) survival.

CONCLUSION—Our study suggests a novel model of combining p16 and subcellular β -catenin for prediction of HPV-associated OPSCC and this finding deserves further validation.

Keywords

HPV; p16; β -catenin; biomarker; model; head and neck cancer

Introduction

Approximately 70%-80% of oropharyngeal squamous cell carcinoma (OPSCC) cases in the U.S. are associated with the human papillomavirus (HPV), with the major subtype being HPV16 (1, 2). The incidence of HPV-associated OPSCC has increased over the last several decades. It is estimated that by 2020, HPV positive OPSCC will surpass cervical cancer as the most common HPV-associated cancer in the United States (2, 3). Increasing evidence shows that HPV-associated OPSCC is a distinct clinical entity that occurs mainly in younger patients who are less often active smokers and demonstrate better prognosis compared to their HPV-negative counterparts, due partially to increased sensitivity to chemotherapy and radiation therapy (4-9). Therefore, the detection of HPV-associated OPSCC has significant clinical implications such as selection of treatment plan and prediction of prognosis.

The HPV oncoproteins E6/E7 play an important role in HPV induced transformation of normal epithelial cells both *in vivo* and *in vitro*. Accumulated data has shown that E6 oncoprotein disrupts p53 synthesis leading to lower p53 protein levels, while E7 degrades the retinoblastoma (Rb) protein resulting in p16 overexpression (1, 10, 11). The abnormally upregulated expression of p16 is a potential substitute biomarker for HPV-associated OPSCC and has been implicated in the molecular classification of HPV-associated OPSCC (12). p16 has been shown to be an independent prognosis factor in OPSCC (13, 14) and although highly sensitive for predicting HPV-associated OPSCC, its specificity remains to be improved as compared to HPV DNA in situ hybridization, varying according to different studies between 76% and 82% (15-17). Identification and testing of potential biomarkers that can be used to improve HPV detection could potentially benefit clinical management of this malignancy.

The dysregulation of the Wnt/ β -catenin signaling pathway has been implicated in many human malignancies, including head and neck cancer (18, 19). β -Catenin is a multifunctional protein that interacts with cadherin complexes to promote cell-cell adhesion and functions as a transcriptional co-activator when entered into the nucleus, where it stimulates the expression of genes such as c-Myc, c-Jun, cyclin D1, and EGFR, leading to tumor proliferation, migration and invasion (20). E-cadherin and β -catenin seem to play a pivotal role in the development of head and neck cancer (21-23). An increased nuclear β -catenin expression has been noted in primary tonsillar cancers with HPV-positive status than

with HPV-negative ones (24). Our recent data shows that HPV E6 oncoprotein induced nuclear translocation of β -catenin, which may be mediated by the EGFR pathway (25). Using different transgenic mouse models, Bulut et al. has demonstrated that the activation of Wnt/ β -catenin enhances HPV-associated cervical cancer progression (26). Further evidence shows that the PDZ binding domain of the HPV16-E6 oncoprotein is essential for inducing nuclear β -catenin accumulation both *in vivo* and *in vitro* (27). However, whether β -catenin can be used as a standalone and/or complementary biomarker to p16 for predicting HPV-associated OPSCC remains unclear.

Currently there is no widely accepted “gold standard” for testing HPV associated OPSCC. The detection of high risk HPV E6/E7 mRNA in tumor tissues by HPV RNA in situ hybridization (ISH) or RT-PCR has gained increasing attention (28). These RNA-based tests, however, remain highly costly, complex, and not widely available in clinical practice (15, 16). Instead, HPV DNA ISH and RT-PCR for detection of HPV-associated OPSCC are more widely used nowadays. In this study, we aimed to test whether the combination of β -catenin (nuclear and membrane) with p16 detection by IHC could better predict HPV-associated OPSCC as compared to single marker. We examined different prediction models with single or multiple biomarkers and compared their predictive powers for HPV status (as determined by DNA ISH) in OPSCC. The overall survival (OS) and disease free survival (DFS) based on the selected model were also assessed.

Methods

OPSCC specimen collection

In this study, 101 primary tumor tissue specimens were obtained from patients diagnosed with OPSCC at the Emory University Hospital from 1994 to 2008. These patients received no prior treatment before surgical removal of the tumors or biopsy. This study was approved by the Institutional Review Board at Emory University and all of the analyses were conducted using de-identified data in compliance with Health Insurance Portability and Accountability Act (HIPAA). The clinical characteristics of the study patients are shown in Table 1.

HPV status confirmation by DNA in situ hybridization (ISH)

Formalin-fixed and paraffin-embedded specimens were evaluated for HPV DNA by the DNA ISH method. ISH was performed using the GenPoint Catalyzed Signal Amplification System (DAKO, Carpinteria, CA) according to the manufacturer's protocols. Two previously defined HPV-positive OPSCC cancer tissues were used as positive controls. This method is able to detect as few as 1-2 copies of HPV DNA, as described previously (29, 30).

Immunohistochemical (IHC) staining

Formalin-fixed, paraffin-embedded (FFPE) tumor tissues were used for IHC staining. Tumor sections were cut to a thickness of 3-4 μ m and stained for p16 and β -catenin using the ABC kit following the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Tumor sections were incubated with primary antibody against p16 (1:100, Delta Biolab, Gilroy, CA) or β -catenin (1:100, BD Pharmingen, San Jose, CA). The staining of the antibody was

observed by diaminobenzidine tetrahydrochloride peroxidase substrate solution (Vector Laboratories). Cell nuclei were counterstained by hematoxylin QS (Vector Laboratories). Immunoglobulin G (IgG) was used as the negative control. p16 positive was determined if > 70% of tumor cells stained with p16 specific antibody at nuclei and cytoplasm (31, 32). Nuclear and membrane β -catenin staining were evaluated separately because the translocation of β -catenin from membrane to nucleus has been related to HPV positivity as mentioned previously (25). Nuclear β -catenin staining was recorded as either negative (no staining) or positive (any level of staining). Membrane expression of β -catenin was evaluated as weighted index (WI = intensity (0, 1+, 2+, or 3+) $\times$ percentage of positive staining). Representative images of p16 and β -catenin staining are shown in Fig. 1.

Statistical analysis

Clinical characteristics including the expression of biomarkers were compared between HPV-positive and HPV-negative cases using chi-square test, Fisher's exact test or Wilcoxon rank-sum test wherever appropriate. A best predictive model was identified by entering all three biomarkers and covariates, only three biomarkers, or only covariates into a logistic model and using a backward variable selection method with an alpha level of removal at 0.1. Logistic regression models were used to estimate the effect of a single biomarker or multiple biomarkers on the prediction of HPV status.

The ability of a biomarker to predict HPV status was further determined by using receiver operating characteristic (ROC) curves and measuring the area under the curve (AUC). Chi-square tests were used to test whether the AUCs of ROC curves were different from 0.5 (no discrimination ability). The cut-off values to obtain 90% sensitivity and 90% specificity levels in the ROC curves were estimated. To obtain the optimal cut-off point for membrane β -catenin with the best discrimination power for HPV status, sensitivity and specificity pairs were obtained in the logistic regression analysis under all the possible thresholds; and the optimal cut-off point was calculated where the maximum sum of sensitivity and specificity was achieved. Membrane β -catenin was dichotomized by the optimal cut-off point, which maximized sum of sensitivity and specificity. The comparisons of AUC for different multivariable models were performed by chi-square test. The Hosmer-Lemeshow test was used to examine goodness-of-fit for models and a likelihood ratio test was used to compare different models. Overall survival (OS) and disease-free survival (DFS) were assessed by the Kaplan-Meier method and compared with the log-rank test.

All analyses were performed using SAS 9.3 software (SAS Institute, Inc., Cary, North Carolina). All statistical tests were 2-tailed and a *P*-value of less than 0.05 was considered statistically significant.

Results

Univariate analysis of the association of HPV status with clinical characteristics and biomarkers

Among 101 patients studied, 74 (73%) patients were determined to be HPV-positive and 27 (27%) patients were HPV-negative by HPV ISH (Table 1). p16 and nuclear β -catenin were

positively associated with HPV status ($P < 0.001$ and $P = 0.002$, respectively) while membrane β -catenin level was lower in HPV-positive than in HPV-negative tumors ($P < 0.001$). HPV-positive patients were more frequently non-smokers or former smokers ($P = 0.084$), with non-keratinizing tumor differentiation ($P = 0.007$), nodal metastasis ($P = 0.083$), and younger age ($P < 0.001$). Gender, race, tumor stage and stage were not associated with HPV status in our patient population.

Multivariable analysis of the association of HPV status with the three biomarkers, clinical characteristics, or both combined

A multivariable model after adjusting for only three biomarkers (p16, nuclear and membrane β -catenin) showed that p16 was a strong and significant predictor of HPV status ($P < 0.001$), with a hazard ratio (HR) (95% CI) of 381.21 (17.14 - 8476.34) (Table 2). Nuclear and membrane β -catenin were marginally significantly related to HPV status ($P = 0.089$ and 0.085 , respectively). The HRs (95% CIs) were 4.93 (0.78 - 31.02) and 0.98 (0.97 - 1.00) for nuclear and membrane β -catenin, respectively.

A multivariable model after adjusting for only covariates (age, smoking, differentiation status, race, stage, gender, nodal metastasis, tumor stage) showed that differentiation status and age were significant predictors of HPV status and smoking was marginally significantly related to HPV status (Table 3). HPV positive patients were more likely to be younger patients ($P = 0.020$) with non-keratinizing morphology ($P = 0.023$) and former or non-smokers ($P = 0.078$).

A multivariable model after adjusting for age, smoking, differentiation status, race, stage, gender, nodal metastasis, tumor stage, and the three biomarkers showed that nuclear β -catenin positivity ($P = 0.015$) and decrease in membrane β -catenin ($P < 0.001$) were significant predictors of HPV status, while younger age was marginally significantly related to HPV ($P = 0.048$) (Table 4).

Predictive power of single biomarkers and combined biomarkers (two or three biomarkers) for HPV status in OPSCC

All three biomarkers (p16, nuclear and membrane β -catenin) had significant predictive discriminatory powers for patients' HPV status ($P < 0.001$ for p16 and membrane β -catenin and $P = 0.002$ for nuclear β -catenin, Table 5). Among them, p16 had the strongest predictive discriminatory power (AUC = 0.9074; Table 5 and Fig. 2a). The AUC of the model with p16 alone was significantly larger than that with nuclear β -catenin alone ($P = 0.001$) or with membrane β -catenin alone ($P = 0.036$), but there was no significant difference between the AUC of models with nuclear or membrane β -catenin alone ($P = 0.274$).

p16 + nuclear β -catenin, p16 + membrane β -catenin, and the combination of all three biomarkers all had a higher predictive discrimination power than any single biomarker, as shown in AUC (Fig. 2a and 2b). The three biomarker model yielded the highest AUC as compared to other models. Since Table 2 suggests that all three biomarkers were significant predictors of HPV status, they were selected as the optimal model. The best predictive probability model for HPV status consisting of p16, nuclear and membrane β -catenin was as follows:

$$\hat{p} = \frac{\exp(0.4175 + 2.9717 \times P16Positive + 0.7976 \times \beta\text{cateninNuclearPositive} - 0.0168 \times \beta\text{cateninMem})}{1 + \exp(0.4175 + 2.9717 \times P16Positive + 0.7976 \times \beta\text{cateninNuclearPositive} - 0.0168 \times \beta\text{cateninMem})}$$

(Note: $\beta\text{cateninNuclear}$ indicates nuclear β -catenin and $\beta\text{cateninMem}$ indicates membrane β -catenin)

The predicted probability for a patient to have HPV positive OPSCC was higher than 50% when the cutoff value of $2.9717 \times \text{p16positive} + 0.7976 \times (\text{nuclear } \beta\text{-catenin positive}) - 0.0168 \times (\text{membrane } \beta\text{-catenin})$ was set at equal and higher than -0.172 (Table 6). The sensitivity and specificity of HPV prediction reached 98.6% and 88.9%, respectively, when the cutoff value of $2.9717 \times \text{p16 positive} + 0.7976 \times (\text{nuclear } \beta\text{-catenin positive}) - 0.0168 \times (\text{membrane } \beta\text{-catenin})$ was set at 0.264 (Table 6).

Moreover, the AUC of the model with all three biomarkers combined (best among the models with multiple biomarkers) was marginally significantly larger than that of the model with p16 alone (best among the models with a single biomarker) ($P = 0.057$).

Comparisons of three best predictive models for HPV status

The ROC curves for the three best predictive models of HPV status listed in Tables 2 through 4 are shown in Fig. 3. The AUC of the best predictive model adjusted for only the three biomarkers was significantly larger than that of the model adjusted for only the clinical characteristics ($P = 0.005$), while marginally significantly larger than the AUC of the model adjusted for three biomarkers and clinical characteristics ($P = 0.051$).

Kaplan–Meier estimates of OS and DFS using the model of p16 + $\beta\text{cateninNuclear}$ + $\beta\text{cateninMem}$

Using the three-biomarker model (p16 + nuclear and membrane β -catenin), the survival analysis showed that patients with high cutoff value (the cutoff value maximizing the sum of specificity and sensitivity: 0.264, Table 6) had significantly longer OS ($P = 0.0002$) and DFS ($P = 0.0158$) compared to those with low cutoff value (< 0.264) (Fig. 4a and 4b).

Discussion

HPV-associated OPSCC has drawn much clinical attention and discussion because of the distinct characteristics of this particular subgroup of disease, such as early onset, good response to chemotherapy and better prognosis. The cost-effective and less laborious prediction of HPV-associated OPSCC is, therefore, of significant clinical implication. In this study we demonstrated that the combination of p16, nuclear and membrane β -catenin had satisfactory power for HPV prediction in OPSCC patients, with sensitivity and specificity being 98.9% and 88.9%, respectively. The new model has two major advantages: first, it increases the specificity from 81% (p16 alone) to 88.9% (the three-marker model); second, p16 and β -catenin are downstream targets of HPV oncoproteins E7 and E6, respectively, and therefore are a good reflection of the biological changes induced by these two oncoproteins.

This model therefore may represent a possible novel approach for more reliably predicting the HPV status in OPSCC using FFPE specimens.

HPV-associated head and neck cancer depends on the biologic roles of *E6* and *E7* oncogenes, which inactivate p53 and RB, respectively, resulting in dysregulation of cell cycle and altered protein expression (10). Although downregulation of p53 has been biologically linked to HPV-associated OPSCC, our data demonstrated that the combination of p53 and p16 did not improve the specificity of HPV prediction compared to p16 alone (data not shown). Decreased RB upregulates p16 expression which is, thus far, the single best marker correlating with HPV status in OPSCC (15). p16 overexpression is strongly correlated with transcriptionally active HPV making it an attractive method of detection (33). In this study, p16 alone was found to be significantly correlated with HPV status. The observed p16 sensitivity (100%) and specificity (81%) are close to those reported in previous studies (Table 6). A study by Smeets *et al* reported that when using E6/E7 mRNA as a comparator gold standard for HPV positive status, p16 IHC is 100% sensitive and 79% specific (34). Singhi and Westra reported a sensitivity of 100% and a specificity of 82% (17). In addition, p16 as an independent prognostic factor was also confirmed in this study (data not shown). However, as illustrated, p16 overexpression is not very specific to HPV positive status (19% false positive in this case), and can also be found in HPV negative sinonasal undifferentiated carcinoma (35) as well as certain types of non-small cell lung cancer (36). Therefore, the non-specificity of p16 IHC for HPV testing remains a major hurdle for clinical application and calls for solutions (15, 16).

Several studies have demonstrated that nuclear translocation of β -catenin was associated with a positive HPV status (determined by real-time quantitative PCR) in human head and neck cancer specimens (24) and cell lines such as 147T and 090 cells (37). HPV E6 oncoprotein was implicated in the increase of nuclear β -catenin at least partially through degradation of PDZ-containing cellular targets (27). Membrane β -catenin functions mainly as cell-adhesion molecules and when the Wnt signal is on, stabilized β -catenin in the cytoplasm will transport to the nuclei where it binds to TCL/LEF and activates Wnt target gene expression. A recent publication indicates that the interaction between E6 and E6AP act together to stabilize β -catenin by protecting it from degradation in proteasome (38). Currently it remains unknown about the sensitivity and specificity of β -catenin for HPV prediction it is likely that variation exists if different HPV detection methods were used. In this study, we found that HPV was inversely correlated with membrane β -catenin expression and positively correlated with nuclear β -catenin accumulation. However, it needs to be noted that nuclear and membrane β -catenin alone only showed moderate sensitivity and specificity for HPV prediction (Table 6), possibly due to its non-specificity as a molecular marker. Therefore, we further tested whether the combination of β -catenin (nuclear and membranous) with p16 could increase specificity for HPV prediction.

Although all three biomarkers examined (p16, nuclear and membrane β -catenin) are significant predictors of HPV status (Table 2), we need to integrate them into a single mathematical model for better HPV prediction. To address this, a logistic regression model with all three biomarkers predicting HPV status was employed. The model constructed can be used to calculate the cutoff value for a specific probability that HPV is correctly

predicted. The ROC analysis, on the other hand, was used to calculate different sensitivities and specificities at different cutoff values. The model maximizing the sum of sensitivity and specificity has achieved a specificity of 88.9% (Table 6), which is significantly higher than that using p16 alone (81%). Importantly, the sensitivity was not obviously reduced (98.9% versus 100%). In comparison of different models, the model adjusting for three biomarkers only had a better prediction power than the model adjusting for both clinical covariates and three biomarkers (AUC = 0.962 versus 0.852, $P = 0.051$) (Fig. 3). One possibility exists that there are potential complicated interactions between clinical characteristics and p16 status. In addition, the model consisting of three biomarkers only showed significant prediction for patients' OS and DFS, further indicating the clinical relevance of this model.

Clinical characteristics such as age, smoking, differentiation status, and nodal metastasis were significantly correlated with HPV status as shown by univariate analysis (Table 1). Multivariable analysis showed that HPV positive OPSCC tends to occur in young non-smoker patients with non-keratinizing morphology (Table 3). This finding is consistent with previous reports (4, 6). Risk factors for HPV negative HNC patients includes tobacco use and alcohol drinking, leading to accumulation of mutation events over time, which may account for the older age in this population while in contrast, HPV positive patients are strongly related to a higher number of lifetime sex partners and not majorly on smoking (39) although an interplay between HPV and smoking may exist (40). Non-keratinizing phenotype is likely due to HPV oncogenes that may affect differentiation of epithelial cells (41), however, the exact molecular mechanism remains unclear. The prediction model adjusting for both clinical characteristics and biomarkers, however, did not give better prediction as mentioned above. In this model age was only marginally correlated with HPV in multivariate analysis indicating that as an independent risk factor age is weakly correlated with HPV, possibly due to dilution of the effect by other clinical covariates. For comparison purpose, the model adjusting for clinical characteristics only was also tested and not surprisingly, it turned out to be less powerful for HPV predication than the other models. A recent study has also demonstrated that the use of clinical characteristics only was not accurate for HPV prediction (42). Therefore, in this study we selected the model adjusting for biomarkers only for HPV predication.

It is noteworthy that the currently advocated method for HPV detection is the initial p16 IHC testing followed by an HPV specific test such as DNA PCR or ISH on the p16 positive cases (17, 28, 34). This method yielded reliable results on archival FFPE tumor specimens (43). However, HPV DNA PCR assays are sensitive assays that can be positive in "productive infection" (in which case the expression of E6 and E7 is confined to the differentiating layers of the squamous epithelium) as well as subject to laboratory artifacts, which has been well documented from cervical cancer screening by HPV testing (44, 45). On the other hand, the detection of transcriptionally active E6/E7 mRNA is a promising yet technically challenging and costly approach for both sensitive and specific HPV detection (16, 33). The application of RNA method to FFPE tissues has been proposed (46) but also needs further validation. The mere presence of HPV DNA is not indicative of a causal role of HPV (47), the perturbation of pRb cell cycle (e.g. p16) and other potential molecular targets (e.g., β -catenin or others), on the other hand, may be more functionally relevant to HPV-associated cancers.

A few limitations of this study include that the determination of sensitivity and specificity of the p16 IHC was based on clinically used HPV DNA ISH method rather than RNA ISH assay (33), RNA RT-PCR assay (34), or HPV DNA PCR mass-array (48). Compared to mRNA based methods, the specificity of p16 for HPV detection varies from 79% (34) to more than 90% (33, 49) while compared to the HPV DNA PCR mass-array method, p16 IHC has a specificity of 85.5% (48). Therefore, our current study may be considered as a pilot study in terms of predicting HPV-associated OPSCC. It is necessary to compare our model to the most specific and sensible HPV-tests with a large sample size to be conclusive. Second, the treatment was not taken into account for the analyses which may limit the interpretation of the survival analysis. Third, a large variation in stage was found in this study which may also affect the survival analysis, however, such an observation is consistent with other similar studies (12, 50), indicating that this finding could be characteristic of the disease in the site.

In summary, we demonstrated that the novel approach of combining p16 with nuclear and membrane β -catenin has a satisfactory prediction power for HPV-associated OPSCC. The three-marker model has showed increased power as compared to single markers and is of significant prognostic value for patient's outcomes. Further validation of the current model is warranted in different clinical settings and prospectively designed studies.

Acknowledgements

The authors thank Dr. Anthea Hammond for her editing of the manuscript. This study was supported by grants from HPV Small Business Innovation Research (SBIR) program Phase I and II Award (HHSN261201000125C and HHSN261201200097C, Overall PI: Wang AY), National Institutes of Health (R33 CA161873, PI: Chen ZG), and Georgia Cancer Coalition Distinguished Scholar Award to Dr. Chen ZG.

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Figure 1.

Representative images of p16 and β-catenin (nuclear and membrane) IHC staining and their overall percentages. (a-c) p16 expression levels with different HPV status. (d-g) Nuclear β-catenin staining with different HPV status. (h-k) Grading of membrane β-catenin staining intensity. Arrows indicate nuclear β-catenin staining. **Bar represents 100 μm.**

Figure 2.

Receiver operating characteristic (ROC) curves for HPV prediction using single or multiple biomarkers. **(a)** Single biomarker ROC curves of p16, nuclear β -catenin (BetacateninNuclear), and membrane β -catenin (BetacateninMem). **(b)** Multiple biomarker ROC curves of p16 + BetacateninNuclear, p16 + BetacateninMem, BetacateninNuclear + BetacateninMem, and p16 + BetacateninNuclear + BetacateninMem.

Figure 3. ROC curves of different models adjusting for only biomarkers, only clinical covariates, or biomarkers and clinical covariates.

Figure 4.

Kaplan–Meier estimates of overall survival and disease-free survival using the model of p16 + BetacateninNuclear + BetacateninMem. The cut-off value 0.264 was obtained by maximizing the sum of sensitivity and specificity. (a) Overall survival curve and (b) disease-free survival curve.

Table 1

Clinical characteristics and biomarker levels in study patients

		HPV Positive (N=74)	HPV Negative (N=27)	P-value
Age	Median (Range)	52 (37 - 72)	61 (32 - 77)	<.001
Gender	Male	64 (86.49)	21 (77.78)	0.357
	Female	10 (13.51)	6 (22.22)	
Race	Africa America	4 (5.41)	4 (14.81)	0.380
	Hispanic	1 (1.35)	1 (3.71)	
	White	59 (79.73)	19 (70.37)	
	Other	10 (13.51)	3 (11.11)	
Smoking	Never	18 (24.32)	3 (11.11)	0.084
	Former	31 (41.89)	8 (29.63)	
	Current	21 (28.38)	15 (55.55)	
	Unknown	4 (5.41)	1 (3.71)	
Differentiation status	Keratinizing	21 (28.38)	16 (59.25)	0.007
	Non- keratinizing	52 (70.27)	10 (37.04)	
	Unknown	1 (1.35)	1 (3.71)	
Tumor Stage	T1	25 (33.78)	10 (37.04)	0.576
	T2	31(41.89)	8 (29.63)	
	T3	4 (5.41)	3 (11.11)	
	T4	12 (16.22)	4 (14.81)	
	Unknown	2 (2.70)	2 (7.41)	
Nodal Metastasis	Negative	5 (6.76)	6 (22.23)	0.083
	Positive	64 (86.48)	19 (70.37)	
	Unknown	5 (6.76)	2 (7.40)	
Stage	I	1 (1.35)	1 (3.71)	0.524
	II	8 (10.81)	3 (11.11)	
	III	7 (9.46)	2 (7.40)	
	IV	57 (77.03)	19 (70.37)	
	Unknown	1 (1.35)	2 (7.41)	
p16	Negative	0 (0)	22 (81.48)	<.001
	Positive	74 (100)	5 (18.52)	
Nuclear β -catenin	Negative	26 (35.14)	19 (70.37)	0.002
	Positive	48 (64.86)	8 (29.63)	
Membrane β -catenin	Median (Range)	30 (0 - 136.5)	115 (0 - 219)	<.001

Data are presented as number of patients (%) or median (range).

* *P*-values were calculated by Wilcoxon rank-sum test for numerical covariates; and chi-square test or Fisher's exact test or categorical covariates, wherever appropriate.

Table 2

Best predictive model of HPV positivity after adjusting for the three biomarkers only

Biomarkers		Odds Ratio	95% CI low	95% CI high	P-value
p16 status	Positive	381.21	17.14	8476.34	<.001
	Negative	1 (Ref)			
Nuclear β -catenin	Positive	4.93	0.78	31.02	0.089
	Negative	1 (Ref)			
Membrane β -catenin		0.98	0.97	1.00	0.085

Hosmer-Lemeshow Goodness-of-Fit test statistic = 5.327; P -value = 0.722 (fitted model is an adequate model). Likelihood Ratio test statistic = 75.65; P -value < 0.0001 (The overall logistic regression model was significant). AUC (area under the Receiver Operating Characteristic (ROC) curve) = 0.935; P -value < 0.001.

Table 3

Best predictive model of HPV positivity after adjusting for clinical characteristics only

Clinical characteristics		Odds Ratio	95% CI low	95% CI high	P-value
Age		0.93	0.88	0.99	0.020
Differentiation status	Keratinizing	0.30	0.11	0.85	0.023
	Non-Keratinizing	1 (Ref)			
Smoking	Never	3.32	0.74	15.02	0.078
	Former	3.33	1.06	10.47	
	Current	1 (Ref)			

Hosmer-Lemeshow Goodness-of-Fit test statistic = 7.0293; P -value = 0.534 (fitted model is an adequate model). Likelihood Ratio test statistic = 19.35; P -value = 0.0007 (The overall logistic regression model was significant). AUC (area under the Receiver Operating Characteristic (ROC) curve) = 0.776; P -value < 0.001.

Table 4

Best predictive model of HPV positivity after adjusting for the three biomarkers and clinical characteristics

Clinical characteristics		Odds Ratio	95% CI low	95% CI high	P-value
Age		0.94	0.88	1	0.048
Nuclear β -catenin	Positive	4.31	1.33	13.91	0.015
	Negative	1 (Ref)			
Membrane β -catenin		0.98	0.97	0.99	<.001

Hosmer-Lemeshow Goodness-of-Fit test statistic = 11.707; P -value = 0.165 (fitted model is an adequate model). Likelihood Ratio test statistic = 40.22; P -value <.0001 (The overall logistic regression model was significant). AUC (area under the Receiver Operating Characteristic (ROC) curve) = 0.852; P -value < 0.001.

Table 5

Predictive accuracy for HPV status using single biomarkers

Biomarker	Level	Negative	Positive	Predicating accuracy
p16				AUC = 0.9074 [†]
	Positive	22 (81.48)	0 (0)	Sensitivity = 100%
	Negative	5 (18.52)	74 (100)	Specificity = 81%
				Overall accuracy = 95%
Nuclear β -catenin	Negative	19 (70.37)	26 (35.14)	AUC = 0.6762 [†]
	Positive	8 (29.63)	48 (64.86)	Sensitivity = 65%
				Specificity = 70%
				Overall accuracy = 66%
Membrane β -catenin				AUC = 0.7635 [†]
	113.979 *	14 (51.85)	3 (4.05)	Sensitivity = 96%
	< 113.979 *	13 (48.15)	71 (95.95)	Specificity = 52%
				Overall accuracy = 84%

Abbreviation: AUC, area under the Receiver Operating Characteristic (ROC) curve. Data are presented as number of patients (%).

* The cut-off point was the value of BetacateninMem, maximizing the sum of sensitivity and specificity.

[†] AUC of ROC curve is different from 0.5 with *P*-value < 0.05.

Table 6

Summary of cut-off points for different sensitivity and specificity with multiple biomarkers

Parameter	Sensitivity = 90%	Specificity = 90%	Maximize sum of specificity and sensitivity	50% to be HPV positive
Cut-off = $-3.1992 * \beta_{16Positive} + 0.8461 * \beta_{cateninNuclearPositive}$ [‡]	Specificity=81.5% Cut-off=-2.353	Sensitivity=100% Cut-off=2.353	Sensitivity=100% Specificity=81.5% Cut-off=2.353	Cut-off 2.353
Cut-off = $2.9981 * \beta_{16Positive} - 0.0203 * \beta_{cateninMem}$ [‡]	Specificity=88.9% Cut-off=1.217	Sensitivity=100.0% Cut-off= 0.235	Sensitivity=97.3% Specificity=88.9% Cut-off=0.69	Cut-off 0.164
Cut-off = $0.8078 * \beta_{cateninNuclearPositive} - 0.0236 * \beta_{cateninMem}$	Specificity=66.7% Cut-off=-1.93	Sensitivity=54.1% Cut-off=-0.728	Sensitivity=89.2% Specificity=66.7% Cut-off=-1.93	Cut-off -2.418
Cut-off = $2.9717 * \beta_{16Positive} + 0.7976 * \beta_{cateninNuclearPositive} - 0.0168 * \beta_{cateninMem}$	Specificity=88.9% Cut-off=1.043	Sensitivity=100% Cut-off=0.013	Sensitivity=98.6% Specificity=88.9% Cut-off=0.264	Cut-off -0.172

[‡] A logistic model was fitted using a penalized likelihood estimation method.