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Targeting Viral DNA and Promoter Hypermethylation in Salivary Rinses for Recurrent HPV-Positive Oropharyngeal Cancer

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Abstract

Objective.—The incidence and survivorship of human papillomavirus (HPV)–associated oropharyngeal squamous cell carcinoma (OPSCC) are increasing. Presence of HPV DNA and epigenetic alterations in salivary rinses are independently associated with clinical prognosis. We evaluated the utility of a combined panel in detecting disease recurrence during surveillance. We also assessed the assay's applicability in screening for HPV+OPSCC.

Study Design.—Retrospective cohort study.

Setting.—Two tertiary academic hospitals.

Subjects and Methods.—Forty-nine patients with posttreatment OPSCC were enrolled. Separately, 21 treatment-naive patients and 40 controls were included in the screening analysis. Salivary rinses were obtained from these cohorts and biomarker levels were quantified. Receiver

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Author Contributions

Sarek Shen, conceived, designed, and performed experiments, developed methodology, analyzed data, composed manuscript; **Yuki Saito**, conceived, designed, and performed experiments, developed methodology, edited manuscript; **Shuling Ren**, conceived, designed, and performed experiments, edited manuscript; **Chao Liu**, contributed materials, designed experiments, edited manuscript; **Theresa Guo**, contributed materials, designed experiments, acquired and interpreted clinical data, edited manuscript; **Jesse Qualliotine**, contributed materials, designed experiments, edited manuscript; **Zubair Khan**, contributed materials, acquired and interpreted clinical data, edited manuscript; **Sayed Sadat**, performed experiments, acquired and interpreted clinical data, edited manuscript; **Joseph A. Califano**, conceived and designed experiments, developed methodology, analyzed data, composed manuscript.

Disclosures

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Supplemental Material

Additional supporting information is available in the online version of the article.

operative characteristic (ROC) curves and multivariate logistic models were used to assess performance of biomarker combinations.

Results.—Eight patients (16.3%) in the posttreatment cohort developed locoregional recurrence. Recurrence was associated with alcohol use (odds ratio [OR], 6.12; 95% confidence interval [CI], 0.26–3.79) and advanced nodal disease (OR, 2.21; 95% CI, 1.52–3.01). A panel of HPV DNA and methylated *EDNRB* improved detection of recurrent disease (area under the curve [AUC], 0.88) compared to single markers (AUC, 0.69–0.78). Positive biomarkers preceded clinical detection by 2.4 ± 1.6 months and was associated with nearly 40-fold risk of recurrence (OR, 36.4; 95% CI, 1.15–45.22). Within the screening analysis, single biomarkers demonstrated moderate sensitivity and specificity (AUC, 0.59–0.83) in the detection of primary disease. A panel combining HPV DNA markers with methylated *EDNRB* and methylated *PAX5* improved AUC to 0.93.

Conclusion.—Detection of high-risk HPV DNA or aberrant hypermethylation in oral rinses is associated with presence and recurrence of OPSCC. Targeting both markers in saliva may have utility in long-term surveillance.

Keywords

oropharyngeal squamous cell carcinoma; human papillomavirus; HPV; saliva; promoter hypermethylation; epigenetics; screening; recurrence

Oropharyngeal squamous cell carcinoma (OPSCC) is a highly lethal cancer that affects over 12,000 people in the United States annually.[1] A distinct subset of OPSCC is associated with infection by high-risk subtypes of human papillomavirus (HPV). Despite a decline in smoking and alcohol consumption, increasing infection rates have contributed to the growing prevalence of OPSCC.[1,2] Even with advances in surgical technique and targeted chemoradiotherapy, recent studies have shown that approximately 50% of these patients will experience recurrence, often in a locoregional pattern.[3,4]

Earlier detection of recurrent tumors could have the potential to improve patient survival rates. Within OPSCC, salivary biomarkers have been widely explored as surrogate indications for premalignant or microscopic disease.[5] Prior efforts to characterize distinct molecular signatures of OPSCC have examined genomic and proteomic targets, including variations in microRNA (miRNA) expression, presence of cytokeratins, and changes in growth factor concentration.[6–8] A panel of salivary screening targets could complement physical examination in a clinical setting, aiding in earlier diagnosis.

Previous studies have identified hypermethylation of tumor-suppressor gene promoter regions as targets for early diagnosis of OPSCC.[9] The covalent addition of methyl groups to CpG islands in promoter regions is a widely studied mechanism of transcriptional regulation and heavily implicated in head and neck cancer oncogenesis. Several differentially methylated regions have been identified as specific biomarkers for OPSCC, including *p16(INK4a), PAX5*, and *EDNRB*.[10,11] A recent review of abnormal methylation markers revealed that salivary panels showed a pooled specificity of 0.89 (0.85–0.91) for 10 studies in the identification of head and neck cancer.[12] Hypermethylation of certain genes has also been shown to exhibit prognostic value in 5-year overall survival and disease-free survival.[10] It is evident that epigenetic modifications play a significant role in disease course; analyzing

these changes in conjunction with traditional HPV markers could improve understanding of longitudinal clinical presentation.

In this study, we describe the discriminatory ability of selected salivary biomarkers, individually and in panels. To assess OPSCC recurrence, salivary samples from posttreatment patients were analyzed for promoter methylation patterns and markers of high-risk HPV. By comparing treatment-naive to control samples, we also demonstrate the utility of these markers in screening for primary disease.

Methods

Patient Information

Saliva from 3 separate cohorts was collected. Posttreatment saliva samples were collected from 53 patients at Johns Hopkins University after primary surgery, chemotherapy, radiotherapy, or multimodality treatment. Samples were taken during postoperative clinical follow-up on a longitudinal basis in accordance with the approved institutional review board (IRB) protocol (#NA_00–36235). The number of samples collected was contingent on patients' adherence to follow-up, length of treatment, and date of treatment completion. Recurrence was determined based on documentation in clinical notes and presence of subsequent treatment. These patients were included in a retrospective cohort analysis, with recurrence as the primary event.

Twenty-one patients from the University of California, San Diego (UCSD) were included in the treatment-naive population. These patients presented with previously untreated primary OPSCC and enrolled in accordance with an IRB-approved UCSD protocol (IRB# 181755). These patients had confirmed primary OPSCC on immunohistopathology at the time of saliva collection. The control group comprised 40 patients enrolled in accordance with a predefined protocol at Johns Hopkins University (JHU).[13] Specimens were obtained from non-age-matched patients undergoing uvulopalatopharyngoplasty surgeries with HPV-negative pathology. These cohorts were included in a retrospective case-control analysis.

DNA Extraction

Salivary rinses were obtained brushing the oral cavity and oropharyngeal surface with an exfoliating brush. Patients then swished 15 mL normal saline and expectorated into a 50-mL conical tube. The saline rinse was centrifuged and the supernatant was discarded. The remaining pellet containing cellular material from the brushing was resuspended in 200 μL normal saline and frozen at −20°C.

Bisulfite Treatment

Unmethylated cytosines in salivary genomic DNA were converted to uracil using the EpiTect Bisulfite Kit (QIAGEN, Hilden, Germany). Then, 500 ng DNA was used as template for the reaction. The samples were eluted in 20 μL Buffer EB for quantitative methylation-specific polymerase chain reaction (PCR).

Quantitative PCR and Quantitative Methylation-Specific PCR

Primers for *PAX5, p16(INK4a),* and *EDNRB* were designed to include CpG dinucleotides in the promoter region, resulting in selective amplification of hypermethylated genes. Specific design parameters have previously been described by our group.[13] Quantitative methylation-specific PCR (QMSP) was performed using the Quant Studio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts). Each reaction used 20 ng bisulfite-converted DNA as template and was performed in triplicate. Bisulfite-converted leukocyte DNA from a healthy individual was used as a negative control; completely methylated DNA was used as a positive control and standard. The absolute copy numbers of methylated genes of interest were calculated. Beta-actin copy numbers were quantified to ensure DNA integrity for each sample.

Presence of high-risk HPV was determined using quantitative real-time PCR (qPCR) with highly selective primer-probe combinations (BR E1–5, HR E5L2–4, and E7) as previously described by our group.[14] Each reaction used 20 ng purified genomic DNA from saliva as template and was performed in triplicate. Reverse-transcribed full-length HPV genome was used as a standard.

For both QMSP and qPCR, a positive result was based on consistent detection in all triplicates. A combination of biomarkers was regarded as positive when at least one of a primer-probe set was positive. “Any HPV” indicates that at least a single HPV primer-probe set had a positive result.[15]

Statistical Analysis

Frequency and percentage, or mean and standard deviation, of participant characteristics were described for the treatment-naive and posttreatment cohort. Within the posttreatment cohort, patient characteristics were compared using Fisher’s exact test for categorical and binary variables and independent *t* tests for continuous variables.

The 3 HPV-associated markers and 3 methylated genes of interest were analyzed both as continuous and binary variables. The presence of targeted biomarkers was described in the 3 cohorts. Prevalence of the biomarkers was compared between the control group and the treatment-naive group, as well as between the recurrent and nonrecurrent patients in the posttreatment group, with Fisher’s exact test. A predictive model was built using logistic regression with subset selection based on Akaike information criteria (AIC). Additional predictive models were tested based on clinical hypotheses. Receiver operating characteristics and corresponding area under curve were used to compare varying combinations of tested biomarkers. The sensitivity and specificity of this model for both screening and detection of recurrence were calculated.

In the posttreatment cohort, univariate logistic regression was performed with presence of recurrence as the dependent variable. Multivariate analysis was performed with associated clinical variables, including age, smoking status, HPV status, T-classification, nodal classification, and treatment type incorporated into the model. All tests were 2-sided with statistical significance determined at a *P* value less than .05. Analyses were done using SPSS

(version 22.0; SPSS, Inc, an IBM Company, Chicago, Illinois) and R (Foundation for Statistical Computing, Vienna, Austria).

Results

Demographic and Clinical Characteristics of the Posttreatment Cohort

Forty-nine patients were included in the posttreatment cohort. Demographically, 95.9% (47/49) of patients were male, 98.0% (48/49) were white, 42.9% (21/49) of patients had a history of tobacco use, and 36.7% (18/49) endorsed alcohol consumption at the time of study. At presentation, most patients had American Joint Committee on Cancer (AJCC; seventh edition) stage IVa (69.4%, 34/49) and stage III (26.5%, 13/49) disease. Twenty patients (40.8%) received primary surgical treatment; within this group, 75% had adjuvant therapy, and 25% did not. Twenty-nine patients (59.2%) underwent chemoradiotherapy without primary resection.

Eight patients (16.3%) experienced recurrence of OPSCC within the study period. Median follow-up time was 41 months (interquartile range, 21–52). Advanced stage at presentation was associated with tumor recurrence. Patient age, sex, ethnicity, smoking status, alcohol use, tumor site, or HPV status did not vary significantly between patients with and without recurrence (Table 1).

Presence of HPV DNA and Promoter Methylation within Posttreatment Saliva Samples

Of the 8 patients who developed locoregional recurrence, 50% had methylated *PAX5*, 25% showed methylated *EDNRB*, and none exhibited methylated *p16(INK4a)*. HR E5L2–4 and E7 DNA were detected in 75% of recurrent patients; BR E1–5 DNA was identified in 25.0%. Other than BR E1–5, all biomarkers were detected at a significantly higher rate in patients who developed recurrence compared to those who did not (see Supplemental Table S1 in the online version of the article).

In differentiating patients who develop recurrence within the posttreatment cohort, HR E5L2–4 had the highest sensitivity (0.76) and specificity (0.81) (area under curve [AUC] = 0.78). Presence of *p16(INK4a)* was equal in recurrent and nonrecurrent patients (AUC = 0.50). Details for each biomarker are listed in Table 2.

Predictive models that combined multiple biomarkers were built using logistic regression. Variable selection was based on 2 approaches: best subset regression and clinical hypothesis. Combinations of an HPV marker with a methylated gene demonstrated improved sensitivity, specificity, and AUC compared to a single HPV marker or methylated gene alone. Notably, a panel of HR E5L2–4 and methylated *EDNRB* demonstrated a sensitivity of 0.90 and specificity of 0.81. The AUC of 0.88 was higher than any HPV or methylated gene alone.

We defined a variable “any HPV” as the presence of BR E1–5, HR E5L2–4, or E7 and repeated analysis. Using this “clinical hypothesis” model of variable selection, we describe the AUC, sensitivity, and specificity of the combinations. There were no significant differences in discriminatory ability among the combinations selected from best subset

regression compared to clinical hypothesis. The top 5 combinations for both methods are summarized in Table 2.

Multivariate Analysis of Variables Associated with Tumor Recurrence

In univariate logistic regression with recurrence as the dependent outcome, we found that alcohol use (odds ratio [OR], 6.05; 95% confidence interval [CI], 0.28–4.11; $P = .03$) and advanced nodal disease (OR, 2.25; 95% CI, 1.64–3.22; $P = .02$) were associated with increased risk of recurrence. Other clinical demographics, including age, sex, race, smoking status, tumor site, and HPV status were not significant. Patients with a positive salivary panel (HR E5L2–4, *EDNRB*) had an unadjusted OR of 22.10 (95% CI, 1.82–31.92; $P = .006$).

When controlling for age, sex, smoking history, and other clinical variables, patients with a positive salivary panel (HR E5L2–4, *EDNRB*) retained increased odds for developing recurrence (OR, 36.4; 95% CI, 1.15–45.22; $P = .01$). Patients with advanced nodal disease (N2/N3) had increased odds of developing recurrence as well (OR, 1.98; 95% CI, 1.31–4.13; $P = .04$) (Table 3).

Demographic and Clinical Characteristics of the Treatment-Naive and Control Cohorts

Twenty-one patients were included in the UCSD treatment-naive cohort. Mean age was 60.2 years, 71.4% (15/21) of patients were male, 90.4% (19/21) of patients were white, 47.7% (10/21) had a history of tobacco use, 9.5% (2/21) presented with early stage OPSCC (AJCC [seventh edition] stage I, stage II), 23.8% (5/21) presented with stage III disease, and the majority presented with stage IV disease (14/21) (Table 4).

Presence of HPV DNA and Promoter Methylation within Treatment-Naive and Control Saliva Samples

Overall, 76.1% of the UCSD treatment-naive cohort had at least 1 methylated gene; 90.5% had at least 1 positive HPV marker. *PAX5* was the most frequently methylated (71.4%), followed by *PAX5* (66.7%) and *p16(INK4a)* (19.0%). In the detection of HPV DNA, 80.9% of patient samples were positive for BR E1–5, 76.2% of samples had E7, and 66.7% of samples had HR E5L2–4. For the control group of saliva samples, we detected promoter methylation in 5 of the 40 patients (12.5%) and HPV DNA in 10%. All biomarkers were detected at a significantly higher rate in the treatment-naive cohort compared to the control; these results are summarized in Supplemental Table S2 (in the online version of the article).

In differentiating treatment-naive samples from controls, *EDNRB* had the highest combination of sensitivity (0.72) and specificity (0.95) (AUC = 0.83); *p16(INK4a)* had the lowest combination with a sensitivity of 0.17 and a specificity of 1.00 (AUC = 0.59). For HPV markers, BR E1–5 exhibited the strongest combination of sensitivity (0.60) and specificity (0.91) (AUC = 0.77); E7 had the lowest combination with a sensitivity of 0.57 and a specificity of 0.91 (AUC = 0.7). Details for each biomarker are summarized in Table 5.

Table 5 also tabulates combinations of biomarkers using both AIC criteria and clinical hypothesis for selection. Targeting both HPV and methylated genes was superior to any

single biomarker. From the subset analysis using AIC, a panel consisting of BR E1–5, HR E5L2–4, *EDNRB*, and *PAX5* had a sensitivity of 0.97 and specificity of 0.87 (AUC = 0.93).

Discussion

Given the increasing prevalence and high recurrence rate of HPV-associated OPSCC, there is a need for enhanced screening methods.[16] Here we show that targeting a combination of HPV-associated targets and aberrantly methylated promoter regions improves identification of high-risk patients. Using salivary samples obtained longitudinally from a posttreatment cohort, we demonstrated a combinatory panel reliably distinguishes patients with OPSCC who developed clinical recurrence. By comparing the salivary samples of patients with histologically confirmed OPSCC to controls, we also validated the combined panel's efficacy as a screening tool for primary disease. To our knowledge, this is the first report demonstrating the efficacy of a combined HPV and promoter hypermethylation panel for detection of primary and recurrent OPSCC.

Combining primer-probe sets targeting different segments of HPV has been shown to decrease false-negative rates in studies of salivary biomarkers in OPSCC.[17] However, the sensitivity of HPV detection alone is limited by variations in copy number, in adequate salivary rinses, or differences in viral sequences.[18–20] Indeed, we found that of the treatment-naive cohort, all of which had p16 immunohistochemistry (IHC)–confirmed biopsies, only 90.5% demonstrated positive HPV DNA in salivary rinses. Aberrant promoter hypermethylation has been proposed as an additional method for detection of OPSCC-specific tumor cells in saliva.[21] Identifying patients with these changes in promoter methylation status could capture patients with OPSCC with nonstandard high-risk HPV subtypes or decreased integration.

Methylation statuses of *EDNRB, PAX5*, and *p16(INK4a)* have been widely studied in OPSCC.[22,23] Hayashi et al[24] have demonstrated that hypermethylation of *EDNRB* is associated with increased risk of locoregional recurrence. Similarly, Pattani et al[25] found that the *EDNRB* promoter hypermethylation was linked to the presence of invasive OPSCC, independent of additional clinical covariates. *PAX5* is hypothesized to play a role in the balance of proliferation and differentiation signals; expression loss is seen in OPSCC,[23] as well as esophageal cancers.[26]

Detection of HPV DNA is saliva samples is a marker for recurrence among patients who have completed treatment.[27] Fakhry et al[16] recently demonstrated that persistent detection of viral DNA was associated with lower 2-year recurrence-free survival (adjusted hazard ratio [HR], 3.7). Indeed, we found 75.0% of patients who had return of disease had a positive HPV saliva sample during clinical follow-up, compared to 22.0% of patients who did not. When combined with a hypermethylated gene target, we found that 87.5% of patients had at least 1 positive biomarker. A combination of an HPV sequence (HR E5L2–4) with a hypermethylated gene (*EDNRB*) demonstrated an AUC of 0.88, significantly higher than any HPV marker or gene target alone. When controlling for patient parameters, including age, smoking status, and stage, a positive panel retained increased odds for developing recurrence (OR, 36.4; 95% CI, 1.15–45.22; $P = .01$). These findings corroborate prior cohort

studies by Rettig et al[28] and Ahn et al[29] demonstrating HPV DNA in posttreatment rinses were associated with an adjusted HR of 35.8 and 10.7, respectively.

Analysis of serial saliva samples in the posttreatment cohort demonstrated that first positive sample preceded clinical detection of recurrence by 2.4 months. Since our treatment-naive patients were drawn from a different cohort, we were unable to determine patient-level changes in biomarkers following treatment. However, the changes in HPV DNA and promoter methylation patterns between treatment-naive and recurrent patients are similar to those of prior studies.[16,27] The decrease in biomarker levels after treatment, followed by an increase in recurrent patients only, suggests persistent or recurrent microscopic disease that may not be detectable by routine surveillance examinations.

We further examined the utility of the combination assay in the detection of primary OPSCC. Presence of hypermethylated *EDNRB* and *PAX5* had high specificity (0.95 and 0.90) and moderate sensitivity in discriminating treatment-naive patients from control, which reflect previously reported values.[30] A combination of the methylated gene markers with HPV DNA targets HR E5L2–4 and BR E1–5 improved the sensitivity while maintaining specificity. In fact, this panel captured all 21 patients with tumors, significantly higher than sensitivities demonstrated by methylation aberration (0.24–0.35)[10] or HPV detection (0.77)[31] alone.

The conclusions of our study are limited by several factors. Our treatment-naive patients and posttreatment patients were recruited from different institutes, which restricts the conclusions we can draw on patient-level changes in biomarkers over time. Two of the 3 HPV-specific primers that we used were not from a standard toolkit. Although these primers have previously been shown to have comparable detection thresholds, weakness of standardization limits the generalizability of our findings. Furthermore, this method is unable to differentiate active HPV infection from integrated HPV DNA in tumor cells. The hypermethylation probes used did not explore the full promoter region, which may affect the sensitivity of the panel. Finally, this is an exploratory study in a small cohort of patients. Our findings will require further validation in an independent cohort.

Conclusion

Independently, detection of high-risk HPV DNA or aberrant promoter hypermethylation in saliva is associated with disease prognosis in OPSCC. Together, they are promising targets for monitoring disease recurrence during the follow-up period. With the increasing incidence and survivorship in OPSCC, these liquid biopsies may play a complementary role to physical exam in long-term patient surveillance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1.

Demographics of JHU Posttreatment Cohort Stratified by Recurrence during the Study Period.

Characteristic	Nonrecurrent (n = 41)	Recurrent (n = 8)	*P* Value[a]
Age, mean ± SD, y	57.1 ± 8.2	58.3 ± 11.3	.08
Male, No. (%)	39 (95.1)	8 (100.0)	.91
Race, No. (%)			1.00
White	40 (97.6)	8 (100.0)	
Nonwhite	1 (2.4)	0 (0.0)	
Smoking status, No. (%)			.06
Never	26 (63.4)	2 (25.0)	
Former	11 (26.8)	3 (37.5)	
Current	4 (9.8)	3 (37.5)	
Alcohol use, No. (%)	12 (29.2)	6 (75.0)	.04
AJCC seventh edition stage, No. (%)			.04
Stage I	0 (0.0)	0 (0.0)	
Stage II	0 (0.0)	1 (12.8)	
Stage III	12 (29.2)	1 (12.8)	
Stage IVa	28 (68.3)	6 (75.0)	
Stage IVb	1 (2.4)	0 (0.0)	
Treatment type, No. (%)			.07
Surgery alone	3 (7.3)	2 (25.0)	
Surgery with adjuvant treatment	12 (29.2)	3 (37.5)	
Chemotherapy, radiotherapy, or CRT	26 (63.4)	3 (37.5)	

Abbreviations: AJCC, American Joint Committee on Cancer; CRT, chemoradiotherapy; JHU, Johns Hopkins University.

[a]Continuous variable *P* values were performed by Student t-tests, and categorical and logistic variables were evaluated by χ^2 test or Fisher's exact test.

Table 2.

AUC, Sensitivity, and Specificity Data for Single Markers and Combined Panels in Detecting Recurrent Disease in Posttreatment Patients.[a]

Models	Recurrent Disease		
	AUC	Sensitivity	Specificity
Single biomarker			
BR E1–5	0.59	0.22	0.97
HR E5L2–4	0.78	0.76	0.81
E7	0.78	0.67	0.89
Any HPV	0.78	0.78	0.77
EDNRB	0.50	0.00	1.00
PAX5	0.71	0.51	0.93
p16(INK4a)	0.65	0.44	0.98
AIC criteria			
HR E5L2–4 + *EDNRB*	0.88	0.90	0.81
HR E5L2–4 + E7 + *EDNRB*	0.83	0.81	0.78
HR E5L2–4 + E7 + *EDNRB* + *PAX5*	0.82	0.86	0.72
HR E5L2–4 + *EDNRB* + *PAX5*	0.80	0.88	0.75
BR E1–5 + HR E5L2–4 + E7 + *EDNRB*	0.82	0.89	0.75
Clinical hypotheses with “any HPV”			
Any HPV + *EDNRB* + *PAX5*	0.82	0.89	0.77
Any HPV + *EDNRB*	0.82	0.81	0.73
Any HPV + *PAX5*	0.82	0.81	0.73
Any HPV + *p16(INK4a)*	0.80	0.78	0.76
Any HPV + any methylation	0.81	0.88	0.71

Abbreviations: AUC, area under the curve; HPV, human papillomavirus.

[a]Top logistic predictive models based on Akaike information criteria and clinical hypotheses involving combined “any HPV” marker.

Table 3.

Multivariable Logistic Analysis of Posttreatment Cohort for Predictors of Oropharyngeal Squamous Cell Carcinoma Recurrence.

Characteristic	Odds Ratio	95% CI	*P* Value
Age	1.02	−0.11 to 0.10	.48
Sex			
Female	Reference		
Male	0.15	−7.22 to 3.11	.21
Race			
White	Reference		
Nonwhite	0.01	NA[a]	.99
Smoking status			
Never	Reference		
Current/former	0.52	−4.11 to 1.93	.59
Alcohol use			
None	Reference		
Yes	1.67	−2.26 to 1.86	.68
T category			
T0	Reference		
T1	0.09	−2.31 to 4.53	.22
T2	0.80	−2.24 to 3.92	.78
T3	<0.001	NA[a]	.99
T4a	<0.001	NA[a]	.99
N category			
N0/N1	Reference		
N2/N3	1.98	1.31 to 4.13	.04
Treatment type			
Surgery alone	Reference		
Surgery with adjuvant	0.62	−1.36 to 2.22	.15
Chemotherapy, radiotherapy, or CRT	0.21	−2.01 to 0.42	.24
Biomarker panel			
Negative	Reference		

Characteristic	Odds Ratio	95% CI	*P* Value
Positive	36.4	1.15 to 45.22	.01

Abbreviations: CI, confidence interval; CRT, chemoradiotherapy; NA, not available.

[a] CI unavailable due to limited sample size.

Table 4.

Clinical Demographics of UCSD Treatment-Naive Oropharyngeal Squamous Cell Carcinoma Cohort.

Characteristic	UCSD Treatment Naive (n = 21)
Age, mean ± SD, y	60.2 ± 11.9
Male, No. (%)	15 (71.4)
Race, No. (%)	
White	19 (90.4)
Nonwhite	2 (9.6)
Smoking status, No. (%)	
Never	11 (52.4)
Former	9 (42.9)
Current	1 (4.8)
Alcohol use, No. (%)	12 (57.1)
AJCC seventh edition stage, No. (%)	
Stage I	1 (4.8)
Stage II	1 (4.8)
Stage III	5 (23.8)
Stage IVa	13 (61.9)
Stage IVb	1 (4.8)

Abbreviations: AJCC, American Joint Committee on Cancer; UCSD, University of California, San Diego.

Table 5.

AUC, Sensitivity, and Specificity Data for Single Markers and Combined Panels in Screening for Primary Disease.

Models	Primary Disease		
	AUC	Sensitivity	Specificity
Single biomarker			
BR E1–5	0.77	0.60	0.91
HR E5L2–4	0.76	0.52	1.00
E7	0.76	0.57	0.91
Any HPV	0.90	0.89	0.90
EDNRB	0.83	0.72	0.95
PAX5	0.78	0.70	0.91
p16(INK4a)	0.59	0.17	1.00
AIC criteria			
BR E1–5 + HR E5L2–4 + *EDNRB + PAX5*	0.93	0.97	0.87
BR E1–5 + HR E5L2–4 + *EDNRB + PAX5 + p16(INK4a)*	0.93	0.97	0.85
BR E1–5 + HR E5L2–4 + E7 + *EDNRB + PAX5*	0.91	0.97	0.85
All markers	0.93	0.97	0.85
BR E1–5 + *PAX5 + EDNRB*	0.91	1.00	0.85
Clinical hypotheses with “any HPV”			
Any HPV + *EDNRB + PAX5*	0.93	1.00	0.85
Any HPV + *EDNRB*	0.93	0.97	0.85
Any HPV + *PAX5*	0.89	0.97	0.90
Any HPV + *p16(INK4a)*	0.89	0.93	0.95
Any HPV + any methylation	0.87	1.00	0.85

Abbreviations: AUC, area under the curve; HPV, human papillomavirus.