IMPORTANCE Clinical trials that deintensify treatment for patients with suspected human papillomavirus (HPV)-positive oropharyngeal squamous cell carcinoma (OPSCC) use p16 expression to identify HPV-mediated tumors and guide treatment. While p16 staining has a strong correlation with good outcomes, approximately 12% of p16-positive patients have recurrent disease. Biomarkers that reveal tumor-specific characteristics, such as nodal involvement, may change therapy decisions.

OBJECTIVE To assess whether if a tumor-specific genetic signature exists for node-negative vs node-positive HPV 16-positive/p16-positive OPSCCs.

DESIGN, SETTING, AND PARTICIPANTS This was a retrospective cohort study with randomized case selection for p16 OPSCCs undertaken at a university-based, tertiary care cancer center. Samples were collected from patients with p16-positive OPSCC. A total of 21 HPV 16/p16-positive tumors were used in this study.

MAIN OUTCOMES AND MEASURES Gene expression profiles of node-negative vs node-positive tumor samples were evaluated using a differential expression analysis approach and the sensitivity and specificity of a molecular signature was determined.

RESULTS Among the 21 patients in the study (3 women, 18 men; mean [SD] age, 54.6 [9.6] years), 6 had node-negative disease and 15 had node-positive disease. Using differential expression analysis, we found 146 genes that were significantly different in patients with node-negative disease vs those with node-positive disease, of which 15 genes were used to create a genetic signature that could distinguish node-negative-like from node-positive-like disease. The resultant molecular signature has a sensitivity of 88.2% (95% CI, 63.6%-98.5%) and specificity of 85.7% (95% CI, 42.1%-99.6%). The positive likelihood ratio of this signature was 6.1 (95% CI, 1.0-38.2) and the negative likelihood ratio was 0.1 (95% CI, 0.04-0.5). Given this population's prevalence of node-positive disease of 70.8%, the positive- and negative-predictive values for this gene signature were 93.7% (95% CI, 70.8%-98.9%) and 75.0% (95% CI, 44.1%-92.0%), respectively. In addition, we developed a gene signature using agnostic, machine learning software that identified a 40-gene profile that predicts node-negative disease from node-positive disease (area under the curve, 0.93; 95% CI, 0.63-1.00).

CONCLUSIONS AND RELEVANCE Many HPV-16 and p16-positive tumors are treated as “lower-risk,” but they do not have similar genetic compositions at the biological level. The identification of subgroups with unique expression patterns, such as those with nodal metastases, may guide physicians toward alternative or more aggressive therapies. In our study, unguided clustering suggested that that the larger biological characteristics of a tumor could be a better prognostic biomarker.
Oropharyngeal squamous cell carcinoma (OPSCC) is now one of the most common cancers of the upper aerodigestive tract, and the rising incidence can primarily be attributed to human papillomavirus (HPV) infection.\textsuperscript{1-3} HPV-driven OPSCC is an epidemiologically and molecularly distinct subset of oropharyngeal cancer.\textsuperscript{4,5} It is associated with the overexpression of the tumor suppressor protein p16 and has markedly better outcomes than non–HPV-driven OPSCC.\textsuperscript{6-8} The HPV oncoproteins E6 and E7 mediate the degradation of the cellular checkpoint proteins p53 and pRB, respectively. The degradation of pRB by E7 results in unregulated p16 expression and has led to the correlation of elevated p16 expression with HPV-positive tumors. This, combined with the ease and inexpensive nature of p16 testing, has led many to recommend the use of p16 expression as a surrogate marker for HPV positivity in OPSCC.\textsuperscript{9-11}

Since HPV-driven OPSCC is associated with markedly better outcomes, multiple trials have been conducted exploring either primary surgical resection or radiotherapy (RT) desescalation for patients with p16-positive malignant neoplasms in an attempt to decrease the toxic effect profile of current standard of care regimens.\textsuperscript{12,13} Despite overall good outcomes, approximately 10% to 15% of p16-positive tumors have consistently failed radiotherapy,\textsuperscript{14} which may stem from radiation resistance pathways\textsuperscript{15} or misdiagnosis of HPV-driven disease, possibly reflecting molecular heterogeneity among p16-positive tumors that is not well understood.\textsuperscript{16} In addition, several studies have demonstrated that primary transoral robotic surgery is effective in selected patient groups, suggesting that a diagnostic for risk-stratifying patients for different treatment modalities could be useful.\textsuperscript{13,17,18} Thus, while p16 staining in OPSCC biopsy specimens has a very high sensitivity for detecting HPV-positive disease, its specificity is a major drawback.\textsuperscript{19}

Dependence of treatment decisions on p16 staining alone may also lead to false identification of a purely HPV-driven process.\textsuperscript{20} For instance, elevated p16 expression can be seen outside of HPV-positive disease, such as in HPV-negative OPSCC tumors\textsuperscript{21} and adenoid cystic carcinoma of the trachea.\textsuperscript{12} Our group and others have published studies on the prognostic significance of p16 overexpression alone in OPSCC\textsuperscript{1-7,13-14} and adenoid cystic carcinoma.\textsuperscript{12} However, aberrant p16 expression in carcinogen-driven (ie, HPV-negative) and non–HPV-16–driven tumors harboring other HPV types\textsuperscript{22} highlights the need for higher specificity, especially when one considers up and coming modifications to standard of care regimens, such as RT deintensification. Furthermore, the ability to successfully identify more aggressive p16-positive tumor subtypes before treatment may lead to treatment escalation and avoidance of recurrence owing to improper deintensification.

Presently, there is not a diagnostic molecular biomarker available that uses both tumor RNA genetics and HPV viral RNA expression to detect p16-positive OPSCCs subtypes. The identification of tumor-specific prognostic gene expression signatures at initial clinical staging could aid in the selection of a directed, patient-specific treatment modality, identify which patients are at risk for recurrent disease, or predict occult lymph node (LN) involvement, which is present in 20% of all OPSCCs.\textsuperscript{21} Furthermore, knowing a tumor’s likelihood to fail RT deintensification at initial clinical staging or following surgery could aid surgical and medical management both preoperatively and postoperatively.

In this study, we analyzed the gene expression levels of 770 oncologic or immunoregulatory genes in banked OPSCC tumor samples and combined the resulting data with DNA sequencing reads for HPV DNA and E6/E7 messenger RNA (mRNA) expression to identify unique oncoimmune phenotypes in p16-positive patients with N0, N1, or N2 disease according to the American Joint Committee on Cancer Staging Manual (AJCC), eighth edition, nodal criteria.\textsuperscript{22} We hypothesized that key tumor gene expression changes vary by degree of LN involvement, and thereby we attempted to develop a tumor immunophenotype by nodal status.

**Methods**

**Biospecimen Identification and Procurement**

The institutional review board and office of human research ethics at the University of North Carolina at Chapel Hill approved the study protocol, and written informed consent was obtained from patients who were treated for oropharyngeal squamous cell carcinoma. The tissue specimens were collected and stored under the Lineberger Comprehensive Cancer Care Center (LCCC) UNCseq initiative. Using a secondary approved protocol, banked, fresh-frozen, paraffin-embedded (FFPE) tumor tissue was procured from 21 of 48 total banked specimens from patients with a diagnosis of OPSCC, confirmed p16-positive immunohistochemical results, and HPV-16–positive DNA status. The 21 OPSCC cases with primary site biopsy were selected according to tumor block availability. Fourteen cases were excluded owing to insufficient tumor specimens available, along with 3 additional cases that were found to be non–HPV-16 genotype. Tumor blocks were sectioned into five 10-μm sections by the Tissue Pathology Core Facility at UNC LCCC and FFPE sections and were then placed onto glass slides. For each tumor, 1 hematoxylin-eosin-stained slide was prepared prior to nucleic acid harvest, analyzed, and marked by a board-certified pathologist to identify the tumor from nontumor prior to macrodissection of each sample.
Clinical Data Elements

Clinical data were collected for all specimens and included primary oropharyngeal subsite, sex, age at diagnosis, race/ethnicity, tumor grade, smoking history, and HPV status. Tumor, Node, and Metastasis (TNM) staging components were confirmed for all cases in the study. A compiled tumor stage using the standard AJCC, eighth edition, staging criteria for TNM was reported as identified by radiographic assessment. Clinical and vital (living or deceased) status as well as tumor status of patients was also recorded. Recurrence was calculated as the number of months to a new tumor event using a no-recurrence cutoff of 36 months. Smoking status was coded as a binary term. Patients who were reported as lifelong nonsmokers were termed “never smokers,” and all other patients were considered smokers. For patients who received RT, or combination chemotherapy and RT (CRT), definitive RT was considered 66 to 70 Gy.

RNA Isolation and Quality Assessment

All RNA was extracted from FFPE samples in which tumor tissue was macrodissected, and resulting samples were extracted using a Maxwell RSC automated extractor (Promega) with Maxwell RSC RNA FFPE kits. RNA was analyzed prior to NanoString preparation work flow to determine RNA quality and fragment size using a standard RNA Screentape/Tapestation Assessment (Agilent Technologies). Prior to sample hybridization, RNA quantitation was determined using Qubit 3.0 nucleic acid fluorimeter (Life Technologies) with a Qubit 3.0 RNA Hi-Sensitivity analysis kit (Life Technologies).

Gene Expression Assays

Validation of mRNA transcripts was performed using nCounter assays. A total of 50 ng of purified RNA was hybridized to target specific probes, as has been previously described using the commercially available PanCancer Immune Profile CodeSets (NanoString Technologies), which contains 730 target genes and 40 “housekeeping” genes (https://www.nanostring.com/download_file/view/436/3808). Counts for each RNA species were extracted and analyzed using NSolver software (version3.0) provided by NanoString.

Viral Quantitative Reverse-Transcription Polymerase Chain Reaction

Reverse-transcription reactions were performed with the Transcriptor First-Strand Synthesis kit (Roche AG) using 1 µg of total RNA, 60 µM of random hexamers, and 2.5 µM of oligo-dT primers, and expression of the indicated genes was analyzed by quantitative reverse transcription polymerase chain reaction using a QuantStudio 6 Flex Real Time PCR System (Applied Biosystems) using SYBR green polymerase chain reaction master mix (Roche AG). Each reaction mixture contained 1 × SYBR green master mix, complementary DNA from 1 µg of RNA, and 0.3 µM of each oligonucleotide primer in a total volume of 20 µL. Primers for E6*I (E6/E7) were as follows: 5′-TAAAACCCCTAAGACACATTGCCA-3′ (sense) and 5′-CAGCCAAACGGCTTTGGATTA-3′ (antisense).

Data and Statistical Analysis

For all patient demographics, descriptive statistics were used to compare the demographic, stage, treatment, and outcomes between node-positive and node-negative patients. Fisher exact tests were used for categorical variables, t tests were used for continuous variables, Wilcoxon-Mann-Whitney tests were used for non-normally distributed variables, and log-rank tests were used to determine recurrence and survival.

RNA expression data generated by NanoString were normalized using the nSolver analysis software and, where indicated in figure legends, log, transformed. For heat map presentation and data clustering, Morpheus (a web-based data analysis tool) was used (https://software.broadinstitute.org/morpheus). Data clustering was accomplished using unguided, hierarchical clustering with complete linkage. Graphs and statistical analyses were generated using Prism 7 (GraphPad Software) and 2-tailed t tests, unless otherwise specified. A gene signature was obtained either by principal component analysis using the 2:1 ratio of upregulated to downregulated genes in LN-negative tumors or by a machine learning program based on a linear predictor score (LPS) equation, where X is equal to gene [X] expression level and β is equal to a gene's weighted value based on its significance. A test set of receiver operating characteristic (ROC) curves were generated by a machine learning program based on the LPS algorithm. The training data sets were split randomly into selected test sets to perform cross-validation. A total of 500 cross-validation tests were performed to generate the mean area under the curve (AUC). Data analysis services were contracted by NanoString Technologies for thorough computational and statistical analysis of our genetic signature model.

Results

Demographics

A total of 48 patients with prospectively collected p16-positive OPSCC samples as part of the UNCSeq cohort were retrospectively analyzed for several clinical variables including age, sex, race/ethnicity, p16 status, HPV status, smoking status and pack-year history, RT history, average RT dose, mortality, incidence of recurrence, and disease-free survival. In total, 21 patients (mean [SD] age, 54.6 [9.6] years; 18 [86%] were men and 3 [14%] were women) with confirmed p16-positive immunohistochemical results and HPV-16 genotype OPSCC were included in the final analysis. The Table gives the demographic and clinical characteristics of all the patients with molecular analysis included in this study, stratified by radiographic LN staging. eTable 1 in the Supplement shows our median follow-up time, 3-year recurrence rates, and 3-year survival estimates for individuals in this study.

NanoString Tumor RNA Profiling

We isolated RNA from FFPE OPSCC samples and performed medium-throughput gene expression analysis using NanoString...
RNA assays to determine if a tumor’s gene expression profile would be predictive of a known clinical correlate of outcomes, such as LN involvement. Following unguided hierarchical clustering of all 770 genes in the array, we found that patients with no nodal involvement typically clustered in the same clade, compared with those with any LN involvement (N1 or N2) (Figure 1). This was independent of overall HPV DNA copy number, E6/E7 mRNA expression, or HPV integration status (Figure 1). Furthermore, there was no significant difference between E6/E7 expression levels (95% CI, −5.09 to 9.23), HPV-16 DNA copies (95% CI, −35 053.0 to 4858.0), or viral integration status when comparing node-negative vs node-positive tumors (Table).

Next, we performed unguided hierarchical clustering of tumor samples based on genes that were differentially expressed either greater than 2-fold or reduced in expression by 50% (Figure 2A). This method of analysis enabled visualization of several “hotspots,” or upregulated gene regions in 4 of 6 patients (67%) with node-negative status. Figure 2B displays the same data stratified by increasing node involvement (n = 0, n = 1, n = 2) and further depicts these highly active gene expression regions in patients with no nodal involvement. In addition, we found a global downregulation of most of the 770 genes analyzed in patients with more advanced LN involvement (Figure 2B).

Following the identification of gene hot spots in node-negative tumors, we sought to determine nodal involvement based on a specific genetic signature. Of the 650 genes that passed quality control metrics, 146 were significantly differentially expressed (Figure 3A). Of these genes with significant

### Table. Demographics and Clinical Characteristic of Patients Stratified by Nodal Status

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients, No. (%)</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P Value</th>
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<td>Age category, y</td>
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<td></td>
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<td>30-40 (n = 1)</td>
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<td>61-70 (n = 6)</td>
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<td>3 (20)</td>
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<tr>
<td>≥71 (n = 1)</td>
<td>0</td>
<td>1 (7)</td>
<td>1 (5)</td>
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<tr>
<td>Total (n = 21)</td>
<td>6 (100)</td>
<td>15 (100)</td>
<td>21 (100)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
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<tr>
<td>Male (n = 18)</td>
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<td>12 (80)</td>
<td>18 (86)</td>
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<td>Smoking status</td>
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<td>Nonsmoker (n = 9)</td>
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<td>7 (47)</td>
<td>9 (43)</td>
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<td>8 (53)</td>
<td>12 (57)</td>
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<td>Pack-years</td>
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<td>Nodal status</td>
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<td>HPV integration</td>
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<td>Nonintegrated (n = 9)</td>
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<td>5 (33)</td>
<td>9 (43)</td>
<td></td>
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<tr>
<td>Integrated (n = 12)</td>
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<td>10 (67)</td>
<td>12 (57)</td>
<td></td>
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<tr>
<td>Total (n = 21)</td>
<td>6 (100)</td>
<td>15 (100)</td>
<td>21 (100)</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
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<td>Surgery alone (n = 4)</td>
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<td>2 (13)</td>
<td>4 (19)</td>
<td></td>
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<tr>
<td>Surgery with adjuvant RT (n = 2)</td>
<td>2 (33)</td>
<td>0</td>
<td>2 (10)</td>
<td></td>
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<tr>
<td>Surgery with adjuvant CRT (n = 2)</td>
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<td>2 (13)</td>
<td>2 (10)</td>
<td></td>
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<td>Radiation alone (n = 2)</td>
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<td>1 (7)</td>
<td>2 (10)</td>
<td>.15c</td>
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<td>10 (67)</td>
<td>11 (52)</td>
<td>.54</td>
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<td>Radiation dosage, Gy</td>
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<td>8 (62)</td>
<td>10 (59)</td>
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<td>66 (n = 2)</td>
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<td>1 (8)</td>
<td>2 (12)</td>
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<td>70 (n = 5)</td>
<td>1 (25)</td>
<td>4 (31)</td>
<td>5 (29)</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Abbreviations: CRT, combination chemotherapy and radiotherapy; HPV, human papillomavirus; NA, not applicable; RT, radiation therapy.

1 P value for difference in mean age.
2 Exact logistic regression test.
3 P value for primary surgical vs nonsurgical treatment.
4 Deintensified dose.
5 t Test.

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RNA Oncoimmune Phenotyping of HPV-Positive Oropharyngeal Cancer by Nodal Status

Research Original Investigation

RNA Oncoimmune Phenotyping of HPV-Positive Oropharyngeal Cancer by Nodal Status

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cant differential expression, 63 were found to be upregulated in LN-negative disease compared with LN-positive disease, and 11 genes were significantly downregulated. The complete list of differentially expressed genes with their associated 95% CIs can be found in Table 2 in the Supplement.

To develop a molecular signature for nodal involvement, we identified 10 that most significantly upregulated and 5 most significantly downregulated genes in tumors that were radiographically node-negative (Figure 3B). We then conducted a principal component analysis based on the gene expression values of these 15 differentially expressed genes, which showed clear discrimination of LN-negative vs LN-negative OPSCC samples along the first 2 principal components and accounted for nearly 70% of the variance in gene expression (Figure 3C).

We then determined the sensitivity and specificity of this test for detecting LN involvement. Using these 15 genes, we found this profile had a sensitivity of 88% (95% CI, 63%-98%) and specificity of 85.7% (95% CI, 42%-99%). Given these test characteristics, we calculated the positive likelihood ratio to be 6.2% (95% CI, 1%-38%) and the negative likelihood ratio to be 0.1% (95% CI, 0.04%-0.52%). Furthermore, we determined that our population's prevalence of LN-positive disease was 70.8%. Using these data, we calculated the positive- and negative-predictive values for this gene signature to be 94% (95% CI, 71%-99%) and 75% (95% CI, 44%-92%), respectively.

In addition to the selection of 10 upregulated and 5 downregulated genes, we also used a machine learning module to compare our method with one that uses a gene signature training algorithm based on the elastic net, demonstrating a mean z-score difference between the 2 groups to be 9.60 (95% CI, 8.20-11.13) (Figure 4A). Using this method, we developed a set of 40 genes that, when measured together, distinguished tumor samples that are biologically node-negative from tumors that are node-positive (AUC = 0.93; 95% CI, 0.63-1.00 by ROC curve analysis) (Figure 4B), independent of clinical N staging.

### Discussion

In this study, we sought to examine the oncoimmune gene expression profile of HPV-positive patients with OPSCC and to determine potential differences in gene expression by nodal metastasis. We identified that the expression of several genes correlated highly with clinical nodal status, as demonstrated by the distinct clustering pattern for node-negative and node-positive patients (Figure 1A and Figure 2B); determined a set of differentially expressed genes between the 2 node stages (Figure 3A); and identified a gene expression signature associated with node-positive vs node-negative tumors (Figure 4B). If further validated, this gene signature has the potential to resolve different tumor phenotypes within the family of p16-positive OPSCCs.

To our knowledge, this is the first study using tumor immune profiling in HPV-driven OPSCC. Despite the widespread use of and recommendation for p16-immunohistochemical analysis as surrogate for HPV-positive OPSCC, there is currently no established molecular test to further stratify treatment or prognosis among known HPV-positive cases. This has led to multiple efforts to develop more refined prognostic biomarkers for OPSCC,
including the use of HPV antibodies, immunohistochemical analysis for multiple immune and tumor cell markers (DEK, PD-L1, PD-L2, EGFR, HER2, and HER3), and radiographic images as surrogates for molecular phenotypes. Work by Keck et al recently suggested a novel gene expression paradigm for HPV-positive OPSCC in which tumors are classified as either classical or inflammatory-mesenchymal. The classical subtype includes expression of detoxification genes and may be associated with tobacco exposure, whereas the HPV inflammatory-mesenchymal subtype is associated with immune gene expression and shares characteristics with the atypical subtype characterized previously. Keck et al demonstrated a trend toward improved survival in the HPV inflammatory-mesenchymal group compared with the HPV classical group.

The findings from our study build on this work by demonstrating a novel immune profile associated with nodal metastasis. Our data suggest that the sensitivity, specificity, and positive and negative predictive values using a tumor-based, molecular approach may supersede current testing methods that rely on p16 immunohistochemical analysis alone (Figure 3C). This is potentially important in the context of treatment selection because several clinical trials are currently evaluating the efficacy of treatment deintensification for HPV-driven OPSCC. If further validated, the method outlined herein could be used to stratify therapy or selection.
Normalized log₂ values of 164 differentially expressed genes (defined as log₂ [node-negative expression/node-positive] expression) were analyzed using nSolver Advanced Analysis Software. A, The top 15 most significant differentially expressed genes (10 upregulated and 5 downregulated) are plotted as Tukey box-and-whisker plots. Horizontal lines in each box represent the median value for the collective group of samples. Whiskers represent the variability outside the upper and lower quartiles. B, Volcano plot showing differentially expressed genes. Genes that are upregulated fall to the right of the plot, and those that are downregulated fall toward the left. Increasing value on the y-axis corresponds to a higher degree of significance. Dashed lines represent increasing P-value stringency. All genes meeting statistical significance have been highlighted in pink. Genes not achieving significance are represented in gray. The 95% CIs for all represented genes can be found in eTable 2 in the Supplement. C, Principal component analysis biplot showing standardized variance for the 2 principal components from the top 15 candidate genes for a gene signature. The ovals represent the confidence regions for either node-negative (gray) or node-positive (orange) tumor samples. Arrows represent the vector for the identified gene and represent the fit of the line to the 21-sample data set. Each tumor sample is represented by a dot. When samples fall within an oval that matches their color, the test is accurately detecting presence of either node-negative or node-positive disease.
prognosis, similarly to the commercially available PAM50-based ProSigna assay developed for breast cancer recurrence risk.\textsuperscript{33,34}

Strengths and Limitations

This study has important strengths and limitations. Given that a clear gene signature is present despite a small sample size demonstrates the potential power of this method to inform us about a tumor’s biological characteristics. Furthermore, this study clearly demonstrates that p16-positive tumors are not necessarily biologically uniform, suggesting that a more refined approach to therapy is warranted. The applicability of our data is limited by the small sample size in this study. It is possible that both our self-selected gene signature and the machine learning gene signature overfit the data and make the profile seem more successful than it would be in clinical practice. Therefore, the use of such a test in the field will likely require extensive, multicenter studies to achieve validation. Another limitation of a small sample size is the imprecision in test characteristics, evidenced by wide confidence intervals for both sensitivity and specificity for each gene signature. We would expect these intervals to narrow by testing these molecular signatures in a larger cohort. Despite these limitations, our study is the first step toward achieving the goal of tumor-specific therapy and suggests that this type of molecular testing is entirely feasible within this patient population.

Conclusions

The use of p16 immunohistochemical analysis for tumor stratification is becoming widely prevalent for patient stratification in the treatment of OPSCC. The data from our study suggest that a more refined molecular analysis of tumors could be beneficial for treatment stratification. Given that not all p16-positive tumors have excellent outcomes, it would seem prudent to stratify therapy and recommend clinical trial deintensification based on tumor biological characteristics. The use of molecular signatures, such as the one we demonstrate herein, may be one step closer to meeting the mandate of precision medicine in the treatment of head and neck cancers.
for the integrity of the data and the accuracy of the data analysis.
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Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: Stepp, Sheth, Hayes, Zevallos.
Critical revision of the manuscript for important intellectual content: All authors.
Statistical analysis: Stepp, Farquhar, Sheth, Mazul, Mandani, Hayes.
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Administrative, technical, or material support: Hayes.
Studysupervision: Stepp, Sheth, Hackman, Hayes, Zevallos.
Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.
Dr Stepp has a patent entitled Molecular signature of metastatic potential in p16-positive oropharyngeal squamous cell carcinoma (OPSCC) pending submission to the USPTO. No other disclosures were reported.
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