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Prediction of survival of HPV16-negative, p16-negative oral cavity cancer patients using a 13-gene signature: A multicenter study using FFPE samples

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ABSTRACT

Objectives: To test the performance of an oral cancer prognostic 13-gene signature for the prediction of survival of patients diagnosed with HPV-negative and p16-negative oral cavity cancer.
Materials and Methods: Diagnostic formalin-fixed paraffin-embedded oral cavity cancer tumor samples were obtained from the Fred Hutchinson Cancer Research Center/University of Washington, University of Calgary, University of Michigan, University of Utah, and seven ARCAGE study centers coordinated by the International Agency of Research on Cancer. RNA from 638 Human Papillomavirus (HPV)-negative and p16-negative samples was analyzed for the 13 genes using a NanoString assay. Ridge-penalized Cox regressions were applied to samples randomly split into discovery and validation sets to build models and evaluate the performance of the

Abbreviations: OSCC, squamous cell carcinoma of the oral cavity and oropharynx; OCC, squamous oral cavity cancer; OPC, squamous cell oropharyngeal cancer; HNC, head and neck squamous cell cancers; FFPE, formalin-fixed paraffin-embedded; HPV, human papillomavirus; FH/UW, Fred Hutchinson Cancer Research Center and the University of Washington; UC, the University of Calgary, Canada; UM, University of Michigan; UU, University of Utah; IARC, International Agency for Research on Cancer; the ARCAGE study, Alcohol-related cancers and genetic susceptibility in Europe study; IHC, immunohistochemistry; H&E, hematoxylin and eosin; AJCC, American Joint Committee on Cancer; PPV, positive predictive value; NPV, negative predictive value; AUC, areas under the curve; pAUC, partial areas under the curve

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13-gene signature in predicting 2-year oral cavity cancer-specific survival overall and separately for patients with early and late stage disease.
Results: Among AJCC stage I/II patients, including the 13-gene signature in the model resulted in substantial improvement in the prediction of 2-year oral cavity cancer-specific survival. For models containing age and sex with and without the 13-gene signature score, the areas under the Receiver Operating Characteristic Curve (AUC) and partial AUC were 0.700 vs. 0.537 ($p < 0.001$), and 0.046 vs. 0.018 ($p < 0.001$), respectively. Improvement in predicting prognosis for AJCC stage III/IV disease also was observed, but to a lesser extent.
Conclusions: If confirmed using tumor samples from a larger number of early stage oral cavity cancer patients, the 13-gene signature may inform personalized treatment of early stage HPV-negative and p16-negative oral cavity cancer patients.

Introduction

Squamous cell carcinoma of the oral cavity and oropharynx (OSCC) represents a considerable public health burden. World-wide, approximately 600,000 new cases and 325,000 deaths occurred in 2012 [1,2]. In the US, the American Cancer Society estimates that 53,000 new cases and 10,860 deaths will occur in 2019 (https://www.cancer.org). OSCC accounts for about 75% of the head and neck squamous cell cancers (HNC); about two thirds of OSCC are oral cavity cancers (OCC) and one third are oropharyngeal cancers (OPC).

HPV status is an important and independent prognostic factor for oropharyngeal cancer. The 5-year survival under the current standard of care, regardless of the specific therapy given, is about 80–85% for HPV-positive but only about 30–35% for HPV-negative OPC patients [3–7]. The discovery that HPV-positivity is associated with better survival for oropharyngeal cancer patients is clearly a breakthrough for the HNC field, in that HPV status can be used to aid clinical management of the oropharyngeal cancer patients. In contrast, for OCC, HPV is relatively uncommon (10 to15%) and the results comparing the survival of HPV-positive vs. HPV-negative OCC patients have been inconsistent [8–12], and thus HPV status is not likely to be useful as a prognostic marker[13–16]. Disease stage judged by clinicopathologic characteristics remains the predominant feature to inform treatment and predict prognosis. However, the ability of staging to predict prognosis in OCC is limited; patients with tumors of the same clinical and pathologic staging have a heterogeneous response to clinical treatment and a different probability of recurrence and survival. Patients with early stage OCC typically undergo unimodality treatment (surgery or radiation), whereas those with late stage disease receive multimodality treatment (some combination of surgery, radiation, and chemotherapy) for late stage disease. For those patients who present with early stage I/II disease treated with unimodality therapy (i.e., surgery) and for whom neck surgery to fully assess high-risk features (multiple positive lymph nodes, or extracapsular spread in positive lymph nodes) may not be warranted, more precise knowledge of whether a patient's tumor is associated with a poor prognosis might justify treatment intensification with a second modality (i.e., radiation +/- chemotherapy). However, at present we lack biomarkers to identify which patients with high risk features will respond to more aggressive treatment, and thus which patients could be spared significant treatment toxicity.

To help improve the prediction of prognosis of OSCC patients, using fresh tumors and Affymetrix U133 2.0 Plus GeneChip arrays, we have previously identified a gene expression profile of 131 probe sets (representing 108 unique known genes), which not only differentiates invasive OSCC from normal oral epithelium[17] but also predicts OSCC-specific survival[18], with an area under the curve (AUC) of around 0.8. Furthermore, we identified a subset of 13 genes from this 131 probe set list for which the gene expression is strongly associated with OSCC-specific survival for patients with HPV-negative OCC and OPC irrespective of treatment modalities, and we validated the performance of this 13-gene signature in predicting survival of OCC patients using an independent dataset [19]. We report here the translation of this finding to a potentially useful clinical test to aid in the management of patients with HPV-negative, p16-negative OCC, by converting the assay to the testing of formalin-fixed paraffin-embedded (FFPE) diagnostic tumor blocks using the simple NanoString platform in a 2-phase study. This study involved analyses of diagnostic tumor blocks from HPV-negative and p16-negative OCC patients from the Fred Hutchinson Cancer Research Center and the University of Washington (FH/UW), the University of Calgary, Canada (UC), University of Michigan (UM), University of Utah (UU), and the ARCAGE study centers in Prague, Czech Republic; Bremen, Germany; Oslo, Norway; Dublin, Ireland; Glasgow, UK; Newcastle, UK; and Barcelona, Spain, that were led by the International Agency of Research on Cancer (IARC), France. This study also evaluated whether the prediction of OCC-specific survival by the expression of the 13-gene signature might be influenced by treatment modalities (surgery alone, or multi-modality such as surgery plus radiation, surgery plus radiation plus chemotherapy).

Materials and methods

Study population

Eligible patients had HPV16 RNA-negative and p16-immunohistochemistry negative primary OCC and were recruited at: FH/UW, UM, UU, UC and seven ARCAGE Study Centers mentioned above. Centralized pathology review for the ARCAGE study was performed by Dr. Abedi-Ardekani of IARC.

The study is approved by the Institution Review Office of the Fred Hutchison Cancer Research Center and that of UM, UU and UC. The ARCAGE study was approved by the Ethical Review Board of IARC as well as the local boards in the individual centers. All participants provided informed consent for the study.

FFPE tumor blocks prior to adjuvant therapy were selected by local pathologists. Two consecutive 20 μm-thick FFPE curls along with two unstained slides, or the diagnostic images of hematoxylin and eosin (H&E) and p16 IHC, if done at local centers, were sent to the FH. H&E staining was performed at FH Experimental Histopathology Shared Resource. p16 IHC was performed at the UW Department of Pathology. All H&E and p16 IHC slides and images were then centrally evaluated by Dr. Upton, who is an anatomic pathologist at UW, to confirm the diagnosis and to interpret p16 IHC results.

De-identified clinical data from each institution were sent to FH. Data were reviewed and harmonized. Vital status for patients from all institutions except IARC was updated during April 2016 to April 2017. IARC conducted one-time retrospective follow up between 2012 and 2015 to obtain last known vital status (alive, death or lost to follow-up) and date of last contact.

Nucleic acid extraction from FFPE samples and quality assessment

Total RNA was extracted from 20 μm-thick curls from FFPE blocks of potentially eligible patients ($N = 736$) with the use of the Qiagen RNeasy® FFPE Kit (Qiagen, Valencia, CA) following manufacturer's protocol. Each extraction batch contained samples from all participating institutions. RNA quantitation and purity were measured using a

Nanodrop ND-8000 spectrophotometer (ThermoFisher Scientific, Waltham, MA). The RNA quality was further tested by qRT-PCR on a Life Technologies 7900 HT using the Qiagen QuantiTect SYBR® Green RT-PCR Kit (Qiagen, Valencia, CA) with primer sets targeting *LAMC2* and *ACTB* transcripts. For samples that failed QC (N = 153) based on assessment on the Agilent 2200 Tapestation (Agilent, Santa Clara, CA), an additional curl was extracted using the RNAStorm™ Kit (Cell Data Sciences, Fremont, CA).

HPV16 RNA test

To screen samples for HPV16 RNA, we designed a probe targeting the HPV16 E6 transcript as part of our NanoString panel. To determine the efficacy of our newly developed NanoString probe test and to identify the cutoff for HPV interpretation, we tested 46 samples with known HPV16 E6 RNA status by other methodologies using our NanoString panel. These include one HPV-positive sample from IARC (tested by RT-PCR[20]), nine HPV-positive samples from UM (tested by RNAscope[21]), and 26 HPV-positive and 10 HPV-negative in-house samples, which HPV status had previously been determined by RT-PCR as follows. Extracted total RNA was tested for HPV16 E6 expression by qRT-PCR on a 7900 HT Sequence Detection System (Applied Biosystems, Foster City, USA). In brief, a 10 μL reaction volume consisted of 200 ng of total RNA, [1X] QuantiTect® SYBR® Green RT-PCR Master Mix (Qiagen, Valencia, USA), 0.1 μL QuantiTect® RT Mix (Qiagen, Valencia, USA), [1 μM] forward primer 5′-GTGTACTGCAAGCAACAG TTA-3′, and [1 μM] reverse primer 5′-TCAGGACACAGTGGCTTT TGA-3′. Cycling conditions were: 50 °C 30 min; 95 °C 15 min; 40 cycles of 94 °C 15 sec, 55 °C 30 sec, 72 °C 30 sec, followed by a dissociation curve of 94 °C 15 sec, 55 °C 30 sec, 94 °C 15 sec. Expression data was analyzed with SDS v 2.3 software (Applied Biosystems, Foster City, USA). Samples with no amplification or the Cycle threshold (Ct) values above 33 were defined as HPV16 RNA-negative.

The range of log2 value of HPV test on NanoString for the 10 HPV-negative samples was 2.8 to 4.4 (mean 3.8) and for the 36 HPV-positive samples was 9.5 to 15.5 (mean 12.9). Thus, we used the value of 7 on log2 scale as a cutoff for HPV positivity on our NanoString test.

p16 testing by immunohistochemistry staining (IHC)

The p16 IHC was performed on unstained slides of cases from UW (n = 203), UU (n = 88), UC (n = 181), and a portion of UM cases (n = 62). It was performed on a Leica Bond III autostainer using a Leica Polymer detection kit (DS-9800). The tissue sections on slides were subjected to Heat Induced Epitope Retrieval for 20 min in EDTA buffer. The primary p16 antibody used was a mouse monoclonal antibody from MTM Labs catalog number 9517 at 1:4 dilution. All p16 IHC slides and the p16 IHC images from UM (n = 85) and IARC (n = 117) were reviewed and interpreted by Dr. Upton independent of other information about the cases.

NanoString assay for HPV16 status and the 13-gene signature

In brief, 600 ng of total RNA was assayed with the NanoString nCounter XT Assay (NanoString, Seattle, WA) using a custom-designed probe sets (see Supplementary Table 1) for target genes (*LIPI*, *C5ORF13*, *CLEC3B, LAMC2, LOC283278, MYH11, OASL, OSMR, SERPINE1, SLC16A1, THBS1, TPPP, ZDHHC11*) as well as six housekeeping genes (*ALAS1, GAPDH, RPL27, RPS18, TBP, TUBA1B)* and *CDKN2A*, and HPV16 E6, following manufacturer's protocol. Samples were assayed in batches of 35 samples plus a positive control reference RNA (XpressRef™ Universal Total RNA – Human Universal RNA (Qiagen, Valencia, CA)) to assess batch to batch variation. To control for technical variation in hybridization efficiencies, RNA spike-in controls provided by the manufacturer were included in each sample tested. Samples were processed on the nCounter Prep Station and read on the nCounter Digital Analyzer (NanoString, Seattle, WA) with FOV set at 555 following manufacturer's instructions at the FH Genomics Shared Resources. The NanoString probes used in this study are shown in Supplementary Table 1.

Data processing

We first evaluated NanoString gene expression data of 724 FFPE samples and excluded data from 23 cases due to low expression of housekeeping genes. Results of the remaining 701 samples were normalized, first according to the gene expression profile of the six spiked-in positive control in each sample, and then to the expression levels of the six housekeeping genes. Within each normalization step, a sample-specific scaling factor was calculated using the geometric mean standardized over the maximum of all sample means. After the spiked-in positive control-based normalization and housekeeping genes-based normalization, expression data were log2 transformed. Gene level expressions were derived using the average expression levels of replicate probes in the same gene.

Statistical analyses

For the current study, we evaluated the predictive ability of the 13-gene signature separately for the prognosis of early stage (American Joint Committee on Cancer (AJCC) stages I/II) and late stage (AJCC stages III/IV) disease. Within each stage, the Ridge-penalized Cox models were built as follows:

Model 1: survival ~ age + sex;
Model 2: survival ~ 13-gene + age + sex;
Model 3: survival ~ 13-gene + age + sex + treatment modality.

The predicted log hazard ratio (on the test data set) according to the fitted models from the training data set were referred to as the prediction scores. Specifically, the prediction scores were calculated as the weighted sum of gene expressions, age, and gender indicator based on the coefficients from the fitted cox regression model with ridge regularization (on the training data set). The performance of these prediction models was assessed through random cross-validations with 100 iterations. In each iteration, samples were first randomly divided into training and testing data sets with equal sample size; then a Ridge-penalized Cox proportional hazard regression model was built based on the training data; and the prediction accuracy (Receiver Operating Characteristic (ROC) curve) of two-year survival outcome based on the fitted models were evaluated on the testing data. Specifically, both the area under the curve (AUC) and partial area under the curve (pAUC) at false positive rate (1-Specificity) of 20% were calculated to quantify the prediction performance. Instead of full AUC, partial AUC considers only those regions of the ROC space where data have been observed, or which correspond to clinically relevant values of test sensitivity or specificity. T-tests were then applied to the cross-validation AUC and pAUC respectively to compare models with and without the 13-gene signature.

For purpose of illustration, we chose one training–testing splitting among the 100 random cross validation data sets, for which the testing AUC is about the median level among all the 100 cross validation results. For the selected training–testing splitting, we divided the testing samples into two clusters based on the predicted risk scores that are above or below the median according to the model fitted on the training samples. Cumulative incidence curves of OCC-specific survival of the two clusters in the testing set were then plotted and compared through a log-rank test. We also calculated the positive predictive value (PPV) and negative predictive value (NPV) for death within two years post-diagnosis for patients with stage I/II OCC in the testing set. The analyses are based on eight patients who died from OCC and 95 patients who remained alive at two years. The analyses did not include patients

who were lost-to-follow up (n = 7) and those who died from causes other than oral cancer (n = 7). All the statistical analyses were conducted using software R version 3.4.3.

Results

We screened FFPE tumor samples of 736 cases (FH/UW, n = 203; UM, n = 147; UU, n = 88; UC, n = 181; IARC, n = 117). Twelve were found to have inadequate or poor-quality RNA, and 23 failed the NanoString assay. Of the samples from the remaining 701 cases, 638 were p16 IHC-negative and HPV16-E6 RNA negative and were retained for the final analyses (see Supplementary Table 2). Selected characteristics of these 638 study participants are shown in Table 1. The mean age at diagnosis was 59–64 years. There were more male than female patients in each study center. The vast majority of the patients were White. Per Canadian law, no information on race/ethnicity was

Table 1
Selected characteristics of study participants.

	FH/UW (n = 185)	U Michigan (n = 138)	U Utah (n = 68)	U Calgary (n = 158)	IARC (n = 89)	Total (n = 638)
Years of Diagnosis	2004–2012	2008–2014	2004–2014	2007–2014	2002–2005	2002–2014
Age						
Range	20–88	30–96	25–89	28–89	28–85	20–96
Mean, SD	60.7, 14.0	64.0, 13.8	64.0, 12.9	62.3, 13.1	58.7, 9.9	61.8, 13.2
Gender						
Male	101 (54.6%)	71 (51.5%)	44 (64.7%)	101 (63.9%)	61 (68.5%)	378 (59.3%)
Female	84 (45.4%)	67 (48.5%)	24 (35.3%)	57 (36.1%)	28 (31.5%)	260 (40.7%)
Race						
White	170 (92.4%)	97 (99.0%)	57 (96.6%)	0	89 (100%)	413 (96.0%)
Other	14 (7.6%)	1 (1.0%)	0	0	0	17 (4.0%)
Unknown	1	40	9	158*	0	208
Ethnicity						
Hispanic	3 (1.8%)	2 (1.5%)	0	0	0	5 (1.1%)
Not Hispanic	165 (98.2%)	131 (98.5%)	57 (100.0%)	0	89 (100%)	442 (98.9%)
Unknown	17	5	11	158	0	191
Cigarette Smoking						
Non-smoker	57 (30.8%)	28 (28.3%)	36 (55.4%)	41 (26.1%)	13 (14.6%)	175 (29.4%)
Former	64 (34.6%)	57 (57.6%)	10 (15.4%)	61 (38.9%)	13 (14.6%)	205 (34.5%)
Current	64 (34.6%)	14 (14.1%)	19 (29.2%)	55 (35.0%)	63 (70.8%)	215 (36.1%)
Unknown	0	39	3	1	0	43
Alcohol Use						
Never	19 (10.3%)	13 (13.3%)	31 (47.7%)	28 (18.7%)	8 (9.0%)	99 (16.9%)
Former	39 (21.2%)	36 (36.7%)	4 (6.2%)	25 (16.7%)	10 (11.2%)	114 (19.5%)
Current	126 (68.5%)	49 (50.0%)	30 (46.1%)	97 (64.6%)	71 (79.8%)	373 (63.6%)
Unknown	1	40	3	8	0	52
Tumor Site						
Tongue	76 (41.1%)	79 (57.3%)	27 (39.7%)	85 (53.8%)	37 (41.6%)	304 (47.6%)
Floor of Mouth	42 (22.7%)	16 (11.6%)	8 (11.8%)	26 (16.4%)	36 (40.4%)	128 (20.1%)
Buccal	14 (7.6%)	9 (6.5%)	10 (14.7%)	10 (6.3%)	1 (1.1%)	44 (6.9%)
Hard Palate	3 (1.6%)	0	2 (2.9%)	2 (1.3%)	1 (1.1%)	8 (1.3%)
Gum	35 (18.9%)	22 (15.9%)	13 (19.1%)	24 (15.2%)	7 (7.9%)	101 (15.8%)
Retromolar Trigone	14 (7.6%)	12 (8.7%)	8 (11.8%)	11 (7.0%)	7 (7.9%)	52 (8.1%)
Overlapping Site of Oral Cavity	1 (0.5%)	0	0	0	0	1 (0.2%)
AJCC Staging**						
I	52 (28.1%)	38 (27.5%)	11 (16.9%)	20 (12.7%)	22 (30.2%)	143 (23.1%)
II	17 (9.2%)	20 (14.5%)	10 (15.4%)	25 (15.8%)	19 (26.0%)	91 (14.7%)
III	20 (10.8%)	25 (18.1%)	12 (18.5%)	23 (14.6%)	12 (16.4%)	92 (14.9%)
IV	96 (51.9%)	55 (39.9%)	32 (49.2%)	90 (56.9%)	20 (27.4%)	293 (47.3%)
Incomplete Data	0	0	3	0	12	19
Tumor content (%)						
Range	1% – 80%	1% – 95%	1% – 90%	1% – 90%	1% – 95%	1% – 95%
Median	30%	15%	40%	30%	35%	30%
Treatment Modality						
Surgery alone	81 (45.5%)	50 (37.6%)	38 (58.5%)	72 (45.6%)	32 (37.6%)	273 (44.1%)
Surgery + RT	43 (24.2%)	44 (33.1%)	16 (24.6%)	58 (36.7%)	35 (41.2%)	196 (31.7%)
Surgery + Chemo	2 (1.1%)	0	0	0	0	2 (0.3%)
Surgery + RT + Chemo	52 (29.2%)	39 (29.3%)	9 (13.8%)	28 (17.7%)	9 (10.6%)	137 (22.1%)
RT + Chemo	0	0	2 (3.1%)	0	4 (4.7%)	6 (1.0%)
RT alone	0	0	0	0	5 (5.9%)	5 (0.8%)
Incomplete Data	7	5	3	0	4	19
Vital Status						
Living	73(39.5%)	90 (65.2%)	23 (33.8%)	82 (51.9%)	44 (49.4%)	312 (48.9%)
Deceased	112 (60.5%)	48 (34.8%)	45 (66.2%)	76 (48.1%)	45 (50.6%)	326 (51.1%)
Cause of Death						
Oral cancer	70 (66.0%)	31 (70.4%)	18 (66.7%)	50 (65.8%)	23 (56.1%)	192 (65.3%)
Other cause	36 (34.0%)	13 (29.6%)	9 (33.3%)	26 (34.2%)	18 (43.9%)	102 (34.7%)
Unknown cause	6	4	18	0	4	32
Follow-up Time (months)						
Range	0.2–145.8	1.3–88.7	0.8–155.8	0.5–114.5	0.5–130.3	0.2–155.8
Median	53.5	28.5	20.2	28.6	57.7	32.6
Median FU time for alive patients	96.1	37.2	32.6	45.8	92.6	58.6

* Per Canadian law, no information on race/ethnicity was collected from participants from the UC.
** Clinical stage used when pathological stage was not available.

collected from participants from the UC. With the exception of UU, most patients from other centers were either former or current cigarette smokers and alcohol drinkers. The tongue and floor of mouth were the most common tumor sites. A greater percentage of patients (62% overall) had late stage (stage III/IV) disease than early stage disease. Surgery was the most common treatment. Oral cancer was the cause of death for about two thirds of the patients who had died prior to the end of follow-up. The median follow-up time was 32.6 months (range 0.2–155.8 months).

Separately for patients with AJCC early stage disease (stage I/II, n = 234) and late stage disease (stage III/IV, n = 385), we evaluated the ability to predict 2-year OCC-specific survival through 100 random cross-validations (see Methods section). For patients with Stage I/II disease, Figure 1 shows the AUC and pAUC values of the testing samples from the 100 cross-validations for the three different models, and Figure 2 illustrates the cumulative incidence curves of high/low gene-signature-score group based on one test data set with p-values pertaining to comparisons between Model 1 and Model 2 (see method section). Compared to a model with the variables age and sex, the AUC and pAUC in a model including age, sex, and the 13-gene risk score was considerably better in predicting 2-year survival (mean AUC 0.700 vs. 0.537, $p < 0.001$; mean pAUC 0.046 vs. 0.018, T test $p < 0.001$). Including treatment modality in the model did not lead to any meaningful change in the result (see Figure 1 for comparison between models 2 and 3). Using the median prediction score as a cut-off, individuals with a high prediction score experienced relatively poorer OCC-specific survival, as illustrated by the cumulative incidence curves. Similar results were observed with 5-year OCC-specific survival.

Among patients with stage I/II disease, the proposed gene signature prediction model showed a sensitivity of 50% in predicting deaths from OCC within 2 years when the specificity was set at 70%; the corresponding positive predictive value (PPV) and negative predictive value (NPV) were 12.5% and 94.4%, respectively. Thus, compared with an unconditional probability of death of about 7.8%, the PPV of 12.5% suggests that the gene signature provided a substantial improvement (increasing PPV by about 60%) in predicting OCC-specific death. The model based on age and sex (Model 1 in Figure 1 legend) without the gene signature yielded a sensitivity of 13% when the specificity was set at 70%; the corresponding PPV and NPV were 3.0% and 90.5%, respectively.

The same analyses were conducted on 385 late stage (AJCC Stage III/IV) patients. The results also showed a statistically significant improvement in the prediction of 2-year OCC-specific survival when comparing the model containing 13-gene risk score plus age and sex to a model containing age and sex alone, though the magnitude of improvement was not as pronounced as in the early stage group. Figure 3 provides the AUC and pAUC values and depicts the comparisons of these values among the various models. Figure 4 shows cumulative incidence curves comparing OCC-specific survival with high and low risk prediction score for late stage patients. A similar observation was obtained when we included treatment modality as an additional covariate in the models (see Figure 3 for comparison between models 2 and 3). The Ridge coefficients used in the testing analyses for overall samples, early stage samples and late stage samples are presented in Table 2.

Discussion

The 13-gene prognostic gene signature was originally discovered and validated using fresh primary tumors obtained at the time of surgical resection prior to any chemotherapy and/or radiation treatment and using the high dimensional whole genome Affymetrix Genechip array. The goal of the current study was to establish a prognostic gene signature test for HPV-negative and p16-negative OCC patients using their diagnostic FFPE tumor samples, with the hope of facilitating the test's eventual adoption to inform precision treatment. Our results suggest that the 13-gene signature may help identify early stage patients who have poor likelihood of survival and who may be considered for more aggressive treatment than surgery alone. Specifically, our observation of modestly improved PPV and NPV values associated with a model composed of the 13-gene-signature plus age and sex suggests that patients who have stage I/II disease and a high risk-score may be more likely, while those with a low risk score may be less likely, to die within two years. While this observation might be useful to physicians and patients to inform treatment choices, the results were based on a relatively small number of deaths due to oral cavity cancer within two years (n = 16) and do not permit any firm conclusion to be drawn. Further confirmation of the findings using diagnostic tumor samples from a larger number of OCC patients with stage I/II disease would be warranted before the adoption of the assay in clinical settings.

The stage classification in the current study was based on AJCC version 7 and not the recently recommended AJCC version 8[22], which requires information on not only tumor size but also on extracapsular spread and depth of tumor invasion (which we did not obtain). To what extent the 13-gene signature can improve the prediction of survival beyond the new AJCC stage is unknown and requires investigation.

Since our discovery of a 131-gene prognostic gene signature for OSCC in general and the 13-gene prognostic gene signature for HPV-negative OSCC, there have been other studies that described prognostic

	AUC			
Model	**Min**	**Max**	**Mean**	**SD**
M1: age+sex	0.289	0.741	0.537	0.082
M2: 13gene+age+sex	0.525	0.877	0.700	0.083
M3: 13gene+age+sex+treat	0.497	0.870	0.695	0.083
	Partial AUC			
Model	**Min**	**Max**	**Mean**	**SD**
M1: age+sex	0	0.053	0.018	0.012
M2: 13gene+age+sex	0.011	0.089	0.046	0.016
M3: 13gene+age+sex+treat	0.014	0.092	0.044	0.016

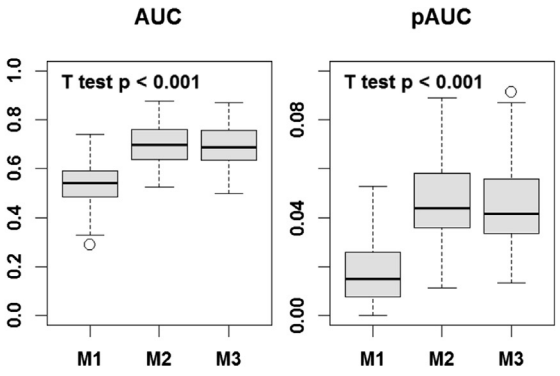

Fig. 1. Comparison of AUC and partial AUC of models for the prediction of 2-year OCC-specific survival among AJCC stage I & II OCC patients (n = 234). M1 (Model 1) contains age and sex; M2 (Model 2) contains expressions of the 13 genes + age + sex; and M3 (Model 3) contains expressions of the 13 genes + age + sex + treatment modality. Cross-validation was performed 100 times with samples randomly split into equal portions for training and testing datasets. The AUC and pAUC values represent values obtained in the 100 testing datasets in the random cross validation process; the p-values in the box plots pertain to comparisons between Model 1 and Model 2.

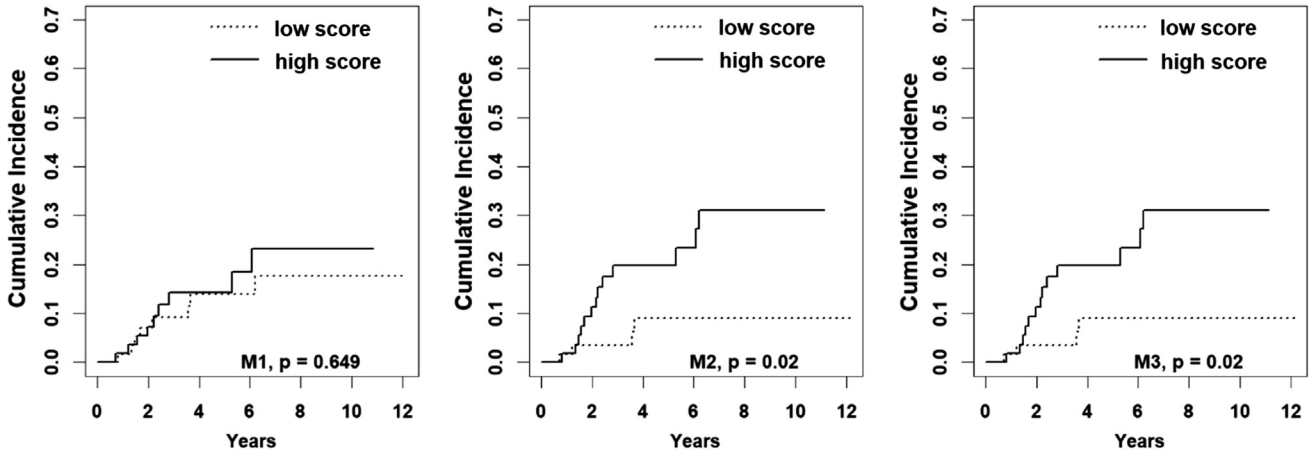

Fig. 2. Results of Cumulative Incidence Curves of AJCC stage I & II OCC patients (n = 234) comparing individuals with low and high prognostic risk score in the three prediction models as described in Fig. 1 legend. The cut-point of the high/low score clusters were the median of the risk score -0.0011 (M1), -0.022 (M2) and -0.018 (M3). The p-values pertain to comparisons made between patients with high vs. patients with low prediction scores in each model.

AUC

Model	Min	Max	Mean	SD
M1: age+sex	0.359	0.607	0.514	0.042
M2: 13gene+age+sex	0.505	0.680	0.592	0.034
M3: 13gene+age+sex+treat	0.506	0.653	0.585	0.034

Partial AUC

Model	Min	Max	Mean	SD
M1: age+sex	0.006	0.054	0.030	0.008
M2: 13gene+age+sex	0.018	0.060	0.035	0.007
M3: 13gene+age+sex+treat	0.023	0.049	0.035	0.006

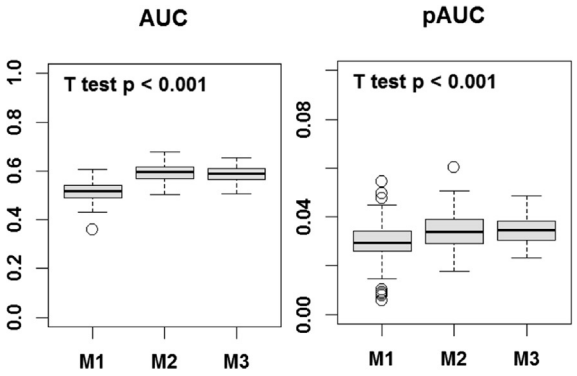

Fig. 3. Comparison of AUC and partial AUC of models for the prediction of 2-year OCC-specific survival among AJCC stage III & IV OCC patients (n = 385). M1 (Model 1) contains age and sex; M2 (Model 2) contains expressions of the 13 genes + age + sex; and M3 (Model 3) contains expressions of the 13 genes + age + sex + treatment modality. Cross-validation was performed 100 times with samples randomly split into equal portions for training and testing datasets. The AUC and pAUC values represent values obtained in the 100 testing datasets in the random cross validation process; the p-values in the box plots pertain to comparisons between Model 1 and Model 2.

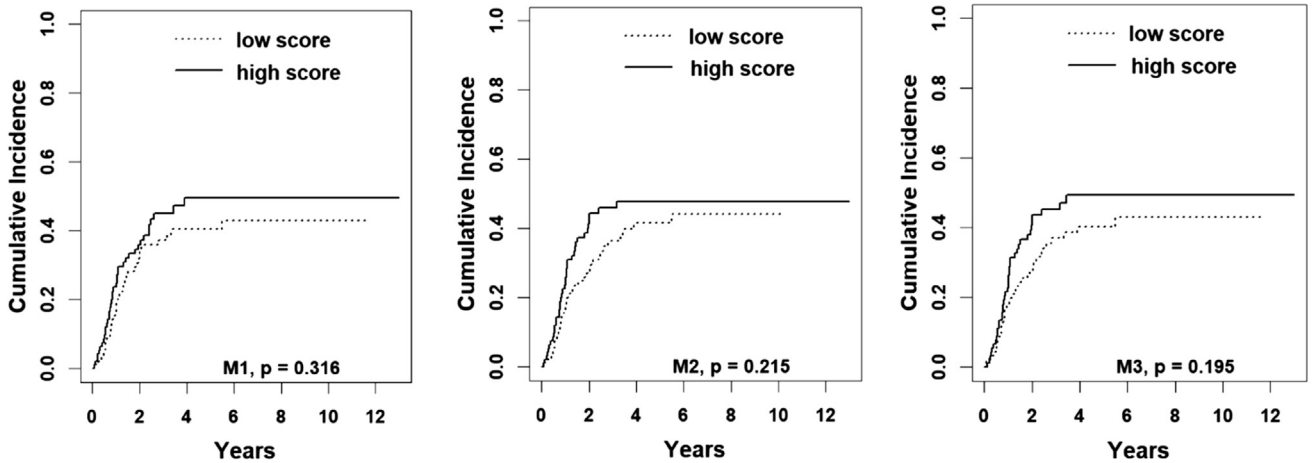

Fig. 4. Results of Cumulative Incidence Curves of AJCC stage III & IV OCC patients (n = 385) comparing individuals with low and high prognostic risk score in the three prediction models as described in Fig. 1 legend. The cut-point of the high/low score clusters were the median of the risk score -0.029 (M1), 0.032 (M2) and 0.0031 (M3). The p-values pertain to comparisons made between patients with high vs. patients with low prediction scores in each model.

Table 2
Ridge coefficients used in the models to predict OCC-specific survival.

	Model Coefficients	
Covariates in Prediction Models	stage I/II	stage III/IV
C5ORF13	0.244933	0.226846893
CLEC3B	-0.069550	-0.375688638
LAMC2	0.155288	0.081598812
OASL	0.195624	-0.031476608
OSMR	0.037876	-0.225751062
SERPINE1	-0.069640	0.315219464
THBS1	0.143375	0.11604339
TPPP	-0.229450	-0.127295638
ZDHHC11	-0.315640	0.019052107
LIPI	0.189138	0.115422795
LOC283278	0.224964	0.156241496
MYH11	0.333253	0.143185048
SLC16A1	0.167736	-0.101888923
Age	0.183348	0.219864623
Sex*	-0.055250	0.1684177

* male = 1, female = 2.

signatures for HNC or OSCC, including some that have included training and testing sets[23–28]. Our previously deposited datasets to the GEO database were used either as training or validation set in some of these studies [23,29]. There were also some prognostic signatures reported for HNC/OSCC based on microRNA[30,31], long non-coding RNA[32], copy number alterations of chromosomal regions[33,34], protein markers[35] or methylation markers [25]. In addition, there have been prognostic signatures reported on oral cancer patients whose primary risk factors included betel quid chewing[36–38]. However, none of these studies have restricted their efforts to HPV-negative and p16-negative OCC, where the need for such a prediction tool is most needed, and none have attempted to show validation of a signature that substantially outperforms clinicopathological features. Given the heterogeneity of head and neck cancer with respect to risk factors, tumor site, HPV involvement, etc., to realize a signature's clinical utility it would be important to evaluate how applicable a gene signature is to the patient population for which a signature test is intended.

Ultimately, if the gene signature we have developed (or a modified version of it) proves to be a strong enough tool in predicting survival, clinical studies could be conducted to determine the utility of modifying treatment recommendations based on the predicted survival: specifically, intensification of treatment in patients with a poor prognosis.

Declaration of Competing Interest

Dr. Melissa P. Upton is now a consultant for Hamamatsu Photonics related to validation of a digital slide scanner, but she has not received any compensation for the study, which has not yet begun. Ms. Kathie Zairns' salary has been partially funded through a grant to University of Michigan with Brooklyn Immuno Therapeutics since Jan 2018. Other authors do not have potential conflict of interest to disclose

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2019.104487.

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