A 13-Gene Signature Prognostic of HPV-Negative OSCC: Discovery and External Validation

Pawadee Lohavanichbutr, Eduardo Méndez, F. Christopher Holsinger, Tessa C. Rue, Yuzheng Zhang, John Houck, Melissa P. Upton, Neal Futran, Stephen M. Schwartz, Pei Wang, and Chu Chen

Abstract

Purpose: To identify a prognostic gene signature for patients with human papilloma virus (HPV)-negative oral squamous cell carcinomas (OSCC).

Experimental Design: Two gene expression datasets were used: a training dataset from the Fred Hutchinson Cancer Research Center (FHCRC, Seattle, WA; \( n = 97 \)) and a validation dataset from the MD Anderson Cancer Center (MDACC, Houston, TX; \( n = 71 \)). We applied L1/L2-penalized Cox regression models to the FHCRC data on the 131-gene signature previously identified to be prognostic in patients with OSCCs to identify a prognostic model specific for patients with high-risk HPV-negative OSCCs. The models were tested with the MDACC dataset using a receiver operating characteristic (ROC) analysis.

Results: A 13-gene model was identified as the best predictor of HPV-negative OSCC-specific survival in the training dataset. The risk score for each patient in the validation dataset was calculated from this model and dichotomized at the median. The estimated 2-year mortality (±SE) of patients with high-risk scores was 47.1% (±9.24%) compared with 6.35% (±4.42) for patients with low-risk scores. ROC analyses showed that the areas under the curve for the age, gender, and treatment modality-adjusted models with risk score [0.78; 95% confidence interval (CI), 0.74–0.86] and risk score plus tumor stage (0.79; 95% CI, 0.75–0.87) were substantially higher than for the model with tumor stage (0.54; 95% CI, 0.48–0.62).

Conclusions: We identified and validated a 13-gene signature that is considerably better than tumor stage in predicting survival of patients with HPV-negative OSCCs. Further evaluation of this gene signature as a prognostic marker in other populations of patients with HPV-negative OSCC is warranted.

Clin Cancer Res; 19(5); 1197–203. ©2012 AACR.
Translational Relevance

Mounting evidence suggests that head and neck squamous cell carcinoma is not a single disease but rather can be further subclassified molecularly. Subsite-specific etiologic and prognostic markers, such as human papillomavirus (HPV), are emerging. Patients with HPV-positive oropharyngeal carcinomas are known to have better treatment response and survival. In contrast, there are no currently defined molecular markers to assess treatment responsiveness for HPV-negative oral squamous cell carcinomas (OSCC). Recent works including our own have shown that gene expression profiling can identify subgroups of patients with poor survival. Thus, gene expression signatures show promise as clinical tools to help guide treatment intensification. In this study, we built on our previous molecular work to subclassify OSCCs and have identified and validated a prognostic 13-gene signature that showed a higher ability than tumor stage in predicting survival for patients with HPV-negative OSCCs. This is the first demonstration of a gene set that provides prognostic information beyond American Joint Committee on Cancer stage for this subgroup of tumors.

determine whether a subset of this signature could aid in predicting the survival of patients with HPV-negative OSCCs.

Materials and Methods

Datasets

Expression profile datasets from the Fred Hutchinson Cancer Research Center (FHCRC, Seattle, WA) and from the University of Texas M.D. Anderson Cancer Center (MDACC, Houston, TX) were used. The FHCRC dataset, used for discovery, comprised the gene expression profiles of 167 tumor samples from patients with OSCCs and 45 normal oral mucosa samples from patients without oral cancer, all treated at the University of Washington Medical Center (Seattle, WA), the Harborview Medical Center (Seattle, WA), or the Veterans Affairs Puget Sound Health Care System during 2003 to 2007. The FHCRC dataset also contained information on patient demographics, medical and lifestyle history, vital status, cause of death, and tumor characteristics, including tumor site, stage, and HPV status. The specimen collection, storage protocols and assays for specimen processing, and generation and normalization of raw expression data were conducted as previously described (8). The MDACC dataset, used for validation, contained the gene expression profiles of 103 samples generated from residual tumor tissue, taken from the MDACC Head and Neck Tumor Bank. Demographic characteristics, tumor site, stage, vital status, and cause of death were abstracted from the medical record and the MDACC Tumor Registry. The gene expression and other data from these tissue samples were analyzed under protocol DR09-0664, and de-identified data were shared with the FHCRC under MT2010-6749. Among 103 samples from the MDACC dataset, 74 were cancer samples from patients with OSCCs, 24 were matched normal oral samples from patients with OSCCs, and 5 were normal oral samples from other patients with cancer treated in the MDACC Head and Neck Center. The gene expression data obtained from the MDACC were extracted and normalized, also using the gcRMA algorithm but as implemented in Partek Genomics Suite software. The microarray data can be found in the Gene Expression Omnibus database under the accession numbers GSE41613 and GSE42743.

Statistical methods

Validation of the 131-gene signature association with survival. We have previously reported that the principal component (PC) scores calculated from the 131 genes were associated with OSCC-specific and overall survival (9). To validate these results in the MDACC dataset, we conducted a principal component analysis (PCA) of the 131 genes for all 103 samples using R software version 2.8.1, function prcomp. We dichotomized the first PC score at the median and compared the high and low PC score groups using Kaplan–Meier curves for overall survival and cumulative incidence curves for OSCC-specific mortality using R packages survival and cmprsk. We then conducted a Cox proportional hazard regression analysis comparing the group with high and low PC score.

Development of a 13-gene signature prediction model for survival in HPV-negative OSCC patients. To build a prediction model for OSCC-specific survival unique to the patients with HPV-negative OSCCs, we conducted regularized survival analysis using the data from 97 patients with high-risk HPV-negative OSCC in the FHCRC dataset. High-risk HPV that we tested included HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 as previously described (10). To select the most useful predictors among the 131 genes, we used the L1-penalized (Lasso) Cox proportional hazard regression model (R package glmnet; ref. 11). In this model, the L1 penalty parameter controls the sparsity (number of non-zero parameters or number of selected predictors) of the final estimated model. The larger this parameter is, the fewer the predictors will be selected. We used 10-fold cross-validation partial likelihood scores to choose the optimal L1 penalty parameter. Specifically, we first randomly split the data into 10 groups of roughly equal size. Then, for a given candidate L1 penalty parameter, we used data from 9 of the 10 groups to fit the model and calculated the corresponding partial likelihood score of the fitted model on the remaining group, and we repeated this for every possible collection of 9 groups. The final performance score of each candidate penalty parameter was the summation of the log partial likelihood scores from all 10 cross-validate iterations. We examined a sequence of candidate L1 penalty parameters and selected the one returning the best cross-validation performance score. We then refitted the model using all the data with the optimal L1 penalty parameter. The resulting model included 13 genes.
While L1-penalized regressions are helpful for variable selection, the coefficient estimation from such models tends to bias toward zero when a large degree of regularization (large value of L1 penalty parameter) is used to identify the best model. Therefore, to obtain good coefficient estimation for the 13 selected transcripts in the Cox regression model, we further fit a L2-penalized Cox regression model (12). Compared with the ordinary Cox regression model, the L2-penalized version provides good tradeoff between biases and variances and usually improves the prediction error. Again, the amount of L2 penalty applied was determined through a 10-fold cross-validation. With the estimated coefficients from the selected L2 regression model, a continuous risk score was generated for each subject based on the expression of the 13 genes. We then fit 3 Cox regression models, each with the following independent variables: (i) age, gender, treatment, 13-gene risk score; (ii) age, gender, treatment, stage; and (iii) age, gender, treatment, stage, 13-gene risk score. Tumor stage was dichotomized as American Joint Committee on Cancer (AJCC) stage I/II and III/IV, treatment was dichotomized as unimodality and multimodality, and age was categorized as <50, 50–59, 60–69, and ≥70 years.

Validation of the 13-gene risk score–based prediction models for oral cavity cancer–specific survival in the MDACC dataset. HPV status was not available in the MDACC dataset. We therefore included only patients with oral cavity cancer–specific survival in the MDACC/C21 and <modality, and age was categorized as

*Joint Committee on Cancer (AJCC) stage I/II and III/IV*, treatment was dichotomized as unimodality and multimodality, and age was categorized as <50, 50–59, 60–69, and ≥70 years.

Validation of the 13-gene risk score–based prediction models for oral cavity cancer–specific survival in the MDACC dataset. The expressions of the 13 genes were further validated by Gray (14).

Patient characteristics

Selected characteristics of the 97 patients with high-risk HPV-negative OSCCs from the FHCRC and 71 patients with oral cavity cancer from the MDACC studies are presented in Table 1. The majority of the patients from both institutions were older than 50 years. The proportions of males and of current smokers were comparable between the institutions. There was a higher proportion of advanced stage cancers than early-stage cancers and a higher proportion of patients treated with multimodality than unimodality in both institutions. The patients with OSCCs from the 2 institutions were followed for similar lengths of time: median of 26.3 months (range, 0.2–64.9 months) at the FHCRC and median of 26.9 months (range, 0.2–92.6 months) at the MDACC, among patients who were known to be alive at the end of the respective studies.

Survival analysis of the 131-gene signature using the MDACC dataset

Figure 1 shows Kaplan–Meier curves of overall survival and cumulative incidence curves for OSCC-specific mortality for the high and low first PC score groups. Patients with high PC scores had significantly poorer overall survival than the patients with low first PC scores ($P = 0.0075$). At 3 years, the estimated proportion dying ($\pm SE$) from all causes
for patients with high PC scores was 57.6% (±10.4%) compared with 20.3% (±7.4%) for the patients with low PC scores. The cumulative OSCC-specific mortality was also significantly higher for the patients with high PC scores than for the patients with low PC scores (P = 0.031). The estimated cumulative OSCC-specific mortality (±SE) at 3 years for patients with high PC scores was 44.6% (±8.7%) compared with 24.1% (±9.8) for patients with high PC scores. Cox proportional hazard regression analyses showed that patients with high PC scores, compared with patients with low PC scores, had significantly higher overall and OSCC-specific mortality, with HRs of 2.98 (95% CI, 1.53–5.81) and 3.41 (95% CI, 1.38–8.40), respectively. The HRs remained significant after adjusting for age, gender, and tumor stage. The respective adjusted HRs were 2.64 (95% CI, 1.34–5.22) and 3.12 (95% CI, 1.24–7.87) for overall and OSCC-specific mortality.

### Development and validation of a 13-gene prediction model for the survival of HPV-negative OSCC patients

Using an L1-penalized Cox proportional hazard regression method on the FHCRC dataset, we identified a prediction model with 13 genes. The gene symbols and coefficients from the L2-penalized Cox regression model of the 13 genes are presented in Table 2.

Figure 2 shows cumulative incidence curves for oral cavity cancer–specific mortality by high- and low-risk scores in the MDACC dataset. Patients with high risk scores had significantly higher oral cavity cancer–specific mortality than patients with low-risk scores (P = 0.0253). The estimated proportions of patients dying from oral cavity cancer (±SE) at 2 years for high- and low-risk scores were 47.1% (±9.24%) and 6.35% (±4.42%), respectively. The unadjusted HR from a Cox proportional hazard regression analysis comparing patients with high- and low-risk scores was 3.61 (95% CI, 1.45–9.01; P = 0.0059). The HR adjusting for age, gender, tumor stage, and treatment modality was also significant (HR, 3.72; 95% CI, 1.37–10.10; P = 0.0099). The ROC curves for survival from patients with oral cavity cancer at 2 years for 3 prediction models (stage, stage plus risk...
score, and risk score) adjusting for age, gender, and treatment modality are shown in Fig. 3. The AUC for the prediction model with tumor stage was 0.54 (95% CI, 0.48–0.62). Incorporating a term for the 13-gene risk score into the model with tumor stage substantially and significantly improved the ability to predict survival from oral cavity cancer at 2 years from 0.54 to 0.79 (95% CI, 0.75–0.87; \( P < 0.001 \)). The risk score itself, without tumor stage, also had a significantly higher predictability of survival in patients with oral cavity cancer than the tumor stage; the AUC for the model with risk score was 0.78 (95% CI, 0.74–0.86) compared with 0.54 for the model with tumor stage (\( P < 0.001 \)).

### Table 2. Thirteen genes used in the model for prediction of survival from oral cavity cancer

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene Symbol</th>
<th>Prediction model coefficient(^a)</th>
<th>Fold change(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1552283_s_at</td>
<td>ZDHHC11</td>
<td>0.000443</td>
<td>0.30</td>
</tr>
<tr>
<td>1554008_at</td>
<td>OSMR</td>
<td>0.231391</td>
<td>2.54</td>
</tr>
<tr>
<td>1568768_at</td>
<td>SERPINE1</td>
<td>0.383316</td>
<td>2.34</td>
</tr>
<tr>
<td>201108_s_at</td>
<td>THBS1</td>
<td>0.205039</td>
<td>2.35</td>
</tr>
<tr>
<td>201497_x_at</td>
<td>MYH11</td>
<td>-0.206856</td>
<td>0.27</td>
</tr>
<tr>
<td>205200_at</td>
<td>CLEC3B (TNA)</td>
<td>-0.131867</td>
<td>0.37</td>
</tr>
<tr>
<td>207517_at</td>
<td>LAMC2</td>
<td>-0.008777</td>
<td>2.80</td>
</tr>
<tr>
<td>209900_s_at</td>
<td>SLC16A1</td>
<td>0.171380</td>
<td>2.08</td>
</tr>
<tr>
<td>210797_s_at</td>
<td>OASL</td>
<td>0.216877</td>
<td>3.76</td>
</tr>
<tr>
<td>222344_at</td>
<td>NREP</td>
<td>0.199328</td>
<td>1.72</td>
</tr>
<tr>
<td>230104_s_at</td>
<td>TPPP</td>
<td>-0.124634</td>
<td>0.47</td>
</tr>
<tr>
<td>240000_at</td>
<td>LIPI</td>
<td>0.864964</td>
<td>0.95</td>
</tr>
<tr>
<td>242417_at</td>
<td>LOC283278</td>
<td>-0.438477</td>
<td>0.67</td>
</tr>
</tbody>
</table>

\(^{a}\)For the genes with positive coefficients, a higher expression level was associated with a higher risk of OSCC-specific mortality; and, for the genes with negative coefficients, a lower expression level was associated with a higher risk of OSCC-specific mortality.

\(^{b}\)Fold changes comparing patients with high-risk score to patients with low-risk score, based on the original expression scale.

### Validation with Nanostring nCounter Gene Expression assay

We found good-to-excellent correlation between gene expression level from Affymetrix array and from Nanostring nCounter assay across samples for all probes except for LIPI. The correlation coefficient for each gene was as follow: 0.96 (OASL), 0.94 (SERPINE1), 0.92 (MYH11), 0.89 (THBS1), 0.86 (SLC16A1), 0.86 (LAMC2), 0.85 (OSMR).
0.80 (TPPP), 0.75 (ZDHHC11), 0.71 (NREP), 0.67 (LOC283278), 0.66 (CLEC3B), and 0.06 (LIPI).

Discussion
We previously reported on a 131-gene signature which identified patients with OSCCs with poor survival and that, when used in combination with AJCC stage, predicted survival better than AJCC stage alone (9). In this study, we showed that this 131-gene signature is also predictive of OSCC survival in an independent dataset from the MDACC. Moreover, in keeping with the fact that HPV is already a useful biomarker for oropharyngeal carcinomas (19) and the that a survival gene signature would be most relevant for HPV-negative OSCCs, we used the 131 genes to identify a subset of genes, which included 13 genes, that was most predictive of OSCC-specific survival in patients with HPV-negative OSCCs. We showed that this 13-gene signature is strongly associated with OSCC-specific survival in the MDACC dataset and that OSCC-specific survival estimates using this signature were substantially better than those using AJCC stage alone. These findings represent the first development and validation of a survival gene signature for HPV-negative OSCCs using independent datasets from 2 institutions and document the generalizability of our previous 131-survival gene signature for patients with OSCCs. If further validated in multicenter studies, the 13-gene signature may help physicians and patients make better personalized treatment choices. For example, patients with stage I/II disease that test in the high-risk category could be considered for treatment intensification with multimodal therapy and vice versa for those with stage III/IV disease.

In our previous study, we had used stepwise regression to identify the following subset of genes from the 131-gene signature that were most strongly associated with OSCC-specific survival while adjusting for age, sex, stage, HPV status, treatment intensity, and comorbidity (9): LAMC2, OSMR, SERPIN1, OASL, SLC16A1, KL7, THBS1, HOMER3, GRP58, PDPN, AKRD35, CDH3, and EPS8L1. In this study, we also sought to narrow our list of genes, but with 2 main analytic differences: (i) we restricted the cohort to patients with HPV-negative OSCC in the training set and (ii) the methodology to identify the subset of 13 genes was substantially different. Still, 6 genes overlapped between these 2 analyses (LAMC2, OSMR, SERPIN1, THBS1, SLC16A1, and OASL) and all but one of these were among the genes most strongly associated with survival in our previous report. These genes all play a role in cell invasion and motility, cell-to-cell signaling, signal transduction, and proliferation, processes essential to metastasis and cancer progression (20–28).

Mutation in this gene has been identified myosin 5A as a potential biomarker of occult metastasis in OSCCs (31). Mutations in this gene have been found to be associated with intestinal, breast, and prostate cancer (32, 33). TPPP is thought to play a role in microtubule assembly and stabilization (34), and a gain in its chromosomal location 5p15.33 has been associated with high-grade bladder cancer (35). Interestingly, ZDHHC11, in our 13-gene signature, shares this location with TPPP.

A limitation of our study is the lack of information on HPV status in the MDACC dataset that we used for validation. As oral cavity tumors are much less likely to be associated with HPV infection (36, 37), we limit the validation set to oral cavity tumors only.

In conclusion, we have identified and validated a 13-gene expression signature that is strongly predictive of survival in patients with HPV-negative OSCCs. Importantly, (i) the signature adds substantial predictive information beyond stage, and (ii) the small number of genes in the signature should make it more amenable to develop a cost-effective clinical test. Together, these data provide strong justification to proceed with multi-institutional trials to determine whether this signature is effective as a prognostic marker.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: P. Lohavanichbutr, E. Méndez, J. Houck, S.M. Schwartz, P. Wang, C. Chen
Development of methodology: J. Houck, S.M. Schwartz, P. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Méndez, F.C. Holsinger, J. Houck, M.P. Upton, N. Futran, S.M. Schwartz, C. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Méndez, F.C. Holsinger, T.C. Rue, Y. Zhang, N. Futran, S.M. Schwartz, P. Wang, C. Chen
Writing, review, and/or revision of the manuscript: P. Lohavanichbutr, E. Méndez, T.C. Rue, M.P. Upton, N. Futran, S.M. Schwartz, P. Wang, C. Chen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases) P. Lohavanichbutr, E. Méndez, C. Chen
Study supervision: S.M. Schwartz, C. Chen
Review of pathology findings that assisted in selection of cases: M.P. Upton

Acknowledgments
The authors express their deepest appreciation to the study participants and their families for their contribution to this study. They also thank Kevin R. Coombes, PhD, of the MD Anderson Cancer Center for his mentorship to FCH and related contributions to this work.

Grant Support
The study at FHCRC was supported by grants from the NIH, National Cancer Institute (NIH NCI R01CA095419), National Center for Research Resources grant 1KL2RR025015-01, Amos Medical Faculty Development Program Award from the Robert Wood Johnson Foundation, and by institutional funds from the Fred Hutchinson Cancer Research Center. The study at the MD Anderson Cancer Center was supported by Specialized Program of Research Excellence in Head and Neck Cancer Grant P50CA97007 from the National Cancer Institute, “Clinician Investigator Program in Translational Research” K12CA088084, Clinical Research Program 2 L30CA117652-02A1, and THANC Foundation Young Investigator Award in Head and Neck Cancer.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

1202 Clin Cancer Res; 19(5) March 1, 2013
Clinical Cancer Research
advertising in accordance with 18 U.S.C. Section 1733 solely to indicate this fact.

References


