A Genetic Expression Profile Associated with Oral Cancer Identifies a Group of Patients at High Risk of Poor Survival

Eduardo Méndez,1,5,6 John R. Houck,6 David R. Doody,6 Wenhong Fan,7 Pawadee Lohavanichbutr,6 Tessa C. Rue,2 Bevan Yueh,6 Neal D. Futran,1 Melissa P. Upton,3 D. Gregory Farwell,9 Patrick J. Heagerty,2 Lue Ping Zhao,2,7 Stephen M. Schwartz,4,6 and Chu Chen1,4,6

Abstract

Purpose: To determine if gene expression signature of invasive oral squamous cell carcinoma (OSCC) can subclassify OSCC based on survival.

Experimental Design: We analyzed the expression of 131 genes in 119 OSCC, 35 normal, and 17 dysplastic mucosa to identify cluster-defined subgroups. Multivariate Cox regression was used to estimate the association between gene expression and survival. By stepwise Cox regression, the top predictive models of OSCC-specific survival were determined and compared by receiver operating characteristic analysis.

Results: The 3-year overall mean ± SE survival for a cluster of 45 OSCC patients was 38.7 ± 0.09% compared with 69.1 ± 0.08% for the remaining patients. Multivariate analysis adjusted for age, sex, and stage showed that the 45 OSCC patient cluster had worse overall and OSCC-specific survival (hazard ratio, 3.31; 95% confidence interval, 1.66-6.58 and hazard ratio, 5.43; 95% confidence interval, 2.32-12.73, respectively). Stepwise Cox regression on the 131 probe sets revealed that a model with a term for LAMC2 (laminin γ2) gene expression best identified patients with worst OSCC-specific survival. We fit a Cox model with a term for a principal component analysis-derived risk score marker and two other models that combined stage with either LAMC2 or PCA. The area under the curve for models combining stage with either LAMC2 or PCA was 0.80 or 0.82, respectively, compared with 0.70 for stage alone (P = 0.013 and 0.008, respectively).

Conclusions: Gene expression and stage combined predict survival of OSCC patients better than stage alone.

Although advances in surgical techniques and the use of adjuvant treatment modalities have led to some site-specific improvements in survival of patients with oral squamous cell carcinoma (OSCC), the overall prognosis for advanced-stage disease has not improved significantly in the past two decades (1). One of the impediments to the effective management of OSCC patients is our limited ability to predict the natural history of individual lesions. Unfortunately, the current head and neck cancer staging system is inadequate for predicting survival outcomes, and there seems to be significant clinical and molecular heterogeneity within stages (2, 3). However, to date, there are no molecular markers that are used clinically to stratify OSCC and other head and neck cancer patients. Recently, many studies have used high-throughput microarray technology in an attempt to identify the different genetic pathways involved in the carcinogenic process and to relate gene expression signatures to clinical outcomes (4–7). Gene expression profiling of OSCC would be most useful if it could add to our existing staging system to predict clinical outcomes more accurately, yet no studies to date have addressed this question.

We recently identified 131 probe sets (corresponding to 108 known genes), which were differentially expressed between OSCC and normal oral mucosa (8). In this article, hierarchical clustering and principal component analyses (PCA) of OSCC, dysplasia, and normal oral mucosa using these 131 probe sets revealed that oral dysplasias appear to have varied expression patterns such that some clustered with OSCC and others with normal oral mucosa. We then tested the hypothesis that there might be a spectrum of oral carcinogenesis based on these 131 probe sets and that OSCC, which are least “dysplasia-like” in

Authors’ Affiliations: Departments of 1Otolaryngology-Head and Neck Surgery, 2Biostatistics, 3Pathology, and 4Epidemiology, University of Washington; 5Surgery and Perioperative Care Service, VA Puget Sound Health Care System; Programs in 6Epidemiology and 7Biostatistics and Biomathematics, Fred Hutchinson Cancer Research Center, Seattle, Washington; 8Department of Otolaryngology-Head and Neck Surgery, University of Minnesota, Minneapolis, Minnesota; and 9Department of Otolaryngology: Head and Neck Surgery, University of California at Davis, Sacramento, California

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Requests for reprints: Chu Chen, Program in Epidemiology, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, M5-C800, P.O. Box 19024, Seattle, WA 98109-1024. Phone: 206-667-6644; Fax: 206-667-2537; E-mail: cchen@fhcrc.org.

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Translational Relevance

We set out to determine if oral squamous cell carcinoma (OSCC) could be further subclassified based on 131 probe sets (108 known genes), which we found previously to be differentially expressed between OSCC and normal controls, and whether this subclassification was associated with survival. In this study, we found that (a) there were significant survival differences in cluster analysis-defined OSCC subgroups; (b) this classification is independently associated with overall and OSCC-specific survival after adjustment for potential confounders such as age, sex, stage, tumor site, human papillomavirus status, and treatment intensity; and (c) genetic expression data and American Joint Committee on Cancer (AJCC) stage combined predict survival of OSCC patients better than AJCC stage alone. To our knowledge, this is the largest study of this kind for oral cancer and the only one that describes an association between gene expression profiling and OSCC-specific survival. This study is prospective and predictive of clinical outcomes and it surpasses other published studies in its effort to address how gene expression data can complement AJCC stage in predicting survival—a key and novel contribution to the field of oral and head and neck cancer.

gene expression, are those that are further along in the carcinogenic process and thus are associated with worse survival.

Materials and Methods

Study population. As described by Chen et al., we identified English-speaking patients ages ≥18 years with a first, primary OSCC or dysplasia undergoing surgery or biopsy between December 16, 2003 and April 17, 2007 at one of the three University of Washington-affiliated hospitals: University of Washington Medical Center, Harborview Medical Center, and VA Puget Sound Health Care System. Eligible controls were patients who were scheduled to undergo surgery of the oral cavity or oropharynx for noncancer treatment, such as tonsillectomy or sleep apnea, at the aforementioned institutions during the same period the cases were recruited. All patients recruited to the study were interviewed in person using a structured lifestyle and medical history questionnaire. Data regarding tumor characteristics, such as stage, were abstracted from medical records. Comorbidity scores were calculated using Adult Comorbidity Evaluation-27 Test (9, 10). Patients were followed actively through telephone contact and passively through review of medical records and linkage to the U.S. Social Security Death Index. If a patient had died, we classified the death as due to OSCC or not due to OSCC based on review of medical records and death certificates. All participants gave informed consent, and all study procedures were approved by the institutional review boards of the Fred Hutchinson Cancer Research Center, University of Washington, and VA Puget Sound Health Care System.

Of the 187 OSCC patients we recruited, we had Affymetrix HG-U133 Plus 2.0 Plus array data that had passed our quality-control criteria and at least 4 months of follow-up time for 150 patients. The requirement for our study participants to have at least 4 months of follow-up refers to the starting point at which we would began to capture events. This was done because we did not want to include any events until participants had completed treatment to avoid capturing deaths from patients who died due to comorbidities rather than tumor biology. We also included array results from 17 dysplasias (11 dysplasia patients and an additional 6 dysplastic lesions from 5 OSCC patients; of these 5 OSCC patients, only 1 invasive tumor tissue was included among the 150 OSCC cases) and 35 normal oral mucosa from controls. All samples were collected, processed, and hybridized onto Affymetrix HG-U133 2.0 Plus oligonucleotide arrays as described in Chen et al. (8). In addition, all cases were typed for human papillomavirus (HPV) using the Linear Array HPV Genotyping Test (Roche) containing complementary sequences to the PCR products for 37 HPV genotypes (including the 13 “high risk” genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) under a research use only agreement as described in Lohavanichbutr et al. (11).

Generation of the 131-probe set list. The 131-probe set list was obtained by comparing the differential gene expression between 119 OSCC cases and 35 normal controls as described in Chen et al. (8).

Hierarchical clustering and PCA. Supervised hierarchical clustering analysis and PCA of the expression data from 119 OSCC and 35 controls used to generate the 131-probe set list in our previous study, plus additional 17 dysplasias, were done using GeneSpring GX Software version 7.3.1 (Silicon Genetics).

Differential gene expression among OSCC. To identify subgroups of the 119 OSCC cases based on differential gene expression values from GC Robust Multiarray Average, we used a regression-based approach implemented in GenePlus software (12). For this comparison, we used the number of false discoveries as the type I error selection criterion (13). Gene ontology and pathway analysis for the resultant list of genes was done using Ingenuity Pathway Analysis software version 6.

Survival analysis. Follow-up time for analyses of survival for the 119 OSCC cases was calculated from the date of surgery to the date of death, loss to follow-up, or April 30, 2007, whichever came first, according to the Kaplan-Meier method. Differences between groups were assessed with the log-rank test. We did not compute OSCC-specific Kaplan-Meier survival estimates because of possible informative censoring due to death from other causes. Rather, we estimated OSCC-specific cumulative mortality using methods described by Kalbfleisch and Prentice, which account for competing risk events (14, 15). Cox proportional hazards regression model was used to estimate overall and OSCC-specific survival associations with cluster-defined OSCC subgroup status, age, sex, stage, HPV status, tumor site, treatment intensity [defined as receiving one, two, or all three different treatment modalities (surgery, radiation, or chemotherapy)], and comorbidity score. Dummy variables were created for cluster-defined OSCC subgroup status, stage, sex, HPV status (none versus positive/low-risk versus positive/high-risk), tumor size, nodal status, tumor site (oral versus oropharyngeal), and comorbidity score. These statistical analyses were conducted using STATA software version 9.2.

Prediction model building for OSCC-specific mortality. For this analysis, we used a total of 150 OSCC cases: 119 cases that have been used to derive the 131 probe sets in our previous study (8) plus an additional 31 cases that were recruited thereafter for which we had vital status information and at least 4 months of follow-up. We used stepwise Cox proportional hazards regression based on the 131 probe sets found previously by us to be differentially expressed between OSCC cases and controls (SAS version 9.2; ref. 8). For the stepwise regression, the significance level for both entrance and exit were each set at $\alpha = 0.01$. To obtain the top 10 models, we conducted 10 sequential stepwise regression procedures, with each successive procedure eliminating the selected probe set(s) from the previous procedure. Individual risk scores from the top probe set Cox regression model were compared graphically with scores from the first and second principal components from PCA of the 131 probe sets using Matlab version R2006b.

Comparing survival prediction models with tumor-node-metastasis stage. To assess whether a survival model that incorporates gene expression data is better than one without it, we used an adapted
receiver operating characteristic (ROC) analysis (16). Risk scores were calculated for five models. The first three models contained the terms: “stage”; “gene(s) from top prediction model”; and “PCA,” a score representing the expression of the entire 131 probe sets as summarized by the combination of the first and second principal components. The other two models combined the term “stage” with either of the other two terms. For each model, we constructed ROC curves for predicting 2-year all-cause survival. At each level of the model-derived risk score, the nearest 10% (using nearest-neighbor estimation) was used to estimate true-positive and false-positive rates. The survival ROC package, available for R project software, was used to implement these methods. The area under the curve (AUC) was calculated to quantify the ability of each model to predict 2-year survival. One thousand bootstrap samples were generated to estimate SE and 95% confidence interval for AUC estimates and to obtain P values for testing the null hypothesis that specific gene expression values or PCA do not add to ability of stage to predict survival.

To reduce the overoptimism of ROC and AUC estimates due to using the same data to both estimate and assess the predictive ability of risk scores, we performed a jackknife leave-one-out analysis (17). Variable estimates for the risk model were obtained excluding one subject, and the resulting risk model was used to estimate a risk score based on the excluded subject’s gene expression and/or stage characteristics. This process was repeated until risk scores were assigned to each subject. ROC and AUC estimates were calculated for these jackknife risk scores as they were for the original risk scores.

**Validation of LAMC2, OSMR, SERPINE1, and OASL by quantitative reverse transcription-PCR.** We used quantitative reverse transcription-PCR (RT-PCR) to validate the expression of the four genes found to be related to survival in our top two models. Sixty samples were chosen at random for testing. Each sample was assayed in triplicate in 10 μL reaction volumes using the Quantitect SYBR Green RT-PCR kit (Qiagen) and bioinformatically validated Quantitect primers (Qiagen) on a 7900HT Sequence Detection System (ABI). The cycling conditions were as follows: 30 min incubation at 50°C, 15 min incubation at 95°C, and 40 cycles each of 15 s at 94°C, 30 s at 55°C, and 30 s at 72°C. The fragment amplified included (a) a 74-bp amplicon spanning exons 18 and 19 for LAMC2 (NM_005562); (b) a 98-bp amplicon spanning exons 4 and 5 for OASL (NM_003733); (c) a 113-bp amplicon spanning exons 13 and 14 for OSMR (NM_003999); (d) a 105-bp amplicon spanning exons 3 and 4 for SERPINE1 (NM_000602); and ACTB, a 146-bp amplicon spanning exons 3 and 4, as the reference gene. Ten point standard curves were generated using Universal Human Reference RNA (Stratagene) for all genes. The linear regression coefficient (\( R^2 \)) was ≥0.99 for all runs. The mean threshold cycles (Ct) values were calculated from the triplicate Ct values. Samples that had Ct values with SD > 0.35 in their triplicate run were repeated. Mean Ct values were standardized to the mean Ct value of ACTB.

**Results**

**Study population.** The characteristics of the study participants are shown in Supplementary Table S1. In general, the OSCC cases tended to be older and male and more likely to be current smokers when compared with controls. The majority of the OSCC cases had advanced-stage disease [approximately two-thirds with American Joint Committee on Cancer (AJCC) stage III and IV].

**Hierarchical cluster and PCA.** Results from a supervised hierarchical cluster analysis of the 119 OSCC cases, 35 normal controls, and 17 dysplastic lesions using the 131 probe sets are shown in Fig. 1. Although OSCC cases largely clustered separately from controls, 7 OSCC cases clustered with the controls. One cluster of genes (Fig. 1, cluster 1) appears to show an increasing gradient of down-regulation progressing from normal to dysplastic to invasive lesions. Notably, neither the dysplasias nor those OSCC that misclassified with the normal controls showed consistent down-regulation of these genes. In particular, this group of 12 probe sets (corresponding to nine genes) was completely down-regulated in a subset of 45 OSCC (Fig. 1, cluster 1; Supplementary Table S2). We therefore hypothesized that the gene expression signature of this group of 45 OSCC represents one end of a continuum of gene expression that is characteristic of increasingly aggressive neoplastic behavior.

Figure 2 shows the results of a PCA on the 131-probe set expression data based on the samples’ phenotype (normal, dysplasia, or cancer). The first principal component, which accounts for the greatest amount of variability, captured 60.26% of the variance, whereas the second principal component captured 5.66%. Based on these two components alone, the controls and OSCC cases are at opposite ends of the spectrum with dysplasia samples in between (Fig. 2). In addition, the same group of 45 OSCC samples identified in the hierarchical cluster analysis is at one extreme based on the first principal component scores (Fig. 2). Although some dysplastic lesions have first principal component scores that overlap with OSCC, none reached the first principal component scores of the group of 45 OSCS samples.

**Differential expression of the 45-sample subcluster.** This cluster-defined OSCC subgroup was initially identified largely based on a qualitative analysis of the expression of a group of 12 down-regulated probe sets (Fig. 1, cluster 1). We used a linear regression model to more rigorously determine which probe sets were differentially expressed in this subcluster compared with the rest of the OSCC cases. After adjusting for age and sex, we detected 62 of the 131 probe sets to be differentially expressed between these two groups (number of false discoveries = 1; Supplementary Table S2). Therefore, although the 12 down-regulated probe sets represent the most obvious change in expression in these 45 samples, nearly one-half of the 131 probe sets shows a distinctive signature in this subcluster.

**Survival analysis.** The patient characteristics for this subcluster compared with those of the rest of the cases are shown in Table 1. Patients in the 45-sample subcluster had more advanced disease, as determined by both tumor size and nodal metastasis, and were less likely to have tumors containing high-risk HPV types. The range of follow-up time for patients known to be alive at the end of the study was 10.7 to 38.7 months with a median of 22.2 months. To test the hypothesis that this 45-sample subcluster had a more aggressive phenotype, we compared Kaplan-Meier survival curves for overall survival (Fig. 3A). The 3-year mean ± SE overall survival for the 45-sample subcluster was 38.7 ± 0.09% compared with 69.1 ± 0.08% (\( P = 0.0001 \)) for the other 74 samples. The estimated cumulative mortality ± SE due to OSCC at 3 years for the 45-sample subcluster was 45.7 ± 0.09% compared with 16.8 ± 0.06% (\( P = 0.0003 \)) for the other 74 samples (Fig. 3B). We estimated hazard ratios for both overall and OSCC-specific mortality, adjusting for AJCC stage, age, and sex. Patients from the 45-sample subcluster had a significantly higher rate of death due to both overall (hazard ratio, 3.31; 95% confidence interval, 0.09% compared with 69.1 ± 0.08% (\( P = 0.0001 \)) for the other 74 samples. The estimated cumulative mortality ± SE due to OSCC at 3 years for the 45-sample subcluster was 45.7 ± 0.09% compared with 16.8 ± 0.06% (\( P = 0.0003 \)) for the other 74 samples (Fig. 3B). We estimated hazard ratios for both overall and OSCC-specific mortality, adjusting for AJCC stage, age, and sex. Patients from the 45-sample subcluster had a significantly higher rate of death due to both overall (hazard ratio, 3.31; 95% confidence interval,
1.66-6.58) and OSCC-specific mortality (hazard ratio, 5.43; 95% confidence interval, 2.32-12.73). These associations continued to be elevated following additional adjustment for HPV status (hazard ratio, 3.43; 95% confidence interval, 1.68-6.99 and hazard ratio, 6.09; 95% confidence interval, 2.48-14.97, respectively). In addition, we adjusted for tumor site and treatment intensity separately and the results did not change appreciably (data not shown). Although a higher comorbidity score was statistically significantly associated with both mortality outcomes, it neither confounded nor improved the precision of the association with OSCC subcluster when included in the Cox regression model (data not shown).

**Prediction models.** We performed a stepwise Cox proportional hazards regression based on these 131 probe sets to determine which, if any, were associated with OSCC-specific survival. Of 150 patients, 109 were alive and 41 had died at the end of the follow-up period (Supplementary Table S1). Among these, there were 27 OSCC-specific deaths, 10 non-OSCC-specific deaths, and 4 deaths of unknown causes. We found that a model containing LAMC2 (laminin g2) alone performed best at identifying patients with the worst OSCC-specific survival. The subsequent nine models that were identified through our stepwise approach are shown in Table 2.

The three-dimensional plot (Supplementary Fig. S1) shows that the risk scores from our top model (0.59151* LAMC2) are highly correlated with the risk scores from models containing terms for the first and second principal components from the analysis of the 131 probe sets. In addition, those patients with the highest risk scores from either the top model or the principal component models are mostly the ones in the cluster-defined group of 45 patients.
Comparing survival prediction models with AJCC stage. Results from the ROC analysis for each of the five models described above are shown in Fig. 4. The AUCs for models with either gene expression alone or in combination with stage were higher than for a model with stage alone (Fig. 4A and B). The differences in the AUCs between models with “stage” plus either “LAMC2” or “PCA” and stage alone were statistically significant ($P = 0.013$ and 0.008, respectively). The AUCs from the jackknife leave-one-out analyses (0.81 for the model with “stage” and “LAMC2” and 0.79 for the model with “stage” and “PCA”) were virtually the same as those estimated using conventional methods.

![PCA using the 131 probe sets. The first principal component is plotted on the $X$ axis and captures 60.26% of the variance. The second principal component is plotted on the $Y$ axis and captures 5.66% of the variance.](image)

**Fig. 2.** PCA using the 131 probe sets. The first principal component is plotted on the $X$ axis and captures 60.26% of the variance. The second principal component is plotted on the $Y$ axis and captures 5.66% of the variance.

### Table 1. Characteristics of two cluster-defined OSCC subgroups

<table>
<thead>
<tr>
<th></th>
<th>Group of 45 ($n = 45$), $n$ (%)</th>
<th>Group of 74 ($n = 74$), $n$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-39</td>
<td>4 (8.9)</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>40-49</td>
<td>8 (17.8)</td>
<td>10 (13.5)</td>
</tr>
<tr>
<td>50-59</td>
<td>12 (26.7)</td>
<td>28 (37.8)</td>
</tr>
<tr>
<td>60-88</td>
<td>21 (46.7)</td>
<td>35 (47.3)</td>
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<tr>
<td><strong>Sex</strong></td>
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</tr>
<tr>
<td>Male</td>
<td>29 (64.4)</td>
<td>55 (74.3)</td>
</tr>
<tr>
<td>Female</td>
<td>16 (35.6)</td>
<td>19 (25.7)</td>
</tr>
<tr>
<td><strong>AJCC stage</strong></td>
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</tr>
<tr>
<td>I</td>
<td>7 (15.6)</td>
<td>21 (28.4)</td>
</tr>
<tr>
<td>II</td>
<td>3 (6.7)</td>
<td>11 (14.9)</td>
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<tr>
<td>III</td>
<td>7 (15.6)</td>
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<tr>
<td>IV</td>
<td>28 (62.2)</td>
<td>33 (44.6)</td>
</tr>
<tr>
<td><strong>Tumor size</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_1/T_2$</td>
<td>22 (48.9)</td>
<td>56 (75.7)</td>
</tr>
<tr>
<td>$T_3/T_4$</td>
<td>23 (51.1)</td>
<td>18 (24.3)</td>
</tr>
<tr>
<td><strong>Nodal status</strong></td>
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<td></td>
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<tr>
<td>$N_0$</td>
<td>15 (33.3)</td>
<td>40 (54.1)</td>
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<tr>
<td>$N_1$</td>
<td>30 (66.7)</td>
<td>34 (45.9)</td>
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<tr>
<td><strong>HPV status</strong></td>
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<td></td>
</tr>
<tr>
<td>HPV negative</td>
<td>35 (77.8)</td>
<td>43 (58.1)</td>
</tr>
<tr>
<td>High-risk HPV positive</td>
<td>9 (20)</td>
<td>30 (40.5)</td>
</tr>
<tr>
<td>Low-risk HPV positive</td>
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<td>1 (1.4)</td>
</tr>
<tr>
<td><strong>Vital status</strong></td>
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<td></td>
</tr>
<tr>
<td>Alive</td>
<td>21 (46.7)</td>
<td>59 (79.7)</td>
</tr>
<tr>
<td>Dead-OSCC</td>
<td>17 (37.8)</td>
<td>9 (12.2)</td>
</tr>
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<td>6 (8.1)</td>
</tr>
<tr>
<td>Dead-unknown cause</td>
<td>4 (8.9)</td>
<td>0 (0)</td>
</tr>
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Validation of LAMC2, OSMR, SERPINE1, and OASL by quantitative RT-PCR. The correlation coefficients for LAMC2, OSMR, SERPINE1, and OASL between microarray and quantitative RT-PCR expression data for the 60 samples assayed were 0.65, 0.14, 0.74, and 0.89, respectively. Thus, with the exception of OSMR, our quantitative RT-PCR results were well correlated with those from microarray analyses.

Discussion

In this study, we focused on the expression of 131 probe sets that we found previously to be highly associated with OSCC (8) and show that OSCC can be further subclassified based on this gene expression signature. Moreover, this classification is independently associated with overall and OSCC-specific survival after adjustment for potential confounders such as age, sex, stage, tumor site, HPV status, and treatment intensity. Interestingly, none of the dysplastic lesions overlapped with the group of 45 OSCC cases based on the first principal component (Fig. 2), suggesting that this 45-sample subcluster represents a more invasive phenotype. This finding suggested to us that there might be a trend of differential expression of these 131 probe sets in OSCC, such that the varying degrees of up-regulation or down-regulation of some genes might be of prognostic significance. The observation that the score ("PCA") that summarized the expression levels of all 131 probe sets as a combination of the first and second principal components was significantly associated with overall survival supports this hypothesis.

There are various risk factors that may be of potential significance in our cohort. First, HPV infection is now emerging as a potentially important predictor of prognosis in patients with oropharyngeal squamous cell carcinoma (18). However, we did not find HPV to either be independently associated with survival (data not shown) or confound the association between gene expression and survival. This is likely due to the fact that the majority of our patients (73%) had oral cavity (as opposed to oropharyngeal) tumors where HPV status has not been shown to play a significant role. Another potential risk factor in our cases that deserves further clarification is treatment. It is possible that treatment modality could modify the association between gene expression data and survival, but our study lacked sufficient numbers to test this hypothesis. However, evidence from randomized clinical trials strongly indicates that the different treatment modalities for advanced head and neck cancer do not show significant differences in survival (19–21). Thus, we would

![Fig. 3. Survival and OSCC-specific mortality estimates in OSCC patients. The two groups were identified with hierarchical clustering analysis using the 131 differentially expressed probe sets in invasive OSCC as described in the text. A, Kaplan-Meier analysis of all-cause mortality. Vertical marks, censored events. B, cumulative incidence of OSCC-specific mortality.](https://www.aacrjournals.org)

Table 2. Top 10 multivariate Cox regression models of OSCC-specific survival

<table>
<thead>
<tr>
<th>Model</th>
<th>Gene symbol (Affymetrix probe set ID)</th>
<th>Model coefficients</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>LAMC2 (207517_at)</td>
<td>0.59151*LAMC2</td>
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<tr>
<td>2</td>
<td>OSMR (1554008_at)</td>
<td>0.42485<em>OSMR + 0.40482</em>SERPINE1 + 0.33483*OASL</td>
</tr>
<tr>
<td>3</td>
<td>SERPINE1 (1568765_at)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>OASL (210797_at)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>SLC16A1 (209900_s_at)</td>
<td>0.81478*SLC16A1</td>
</tr>
<tr>
<td>6</td>
<td>KLF7 (1555420_at)</td>
<td>0.60694*KLF7</td>
</tr>
<tr>
<td>7</td>
<td>THBS1 (201108_s_at)</td>
<td>0.44241<em>THBS1 + 0.43257</em>SLC16A1</td>
</tr>
<tr>
<td>8</td>
<td>SLC16A1 (202235_at)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>HOMER3 (204647_at)</td>
<td>0.66632*HOMER3</td>
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<tr>
<td>10</td>
<td>GRP68 (229055_at)</td>
<td>0.63313*GRP68</td>
</tr>
<tr>
<td>11</td>
<td>PDPN (204879_at)</td>
<td>0.51904*PDPN</td>
</tr>
<tr>
<td>12</td>
<td>ANKRD3S (231118_at)</td>
<td>0.58503*ANKRD3S</td>
</tr>
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<td>13</td>
<td>CDH3 (203256_at)</td>
<td>0.75146<em>CDH3 - 0.50956</em>EPS8L1</td>
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</tbody>
</table>

Fig. 3. Survival and OSCC-specific mortality estimates in OSCC patients. The two groups were identified with hierarchical clustering analysis using the 131 differentially expressed probe sets in invasive OSCC as described in the text. A, Kaplan-Meier analysis of all-cause mortality. Vertical marks, censored events. B, cumulative incidence of OSCC-specific mortality.
think it is unlikely that, had we sufficient numbers of subjects with each treatment type to conduct stratum-specific analyses, meaningful differences would have been observed. Another important finding of this study is that similar results were obtained between summary measures of all 131 probe sets and our top model containing only one gene. The risk scores from models with each of the first two principal components and our top gene-specific model (0.59151* LAMC2) are highly correlated (Supplementary Fig. S1). This underscores the possibility for reducing the dimensionality of the data not only to the summary of principal components but also to one single probe set without substantial loss of information. This is important because it will be easier to implement molecular tests for clinical use if fewer molecules need to be measured.

Two previous studies by Chung et al. have shown an association between microarray-derived expression data and clinical outcomes in head and neck squamous cell carcinomas (4, 5). In the first study, a 582 gene set from 60 head and neck squamous cell carcinomas classified the tumors into four different subclasses with statistically significant differences in recurrence-free survival. In a separate study using formalin-fixed tissues, the authors identified a second 950-gene signature from unsupervised analysis and a 75-gene list from supervised PCA that was predictive of recurrence. Using the Unigene ID numbers for each gene, we determined that these two previous signatures have 39 genes in common. In contrast, comparison of our 131 probe sets with the gene lists from the previous two studies revealed only one gene shared in all three lists: SLC16A1. This gene comprises our third top model for OSCC-specific survival (Table 2). Subsequently, Pramana et al. tested 42 genes with known function out of the 75-gene list from Chung et al. and showed that these genes were predictive of locoregional control in their own data set (5, 6). Only two genes overlapped between our 131-probe set list and these 42 genes: GRP38 (MACF1) and COL5A1.

There are likely to be many reasons for the lack of more substantial overlap between these gene lists. For example, the 950 and 75 gene lists were derived from formalin-fixed samples and a different array platform (5). In addition, the samples in the studies of Chung et al. were from multiple head and neck sites, whereas ours were limited to the oral cavity and oropharynx. The endpoints also differed between studies because we analyzed overall and OSCC-specific survival and Chung et al. examined recurrence-free survival (5, 8). The statistical approaches to derive these gene signatures were also substantially different (5, 8, 12). Given all these issues, overlapping genes in particular should be further investigated for their potential generalizability in predicting clinical outcomes.

Among the 131 genes used in the supervised cluster analysis, 62 probe sets were differentially expressed between the group of 45 patients and the remaining OSCC. Ingenuity Pathway Analysis of the 62 probe sets showed an overrepresentation of genes involved in cell migration, cell-to-cell signaling and interaction, and cellular growth and proliferation. In addition, five of the genes in our top 10 models predictive of OSCC-specific mortality, such as LAMC2, SERPINE1, THBS1, PDPPN, and CDH3, play a role in cell motility and cell-to-cell signaling, implying that expression of genes involved in the process of invasion and metastasis is an important determinant of outcome in patients with these malignancies (22–28).

Specifically, the proteins encoded by these genes reside in the extracellular matrix and function in cell adhesion and migration. For example, LAMC2, the gene comprising our top model, encodes the g2 subunit of laminin-5, which on cleavage by matrix metalloproteinase-2 appears to release a domain with EGD-like repeats that has been shown to bind to the epidermal growth factor receptor, activate epidermal growth factor receptor signaling, and promote cell motility (22). In addition, this laminin-5 g2 subunit has been found overexpressed at the invasive front of several tumors and, in some, this has been associated with poor prognosis (29–31). Two other genes in
our top models, THBS1 and PDPN, have both been ascribed a role in platelet aggregation and may be involved in tumor metastasis by facilitating tumor cell-platelet interactions and platelet-facilitated tumor cell metastasis (25). It is also known that THBS1 binds with members of the tenacin family and SPARC/osteonectin (25). In fact, tenacin C and SPARC were found to be significantly up-regulated at both gene expression and protein levels in this and other studies by our group (2, 32). In addition, P-cadherin (CDH3), a component of our 10th model, is associated with cell-to-cell signaling and we have previously found this gene to be significantly down-regulated in metastatic tumor cell isolated from lymph nodes (33). The findings in this study showing that the deregulation of the expression of these genes is associated with OSCC-specific survival are consistent with the growing body of literature that suggests that tumor proliferation and metastasis may be in great part mediated by the complex interactions between extracellular matrix proteins and cell-surface receptors.

The functions of other genes in our top 10 models are less understood. OASL appears to be a member of a family of thyroid hormone-interacting proteins and may thus be involved in signal transduction in the presence of thyroid hormone (34). OSMR is a member of the interleukin-6 cytokine family and is thought to be involved in signal transduction and proliferation (35, 36). However, we could not validate the expression of this gene with quantitative RT-PCR. It is possible that this finding is a false-positive and/or the oligonucleotides on the Affymetrix U133 Plus 2.0 arrays are not specific for this gene.

This is the first study we are aware of showing an association between a gene signature and OSCC-specific survival and, in particular, how the use of gene expression data can improve on AJCC stage in predicting survival. We showed that regression models that combined stage with gene expression had significantly higher AUCs than stage alone (Fig. 4). We acknowledge the potential for overoptimism in the estimated AUCs for the models containing gene expression covariates because we used the same sample set to select the top models, estimate risk scores, and assess the association of risk scores with survival. Although the results were essentially unchanged when we used a jackknife leave-one-out analysis, it is important to recognize that this only addresses one portion of the potential overestimation of the AUC estimates because the underlying data remain the same. Further studies with large independent data sets will be needed to validate our models, but such gene expression data sets for oral cancer of sufficient size and follow-up time are not yet publicly available. As we enroll more subjects and accumulate longer follow-up time, we will also be able to address whether these results are stage-specific. This will in turn allow us to determine which patients would benefit most from improvements of these AUCs and how to best implement gene expression into clinical practice. Nevertheless, this is the first study in head and neck cancer that begins to address how gene expression data can complement AJCC stage in predicting survival. Given the recent emphasis on genome-wide gene expression studies to find signatures predictive of clinical outcomes, and the abundance of potential predictors emerging, studies such as this will be needed to determine how to integrate genetic data into clinical practice.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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