Original contribution

Four-protein signature accurately predicts lymph node metastasis and survival in oral squamous cell carcinoma

Sharifah Nurain Syed Zanaruddin BSc\textsuperscript{a}, Amyza Saleh PhD\textsuperscript{a}, Yi-Hsin Yang PhD\textsuperscript{b}, Sharifah Hamid BSc\textsuperscript{a}, Wan Mahadzir Wan Mustafa FDSRCPSc, A. A. N. Khairul Bariah PhD\textsuperscript{d,e}, Rosnah Binti Zain MS\textsuperscript{f,g}, Shin Hin Lau FDSRCS\textsuperscript{d}, Sok Ching Cheong PhD\textsuperscript{a,h,*}

\textsuperscript{a}Oral Cancer Research Team, Cancer Research Initiatives Foundation, 2nd Floor Outpatient Centre, Sime Darby Medical Centre, 47500 Subang Jaya, Selangor, Malaysia
\textsuperscript{b}Statistical Analysis Laboratory, Department of Clinical Research, Kaohsiung Medical University, Chung Hon Memorial Hospital, Kaohsiung 807, Taiwan
\textsuperscript{c}Department of Oral and Maxillofacial Surgery, Hospital Kuala Lumpur, 50586 Kuala Lumpur, Malaysia
\textsuperscript{d}Stomatology Unit, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia
\textsuperscript{e}School of Medical Sciences, Medical Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
\textsuperscript{f}Department of Oral Pathology, Oral Medicine and Periodontology, Faculty of Dentistry, Universiti Malaya, 50603 Kuala Lumpur, Malaysia
\textsuperscript{g}Oral Cancer Research and Coordinating Centre, Universiti Malaya, 50603 Kuala Lumpur, Malaysia
\textsuperscript{h}Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Universiti Malaya, 50603 Kuala Lumpur, Malaysia

Received 13 February 2012; revised 25 May 2012; accepted 20 June 2012

Keywords: Lymph node metastasis; Oral squamous cell carcinoma; Biomarkers; Prognostic signature; Survival

Summary The presence of lymph node (LN) metastasis significantly affects the survival of patients with oral squamous cell carcinoma (OSCC). Successful detection and removal of positive LNs are crucial in the treatment of this disease. Current evaluation methods still have their limitations in detecting the presence of tumor cells in the LNs, where up to a third of clinically diagnosed metastasis-negative (N0) patients actually have metastasis-positive LNs in the neck. We developed a molecular signature in the primary tumor that could predict LN metastasis in OSCC. A total of 211 cores from 55 individuals were included in the study. Eleven proteins were evaluated using immunohistochemical analysis in a tissue microarray. Of the 11 biomarkers evaluated using receiver operating curve analysis, epidermal growth factor receptor (EGFR), v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (HER-2/neu), laminin, gamma 2 (LAMC2), and ras homolog family member C (RHOC) were found to be significantly associated with the presence of LN metastasis. Unsupervised hierarchical clustering–demonstrated expression patterns of these 4 proteins could be used to differentiate specimens that have positive LN metastasis from those that are negative for LN metastasis. Collectively, EGFR, HER-2/neu, LAMC2, and RHOC have a specificity...
of 87.5% and a sensitivity of 70%, with a prognostic accuracy of 83.4% for LN metastasis. We also demonstrated that the LN signature could independently predict disease-specific survival ($P = .036$). The 4-protein LN signature validated in an independent set of samples strongly suggests that it could reliably distinguish patients with LN metastasis from those who were metastasis-free and therefore could be a prognostic tool for the management of patients with OSCC.

© 2013 Elsevier Inc. All rights reserved.

### 1. Introduction

Metastasis involves the migration and colonization of cancer cells beyond the primary tumor and is governed by molecular mechanisms that facilitate the multiple steps required for this process. Despite a better understanding of some of the factors influencing metastasis through in vitro and in vivo studies, metastatic disease still remains the principal event leading to death in patients with cancer. In oral squamous cell carcinoma (OSCC), metastasis spreads predominantly via a lymphatic route, where the cervical lymph nodes (LN) are the first location [1], whereas metastasis to distant sites is relatively uncommon. Cervical LN metastasis is an important determinant of the outcome of OSCC [2] and the status is correlated with patient survival [3]. An accurate assessment of the cervical LN metastasis status in OSCC helps to predict the prognosis of patients and also helps clinicians perform the appropriate treatment. Currently, the clinical assessment of cervical LNs is conducted by clinical examination of the neck region or by ultrasound, computed tomography, and magnetic resonance imaging. Notably, postoperative histology examination has demonstrated that approximately 30% of clinically diagnosed metastasis-negative (N0) patients have metastasis-positive LNs in the neck. Furthermore, 10% to 20% of clinically diagnosed metastasis-positive (N+) patients were actually metastasis-free, where the highest overall accuracy for current preoperative neck assessment is 76%, strongly indicating that the sensitivity of these methods is still limited [4-6]. Clearly, the development of other parameters that could help with the assessment of LN metastasis is urgently needed.

Recent evidence has demonstrated that primary tumors are preconfigured to metastasize, and this propensity is detectable at the time of initial diagnosis [7-9]. Despite the large amount of information from these studies, these markers have not yet been validated as clinical tools to predict LN status in patients. In this study, we used high-throughput tissue microarrays (TMAs) to validate biomarkers that could accurately predict LN metastasis in a cohort of patients with OSCC. We identified a 4-protein signature that could reliably distinguish patients with LN metastasis from those who were metastasis-free. The sensitivity and specificity of this gene signature suggest that it could be a useful tool to complement current diagnostic procedures for managing patients with OSCC.

### 2. Materials and methods

#### 2.1. Case materials and the construction of the TMA

Two hundred eleven tissue cores obtained from 55 individuals, comprising 47 patients and 8 nonmalignant controls, were included in the training and validation sets in this retrospective study. OSCCs that were diagnosed at the Kuala Lumpur General Hospital, Malaysia, between 2003 and 2010 were included. Non–squamous cell carcinomas and verrucous carcinomas were excluded. The histopathologic examination of specimens and neck node metastases was performed by an oral pathologist according to the World Health Organization criteria [10]. This study was approved by the institutional review board (ethics approval code: DF OP 03/06/0018/(L)).

Four tissue cores were taken for each patient representing the histologically normal tissue (nonmalignant tissues not immediately adjacent to the tumor), tumor margin (nonmalignant tissues that were adjacent to the tumor), tumor center, invasive front, and LN (where possible). Eight denture hyperplasia tissues (nonmalignant controls) were also included as controls for immunohistochemistry (IHC) analysis. The TMA was constructed on the ATA 100 tissue arrayer (Chemicon, Ternecula, CA, USA) using a 1.5-mm needle. Two breast cancer tissues were also included as positive controls for the antibodies that were tested. The TMA was sectioned at 5 μm and adhered to SuperFrost Plus (Fisher Scientific, Waltham, MA, USA) glass slides for further experiments.

#### 2.2. Selection of candidate biomarkers

Proteins that were investigated in this study were those previously shown to have a prognostic significance in head and neck cancers [11,12]. In addition, these were genes that promote migration and invasion, or contribute to the loss of adhesive property in tumor cells [13]. After a comprehensive review of the literature, 11 proteins were selected (ITGB4, RHOC, c-MET, LAMC2, EGFR, MMP1, HER-2/neu, Ki-67, CDH1, LGALS3, and INHBA).

#### 2.3. Immunohistochemistry

IHC was performed using the DakoCytomation REAL EnVision Detection System-HRP or the Universal
Biotinylated Link LSAB System-HRP (Dako, Glostrup, Denmark) according to the manufacturer’s protocol. Information on the use of these antibodies is summarized in Supplementary Table S1. IHC methods were performed as we have previously described [14]. Using 7 randomly selected cases, concordance analysis was also performed to validate that the staining of the TMA core was representative of the corresponding whole tissue.

2.4. Scoring of the immunohistochemical data

Each TMA core was considered suitable for semiquantitative evaluation if the core contained epithelium. Staining results were assessed by 3 independent investigators including a pathologist, and any discrepancies were discussed to reach a consensus agreement to a definitive score. The assessors were blinded to the diagnosis and clinicopathologic data associated with the tissues. Evaluation of the immunohistochemical data was performed by using a 4-point intensity scoring system, with 0 indicating negative expression; 1, weak positive expression; 2, moderate positive expression; and 3, strong positive expression. Spatial and cellular localization of the proteins was taken into consideration to verify proper protein localization and staining of each biomarker, as described by others in the literature, or the Human Protein Atlas (http://www.proteinatlas.org/).

2.5. Unsupervised hierarchical cluster analysis

Immunohistochemical and clinicopathologic data were tabulated into a Microsoft Excel worksheet program, exported to TMA-Deconvoluter (http://genome-www.standford.edu/TMA) for hierarchical cluster analysis and subjected to average linkage hierarchical clustering analysis and visualized using the TreeView program as previously described [15].

2.6. Statistical analysis

The statistical analyses were performed using SPSS for Windows software version 16.0 (SPSS, Chicago, IL). The $\chi^2$ test was performed to determine the statistical differences between the expression patterns of LAMC2 against LN metastasis status. The paired t test was performed to determine significant differences in the expression of biomarkers between tumor and nonmalignant cores.

The receiver operating characteristic (ROC) curve [16] was used to identify the significant biomarkers in relation to LN metastasis, and these were further analyzed by hierarchical cluster analysis. The ROC curve was also used to identify the best cutoff points in scoring the expression of the biomarkers and to determine the prognostic accuracy (specificity and sensitivity) of the biomarker’s expression to LN metastasis. Kaplan-Meier survival analysis was used to correlate survival rate with LN status and biomarker expression, and the survival probability differences were compared by the log-rank test. Cox regression multivariate analysis was also performed to adjust for other factors that could influence survival including TNM stage, age, and pattern of invasion. The Fisher exact test was used to analyze the validation set to confirm if the protein signature can robustly differentiate cores that are LN positive against those that are LN negative. A $P$ value less than .05 was considered statistically significant.

3. Results

3.1. Demographics and clinicopathologic characteristics of patients recruited

Tissue specimens in excess of diagnostic value obtained from patients who were treated surgically were used in this study. Twenty-eight patients with OSCC diagnosed from 2003 to 2008 were used for the training set. The demographics and clinicopathologic parameters of these patients are included in Table 1. According to histopathologic assessment, 9 of 28 of the patients had LN metastasis. Survival information was available for 25 of 28 patients, in which the length of follow-up ranged from 1 to 54 months until the year 2009, with a median survival of 31 months.

An independent validation set of 19 patients with OSCC diagnosed between 2008 and 2010 was used to confirm our initial findings (Table 1). Eleven of these patients had LN metastasis, and follow-up disease-free survival information was available up to the year 2010 and the length of disease-free survival ranged from 1 to 23 months, with a survival median of 9 months.

3.2. Immunohistochemical assessment

Overall, an average of 79 (71%) cores was deemed as interpretable for analysis. Noninterpretable data were caused by the loss of the tissue core from the TMA and also by the exhaustion of the epithelium from within the core as the tissue block was sectioned deeper. The individual tumor cores (tumor center, invasive front, and LN tissues) showed similar scoring intensity for all of the biomarkers except for 2, in which the invasive front had a higher intensity for LAMC2 and a loss of expression for CDH1. Notably, there was no difference in the expression intensity for all biomarkers between the nonmalignant and margin tissues from the same individual. The staining for ITGB4, RHOC, LAMC2, EGFR, MMP1, HER-2/neu, LGALS3, and INHBA was localized in the cytoplasm of the cancer cells; however, nuclear (c-MET and Ki-67) and membranous (EGFR, CDH1, HER-2/neu) protein localization was observed as well. Representative images on the immunohistochemical staining are shown in Fig. 1. The staining on the TMA and on whole tissue sections were at 100% concordance (data not shown).

Notably, 2 distinct patterns of cytoplasmic LAMC2 expression that were peripheral or diffused were observed.
(Supplementary Fig. S1), and the diffuse staining was significantly associated with LN metastasis ($P = .003$; Supplementary Table S2). Interestingly, c-MET was seen to be expressed only in the nucleus of OSCC cells. Using the 2 antibodies used in this study, c-MET was confirmed to be expressed in both nucleus and cytoplasm in breast cancer tissues; therefore, it appears that the nuclear expression is inherent in OSCC tissues. We found no significant difference between the expression intensity of EGFR between non-malignant and OSCC cores; however, the location of EGFR expression was exclusively confined to the basal and parabasal layers in the nonmalignant tissues but expressed in all layers of the tumor epithelium.

3.3. Immunoprofile of nonmalignant tissues and OSCC

Unsupervised hierarchical clustering analysis was applied to the IHC data to obtain an overview of the expression profiles of the 11 proteins in all samples (Fig. 2A). Paired t test analysis demonstrated that 9 proteins (MMP1, RHOC, LAMC2, HER-2/neu, INHBA, ITGB4, CDH1, LGALS3, and c-MET) were significantly differentially expressed between tumor and nonmalignant cores ($P = .0001-.0287$; Supplementary Table S3), and these could distinctively separate the tumor and nonmalignant cores (Fig. 2B).

3.4. Immunoprofile associated with LN metastasis

We used the expression of the 11 biomarkers in OSCC cores to identify a specific immunoprofile that was associated with cervical LN metastasis. The area under the curve (AUC) values (Supplementary Table S4) demonstrated that the top 4 proteins (HER-2/neu, RHOC, LAMC2, and EGFR) with AUC larger than 0.60 had a high overall diagnostic accuracy. These 4 proteins could distinctly separate the cores into 3 categories: (1) nonmalignant, (2) LN metastasis positive, and (3) LN metastasis negative (Fig. 3A). The nonmalignant cluster (inclusive of margins) exhibits a molecular profile of negative expression of LAMC2, RHOC, and HER-2/neu and positive expression of EGFR. The LN metastasis–positive cores exhibited a molecular profile of strong positive expression for all 4 biomarkers, and 12 (85%) of 14 LN-positive cores were grouped correctly within this cluster. The LN-negative cluster shared a molecular profile of weak positive expression for all 4 biomarkers (Fig. 3B) where 16 (62%) of 26 LN-negative cores are correctly clustered here. To ensure that the immunoprofile separating the tumors with LN metastasis from those with negative nodes is not due to chance, clustering analysis using the top 5 biomarkers including c-MET (Fig. 3C) was performed. With the inclusion of c-MET, the ability to segregate tumors with positive nodes from those with negative nodes was disrupted, indicating that the immunoprofile consisting of LAMC2, HER-2/neu, EGFR, and RHOC in the primary tumor was not random and could indeed predict LN metastasis reliably.

3.5. Scoring cutoff points, prognosis accuracy, sensitivity, and specificity of the 4-protein signature

For the 4 chosen biomarkers, a cutoff point, which yielded the largest sum of sensitivity and specificity, was identified from the intensity scoring system of each biomarker. The cutoff points were then used to dichotomize the expression

<table>
<thead>
<tr>
<th>Variable</th>
<th>Training samples</th>
<th>Validation samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 28</td>
<td>%</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
<td>46</td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
<td>54</td>
</tr>
<tr>
<td>Age (y)</td>
<td>Mean</td>
<td>59</td>
</tr>
<tr>
<td>Tumor site</td>
<td>Buccal mucosa</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Tongue</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Gingiva</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Others (lip and palate)</td>
<td>2</td>
</tr>
<tr>
<td>Tumor stage</td>
<td>T1 and T2</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>T3 and T4</td>
<td>20</td>
</tr>
<tr>
<td>Node stage</td>
<td>NX</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>N0</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>N1, N2, N3</td>
<td>9</td>
</tr>
<tr>
<td>pTNM stage</td>
<td>I and II (early)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>III and IV (late)</td>
<td>20</td>
</tr>
<tr>
<td>Disease-free survival (mo)</td>
<td>Range</td>
<td>1-54</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 1 Demographics and clinicopathologic characteristics of patients recruited
intensity of the 4 biomarkers. IHC scores of 2 and 3 for LAMC2, EGFR, RHOC, and HER-2/neu were allocated a score of 1, indicating positive LN metastasis, whereas an IHC score of 0 or 1 was allocated a score of 0, indicating no metastasis (Supplementary Table S5). Using the 2-point scoring system, the scores from the 4 molecules were then summed up collectively and were subjected to ROC analysis again to determine the optimal number of molecules needed to reflect the highest sensitivity and specificity in correlation with LN metastasis. Based on this, the cutoff point with a cumulative score of 2 (at least 2 biomarkers) reflected the best combination of sensitivity and specificity. From this, the signature would have a sensitivity (true-positive rate) of 70% and a specificity (true-negative rate) of 87.5% (Fig. 4A). The expression profile of the 4 proteins collectively have the best prognostic accuracy in correlation with LN metastasis (AUC, 0.834; \(P = .005\)) in comparison with any of the proteins individually (Fig. 4B). Validating the 4-protein signature on an independent set, 10 of 13 patients who had a cumulative score of 2, 3, or 4 were positive for LN metastasis, whereas 4 of 4 of patients who had a cumulative score of 0 or 1 were negative for LN metastasis (\(P = .015\); Table 2), demonstrating that the 4-protein LN signature is indeed reliable in segregating node-positive patients from node-negative patients, reflecting a positive predictive value of 10 of 13 (77%) and a negative predictive value of 4 of 4 (100%) (specificity, 4/7 [57%]; sensitivity, 10/10 [100%]).

### 3.6. LN metastasis and biomarker expression in correlation to survival status

Using disease-specific Kaplan-Meier analysis, we found a significant survival probability difference between patients with LN metastasis compared with those without (\(P = .008\); Fig. 5A), and this suggests that the 4-protein LN signature should also be able to predict patient survival. To test this, we used the cutoff values calculated in Fig. 4A and divided the patients into those who have a cumulative score of 0 or 1 (group 1) versus those with a cumulative score of 2, 3, or 4 (group 2). The log-rank (Mantel-Cox) test of the Kaplan-Meier survival curves showed a significant survival probability difference between patients in groups 1 and 2 (\(P = .020\); Fig. 5B). Furthermore, The Cox regression multivariate model revealed that the LN signature is an independent prognostic factor with a relative risk of 5.51 for shorter survival, in patients with positive expression of 2 or more markers (Fig. 5C). Taken together, the LN signature is

![Fig. 1](image.png)  
**Fig. 1**  IHC representation of all selected 11 biomarkers in nonmalignant and tumor cores. Original magnification ×100 to ×200 as indicated by scale bars in each individual image.
not only indicative of LN metastasis but is also an independent predictor of disease-specific survival.

4. Discussion

Cervical LN metastasis is a major determinant in predicting patient survival in OSCC [2], and the accurate removal of positive LNs is one of the most important determinants of OSCC treatment. Therefore, the ability to predict LN metastasis at the first biopsy of the primary tumor will significantly improve the management of patients with OSCC. Patients with similar pathological disease stage can exhibit varying survival outcomes, in part, due to the molecular heterogeneity within each of the individual stages [17-19]. Considering the limitations of current diagnostic methods, we used 11 biomarkers that were previously...
Signature for OSCC metastasis and survival
reported to be important in oral carcinogenesis to determine if they were able to separate primary tumors with LN metastasis from those that did not. We identified an “LN signature” consisting of EGFR, HER-2/neu, LAMC2, and RHOC that could distinguish primary tumors with LN positivity from those without, with a high sensitivity and specificity of 70% and 87.5%, respectively, and an overall prognostic accuracy of 83.4%. Although this is still a little lower than that of ultrasound-guided fine needle aspiration cytology (diagnostic accuracy, 86.3%) [20], it is a significant improvement from techniques that are more commonly used for the preoperative clinical assessment of OSCC such as magnetic resonance imaging, computed tomography, and positron emission tomography where the overall accuracy is 76% (sensitivity, 64%-70%; specificity, 68%-82%) [4]. Notably, we further demonstrated that the LN signature is also an independent prognostic predictor for disease-specific survival and therefore would be a useful tool for prognostication. The LN signature here remained significantly predictive of disease-specific survival in the multivariate analysis, taking into account the other major risk factors that are associated with OSCC survival.

Although gene signatures predicting LN metastasis and survival have been identified through microarray analyses previously [8,19,21], they currently have limited use in the clinical setting because the methods used to identify these genes, including microarray assays, cannot be performed routinely in the diagnostic setting, hence preventing the direct translation of these gene expression signatures into clinically useful assays [22]. To the best of our knowledge, this is the first study to demonstrate the translational effort from high-throughput discoveries in OSCC to the selection of suitable biomarkers using methods that are familiar to a typical diagnostic laboratory. IHC is routinely used in diagnostic laboratories to aid in differential diagnosis for the management of patients, and pathologists already have considerable experience using at least several markers including estrogen receptor, progesterone receptor, and HER-2/neu for adjuvant therapy in breast cancer and cytokeratin markers for the detection of micrometastasis. The LN signature presented in our study sets the stage for the development and validation of immunohistochemical prognostic assays from information derived from gene expression profiles and improves the prognostic accuracy of single proteins previously shown to be of prognostic value. Indeed, studies in other cancers have clearly demonstrated the role of multimarker signatures in complementing the current diagnosis and prognosis methods [23,24].

The LN signature identified here provides prognostic information beyond routine clinical utility by shedding light on the molecular mechanisms underlying LN metastasis in OSCC that could be targeted for cancer treatment. Both EGFR and HER2 are members of the epidermal growth factor receptor family, and individually, each has been shown to be associated with LN metastasis of the head and neck [25-27]. Aberrant expression of LAMC2, an adhesion molecule, induces cell migration of many various types of invasive carcinomas including oral carcinoma [28,29] and, more recently, has been shown to be predictive of disease-free survival [21]. Of note, LAMC2 was the only marker within the 4-protein signature presenting heterogeneous expression within the tumor, and in this study, the expression of the invasive front was evaluated because this area is accepted to be the most aggressive part of the tumor. RHOC, a member of the Ras superfamily, plays a major role in the control of cellular

<table>
<thead>
<tr>
<th>Table 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Validation of 4-protein LN signature in relation to LN metastasis status</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LAMC2 + EGFR + RHOC + HER-2/neu</th>
<th>Patients (n)</th>
<th>LN metastasis status</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative, n (%)</td>
<td>Positive, n (%)</td>
</tr>
<tr>
<td>Positive expression of &lt;2 markers</td>
<td>4</td>
<td>4 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Positive expression of ≥2 markers</td>
<td>13</td>
<td>3 (23)</td>
<td>10 (77)</td>
</tr>
</tbody>
</table>
motility [30,31]. RHOC overexpression also promotes proliferation, anchorage independent growth, and metastatic behavior through the mitogen-activated protein kinase and phosphatidylinositol-2-kinase/AKT pathway [32], and most notably, RHOC expression could accurately identify early-stage tumors that have already metastasized [33].

In summary, following closely to the Reporting Recommendations for Tumor Marker Prognostic Studies guidelines [34], we have described the identification of a 4-protein signature from the primary tumor that is indicative of LN metastasis. Furthermore, we validated this LN signature in an independent set of samples demonstrating the robustness of this signature in predicting LN metastasis in OSCC. Given that there is currently an emphasis on using molecular information for the classification of cancers and the abundance of data on differentially expressed genes, this study, first, demonstrates the feasibility of using biomarkers as potential predictors of LN metastasis and, second, has developed these biomarkers using methods that could be directly used in the clinical setting to facilitate the translation of molecular-based information into clinically valid prognostic biomarkers. We acknowledge the sample size limitations and the potential for overoptimism in the estimated AUC of this gene signature and the requirement to continue testing this signature in even larger numbers of OSCC to determine which subgroups would benefit most and to refine the grading of the expression of these proteins to include an element of objectivity. Nevertheless, this study could be used as a model for the development of IHC prognostic assays derived from gene expression studies.

**Supplementary data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.humpath.2012.06.007.

**Acknowledgment**

We are grateful to Mr David Lyn and Ms Tan Bee Ying from Matrix Optics (M) for their technical assistance. The Cancer Research Initiatives Foundation (CARIF) is a nonprofit research organization. We are committed to an understanding of cancer prevention, diagnosis, and treatment through a fundamental research program.

**References**


