Molecular Profiling and the Identification of Genes Associated With Metastatic Oral Cavity/Pharynx Squamous Cell Carcinoma

Cecelia E. Schmalbach, MD, MS; Douglas B. Chepeha, MD, MSPH; Thomas J. Giordano, MD, PhD; Mark A. Rubin, MD; Theodoros N. Teknos, MD; Carol R. Bradford, MD; Gregory T. Wolf, MD; Rork Kuich, MS; David E. Misek, PhD; Douglas K. Trask, MD, PhD; Samir Hanash, MD, PhD

Objective: To investigate differences in gene expression profiles between oral cavity/oropharynx squamous cell carcinoma (OC/OP SCC) primary tumors that have metastasized to cervical lymph nodes and nonmetastatic OC/OP SCC tumors.

Design: Oligonucleotide microarray analysis of primary tumors was used to produce gene expression profiles. Profile comparisons between metastatic and nonmetastatic tumors were performed using principal component analysis, t test, and fold change differences. A similar comparison between metastatic tumors and noncancer oral mucosa samples was performed to ensure tumor origin.

Subjects: A prospective cohort of 20 patients with previously untreated OC/OP SCC who underwent pathologic staging following surgical resection and lymphadenectomy.

Results: Of the approximately 9600 genes profiled, 101 demonstrated significant expression differences between the metastatic and nonmetastatic tumors (fold change ≥1.5; P<.01). Among this subset, 57 genes also exhibited significant differences between metastatic tumors and normal mucosa samples (fold change ≥1.5; P<.05). This profile included genes related to the extracellular matrix, adhesion, motility, inflammation, and protease inhibition. Collagen type 11 alpha-1 (COL11A1) demonstrated the greatest differential expression between metastatic and nonmetastatic OC/OP SCC tumors (fold change = 7.61; P = .002). Tissue inhibitor of metalloproteinase 1 (TIMP-1) also demonstrated increased expression in metastatic tumors (fold change = 3.3; P = .003).

Conclusions: Metastatic OC/OP SCC has a distinct gene expression profile compared with nonmetastatic OC/OP SCC and normal oral mucosa. This metastatic profile includes genes related to the extracellular matrix, adhesion, motility, and protease inhibition. Knowledge gained through tumor gene expression profiling may facilitate early detection of aggressive tumors and targeted therapeutic interventions.
Methods

Tissue Samples and Cell Lines

Tissue was obtained from a prospective cohort of 20 patients with newly diagnosed, previously untreated OC/OP SCC who presented to the University of Michigan Hospital, Ann Arbor, between February and December 2001. The University of Michigan institutional review board approved the study, and written consent was obtained. All patients underwent pathologic staging following surgical tumor resection and lymphadenectomy (13 metastatic and 7 nonmetastatic tumors). Demographic information for this prospective cohort (Table 1) demonstrates that the 20 patients sampled are representative of the HNSCC population.

At the time of surgical resection, a sample of the primary tumor was snap frozen in liquid nitrogen. Samples were embedded in OCT freezing media (Miles Scientific, Naperville, Ill) and stored at –80°C for RNA extraction. A surgical pathologist evaluated a cryotome section (5 µm) from each block using hematoxylin-eosin (H&E) staining. RNA was isolated from areas of tissue containing at least 70% tumor cellularity.

Oral cavity SCC cell lines SCC-15 and SCC-25 were obtained from the American Type Tissue Collection, Manassas, Va. The UM-SCC-1 and UM-SCC-17A cell lines were obtained from Thomas Carey, PhD, at the University of Michigan. Normal controls included oral cavity mucosa samples from 4 patients without cancer who underwent surgical treatment for obstructive sleep apnea and primary cell explants of oral cavity keratinocytes.

RNA Isolation

Total cellular RNA was extracted from the tumor samples and cell lines as previously described.4,13 Samples were homogenized using Trizol reagent (Life Technologies, Gaithersburg, Md) and purified according to the manufacturer’s instructions. RNA samples were further purified on RNeasy spin columns (Qiagen, Valencia, Calif). RNA quality was assessed by 1% agarose gel electrophoresis in the presence of ethidium bromide.

Microarray Analysis

Preparation of RNA and hybridization to commercially available HG_U95Av2 microarrays (Affymetrix, Santa Clara, Calif) were performed according to the manufacturer’s protocols as previously described.4,13 Microarrays were scanned, and probe intensities were extracted from the image (GeneArray scanner and Microarray Suite 4.0; Affymetrix). Each probe set on the array represented a gene and typically consisted of 16 individual 25-base oligonucleotide features complementary to specific DNA, called perfect match (PM) probes, and 16 identical probes whose sequence had been altered at the central base, called mismatch (MM) probes. Publicly available software was used to process the probe intensities to obtain normalized results (software and documentation available as “Supplementary Material” at http://dot.ped.med.umich.edu:2000/pub/HeadNeck/index.html).4,13

The microarray from a T2 N2 c floor of mouth tumor was selected as the standard because it was generalizable to the sample population, had low background measures, and had bright signals. Probe pairs for which the PM-MM difference was less than –200 on the standard were removed from the analysis. The remaining PM-MM differences were averaged for each probe set on each microarray by discarding the 23% highest and lowest differences and averaging the remaining differences. The intensities for each microarray were normalized to the standard using a piece-wise linear function that made 99 evenly spaced quantiles agree with the corresponding quantiles in the distribution of the standard. Fold changes were computed as the ratio of group means, after first replacing the means that were less than 100 with 100. Normalized intensities were log-transformed by mapping \( x \) to \( \log(\max[x+100,0]+100) \) prior
to performing statistical tests. Unigene Cluster identifiers were computed using homology of the probe set sequences to the sequences of Unigene Cluster members (software and documentation available as “Supplementary Material” at http://dot.ped.med.umich.edu:2000/pub/HeadNeck/index.html). One-sided signed-rank tests of PM-MM differences for each probe set on each array were used to determine the number of detectable transcripts.

IMMUNOHISTOCHEMICAL ANALYSIS OF TIMP-1

Expression of increased tissue inhibitor of metalloproteinase 1 (TIMP-1) in primary metastatic and nonmetastatic tumors was investigated using immunohistochemical analysis on a tissue microarray comprised 102 independent tumor samples. All patients for this validation group presented to the University of Michigan Hospital between 1997 and 2000 with newly diagnosed, previously untreated HNSCC. University of Michigan institutional review board approval and written consent were obtained. Patients underwent pathologic staging following surgical tumor resection and lymphadenectomy (70 had metastatic SCC and 32 had nonmetastatic SCC). The mean age for the metastatic and nonmetastatic validation group was 57 years and 58 years, respectively. The mean disease-free interval for the metastatic group was 20 months, with a mean follow-up of 22 months. The nonmetastatic group had a mean disease-free interval of 28 months, with a mean follow-up of 29 months.

A head and neck pathologist used H&E staining to evaluate cryotome sections (5 μm) from each paraffin-embedded primary tumor block. Representative areas of tumor and normal squamous cell mucosa were marked. A high-density tissue microarray was constructed from the marked areas using 3 replicate tumor cores (0.6-mm diameter) and 1 normal mucosa core per patient.

Immunohistochemical analysis of the paraffin-embedded tissue microarray was performed using standard avidin-biotin complex method.13 Protein expression of TIMP-1 was evaluated with a rabbit polyclonal anti–TIMP-1 antibody (sc-5538; Santa Cruz Biotechnology, Santa Cruz, Calif) (1:50). A pathologist, blinded to the metastatic status of the tumors, scored the nuclear protein expression as negative (1), weak (2), moderate (3), and strong (4). The mean TIMP-1 score for each tumor was compared with normal controls using a Wilcoxon rank sum test. Because the gene microarray analysis revealed increased levels of TIMP-1 in metastatic OC/OP SCC, tissue microarray tumors were categorized into an abundant nuclear staining group (mean expression score ≥3.0) and a nonabundant nuclear staining group (mean expression score <3.0). The Fisher exact test was then used to study the relationship of TIMP-1 and cervical lymph node metastasis, sex, primary tumor site, histologic differentiation, pathologic stage, tumor size, presence of extracapsular spread, and tumor recurrence. Linear regression was used to investigate the relationship of TIMP-1 and age. The Kaplan-Meier method was used to investigate the relationship between TIMP-1 and disease-free survival.

RESULTS

Using the Affymetrix HG_U95Av2 GeneChip (12625 probe sets representing approximately 9600 unique genes), we obtained gene expression profiles for 13 OC/OP SCC primary tumors from patients with cervical lymph node metastasis, 7 nonmetastatic OC/OP SCC tumors, 4 SCC cell lines, 4 normal oral cavity mucosa samples, and normal oral cavity keratinocyte explants. Using the comparison outlined in the “Methods” section, tumor samples averaged 7731 detectable probe sets and normal samples averaged 7798 detectable probe sets (P < .05).

A pictorial representation of the gene expression profiles was constructed using principal component analysis. The greatest variance between sample gene profiles was represented by the first 2 principal components, which we graphed on the x- and y-axis of Figure 1A. Similarly, a dendrogram based on the hierarchical clustering is depicted in Figure 1B. The normal mucosa samples, 4 SCC cell lines, and OC/OP tumors clustered into 3 dis-
Tumors did not cluster into 2 distinct groups based on samples, irrespective of cervical lymph node status. The greatest degree of variance between the 20 tumor static and nonmetastatic tumors. Two of the OC tumors (tongue and floor of mouth) were included in the final comparison of metastatic and nonmetastatic OC tumors did not separate into 2 distinct clusters, with tumors having the greatest degree of heterogeneity in expression profiles. To gain further insight into the gene expression profile specifically associated with tumor metastasis, the effects of cell line profiles and normal mucosa profiles were removed, and a principal component analysis using only OC/OP SCC samples was repeated. This exploratory data analysis did not uncover a clear pattern in gene expression profiles related to primary tumor site (Figure 2A) or histologic differentiation. Specifically, OC and OP tumors did not separate into 2 distinct clusters. Two of the OC tumors (tongue and floor of mouth) shared greater profile similarity to the OP tumors than their OC counterparts. For this reason, both OC and OP tumors were included in the final comparison of metastatic and nonmetastatic tumors. Figure 2 was constructed using the 2036 probe sets that demonstrated the greatest degree of variance between the 20 tumor samples, irrespective of cervical lymph node status. Tumors did not cluster into 2 distinct groups based on metastatic nodal status. Therefore, the variation between tumor profiles could not be explained solely by genes that were differentially expressed with respect to metastasis. However, the nonmetastatic tumors clustered closer together as a group, indicating greater homogeneity in their gene expression compared with the metastatic tumors (Figure 2B).

A 2-sample t test using log-transformed data from metastatic and nonmetastatic tumors was performed for all 12625 probe sets represented on the Affymetrix HG_U95Av2 GeneChip to identify genes associated with metastatic OC/OP SCC. 160 probe sets met statistical significance at $P<.01$. To reduce the proportion of false-positives among the selected 160 probe set list, the means of the metastatic and nonmetastatic tumors were required to differ by at least 1.5-fold. This fold change criterion was met by 101 of the 160 probe sets.

To exclude genes that may have differed in expression level between metastatic and nonmetastatic tumors owing to differences in the proportion of normal tissue elements, gene profiles were also compared between the metastatic and normal mucosa samples. Within the 101 probe sets, we identified genes with both an expression level difference between metastatic tumors and normal samples at $P<.05$ (2-sample t test) and a minimum difference in mean expression of 1.5-fold (the direction of change being the same as the metastatic and nonmetastatic comparison). Overall, 61 probe sets representing 57 genes fulfilled all selection criteria. Four of the genes were represented in duplicate on the Affymetrix GeneChip, 50 represented classified genes, and 7 are currently unnamed genes. The expression values for each tumor and normal sample are provided in Figure 3, along with $t$ test and fold-change results. Among the list were genes related to cellular adhesion, cell growth and differentiation, motility, and angiogenesis (Table 2).

To estimate the overall number of false-positives, a computer program randomly relabeled the 20 OC/OP gene profiles 1000 times, irrespective of nodal status. Each of the 1000 permutations was statistically analyzed using the identical selection criteria for obtaining the 61 probe sets in Figure 3. On average, only 18 of the 12625 probe sets met all selection criteria by chance alone, and only 31 of the 1000 random computer trials yielded 61 or more probe sets meeting the selection criteria. This permutation test overestimated the false-positive rate because analysis was based only on tumors. When all samples (OC/OP tumors and normal mucosa) were randomly permuted 1000 times, an average of 8 probe sets met the selection criteria by chance alone. The overall false-positive rate of this study falls between these 2 estimates of 8 and 18 probe sets.

Immunohistochemical analysis was used to confirm protein expression and tumor cell origin of the gene that exhibited the greatest degree of differential expression, for which a commercial antibody was available. Immunohistochemical analysis for nuclear TIMP-1 pro-

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Figure 2. Results of principal component analysis for the 20 primary tumors. A total of 2036 probe sets (mean >100 and coefficient of variance [SD/mean] >0.6) were standardized by subtracting the mean and dividing by the SD for each probe set. The first 2 principal components were plotted. Samples are labeled according to primary tumor site (A) and according to metastatic nodal status (B) (3-dimensional version of principal component analysis is available at http://otol.ped.med.umich.edu:2000/pub/HeadNeck/index.html). Met OC/OP SCC indicates metastatic oral cavity/oropharynx squamous cell carcinoma (tumors metastatic to cervical lymph nodes); Nonmet, nonmetastatic; RMT, retromolar trigone.

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tein expression revealed a statistically significant difference in mean expression between tumor and normal controls (Wilcoxon rank sum test; P<.0001). While most of the tumors demonstrating abundant TIMP-1 expression were metastatic (37/48), the difference between TIMP-1 abundance and metastatic nodal status did not achieve statistical significance (P=.06). Similarly, differences in TIMP-1 abundance based on age, sex, primary tumor site, histologic differentiation, pathologic stage, tumor size, extracapsular spread, and tumor recurrence were not statistically significant. Expression of TIMP-1 did not correlate with disease-free survival.

Various prognostic markers for OC/OP SCC have been reported. These include amplification of cyclin D1 (PRAD1) on chromosome 11q13, overexpression of the MYC family of oncogenes, mutations of p53, loss of p16 protein function, and overexpression of epidermal growth factor receptor and transforming growth factor α.1,2,5 Current no individual marker has emerged as a fully informative prognostic marker, and treatment decisions for OC/OP SCC patients continue to rely exclusively on the

**Figure 3.** Molecular profile for metastatic oral cavity/oropharynx squamous cell carcinoma (OC/OP SCC; tumors metastatic to cervical lymph nodes) demonstrated using Treeview software (Eisen Lab, Stanford University, Stanford, Calif).46 Sixty-one probe sets representing 57 genes were differentially expressed by metastatic nodal status. Each column represents an individual tissue sample; each row represents 1 of the 61 probe sets that differed simultaneously between metastatic and nonmetastatic tumors. Fold change >= 1.5; P<.05). The color of each cell represents the fold change above (red) and below (green) the median of all 24 tissue samples. ATPase indicates adenosine triphosphatase; cDNA, complementary DNA; ESTs, expression sequence tags; mRNA, messenger RNA; Nonmet, nonmetastatic.
clinical TNM system. Overall, cervical lymph node metastasis remains the most important prognostic indicator for tumor recurrence and survival. We used oligonucleotide microarrays to determine if regionally metastatic OC/OP SCC exhibits a distinct gene expression profile. Increased understanding of the molecular biology of metastasis may allow aggressive tumors, which warrant adjuvant therapy despite their early stage, to be recognized at the time of diagnosis. A molecular profile for metastasis may also lead to the development of novel targeted therapies for these aggressive tumors.

We compared our metastatic profile with previously reported profiles of genes unique to HNSCC.6-12 As expected, the genes differentiating metastatic from nonmetastatic tumor had limited overlap with the genes differentiating tumor from normal mucosa. Although we identified a set of genes differentially expressed with respect to cervical lymph node metastasis, a previous study did not detect such a difference.12 This discrepancy can be explained by variations in study design. The most compelling reason is that we used a more recent version of the Affymetrix GeneChip, which consists of almost twice as many probe sets compared with the older HuGeneFL chip. Patient selection may also have contributed to differences observed. We only profiled patients with newly diagnosed, previously untreated SCC to eliminate potential gene profile alterations resulting from radiation and chemotherapy. In addition, we only profiled patients who underwent lymphadenectomy so that pathologic staging for cervical lymph node metastasis could be achieved. Further reasons for the lack of similarity between the profiles include differences in gene selection criteria and statistical analysis. One criticism of our study is the use of a 1.5-fold change selection criterion, which is smaller than that applied in other studies. However, we believe that a smaller fold change is appropriate when comparing histologically identical tissue types, such as OC/OP SCC, with one another.

One of the strengths of our study design was that our comparisons did not rely on cell line profiles. Instead, we compared metastatic and normal mucosa samples to ensure that expression differences were due to genes from OC/OP SCC tumors as opposed to surrounding normal epithelium. Although cell lines provide a more definitive means to confirm tumor cell origin, an in vitro setting does not mirror the natural milieu of tumors because inflammatory and stromal components are lacking. Consequently, genes related to cellular adhesion, mobility, and inflammation and genes requiring activation from surrounding nontumor cells would not be observed in a model incorporating cell line criteria. Indeed, in our study collagen type XI α-1 (COL11A1) demonstrated the greatest difference between metastatic and nonmetastatic tumors. This difference would not be predictable from our cell line profiles (data not shown). Previous studies have demonstrated that increased COL11A1 expression requires paracrine activation from surrounding stroma.17,18 Our use of primary tumors may also account for the limited overlap of only 2 genes from our metastatic profile (IgG Fc receptor gene and serine protease inhibitor-2) with the findings by Dong et al,10 who used cell lines to identify genes differentially expressed between metastatic and nonmetastatic tumors.

Molecular profiling provides complex data, which may be difficult to interpret. We recognize that genes do not function independently and that gene interactions play a critical role in tumor progression. However, examination of individual gene functions and related pathways is still informative. For example, several genes from our profile of metastatic OC/OP SCC are involved in pathways related to the extracellular matrix (ECM). This finding is plausible given the defined role of the ECM in cellular morphogenesis, proliferation, differentiation, apoptosis, and migration.19 Collagen is a major structural component of the ECM. In our study, the mean expression of COL11A1 was more than 7.5 times higher in metastatic vs nonmetastatic tumors. Increased COL11A1 expression is related to uncontrolled epithelial proliferation through the β-cadherin/Wnt/WISP-1 pathway.17 An association between COL11A1 and colorectal carcinoma progression has previously been reported.18

In addition, serine protease inhibitor-2 (SERPINB2) is related to the ECM. This gene regulates ECM remodeling by inhibiting urokinase-type plasminogen activator, a protein involved in tumor invasion and metastasis.20 SERPINB2 expression was decreased in our metastatic OC/OP SCC tumors relative to the nonmetastatic tumors. Dong et al19 demonstrated similar findings using SCC cell lines, and decreased SERPINB2 expression was observed in studies involving metastatic lung,21 breast,22 and gastric carcinoma.23

**Table 2. Classification of Gene Subset Differentially Expressed Between Metastatic and Nonmetastatic OC/OP SCC***

<table>
<thead>
<tr>
<th>Extracellular matrix/adhesion molecules</th>
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<tr>
<td>Tissue inhibitor of metalloproteinase 1 (TIMP-1)</td>
<td>↑</td>
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<tr>
<td>Nerve injury induced protein (MIN1)</td>
<td>↑</td>
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<tr>
<td>Chondroitin sulfate proteoglycan 2 (CSPG2)</td>
<td>↑</td>
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<tr>
<td>Osteoblast-specific factor 2 (OSF-2)</td>
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<tr>
<td>Cadherin 11, type 2 (CDH11)</td>
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<tr>
<td>Partitioning defective 3 (PARD3)</td>
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<tr>
<td>Cell cycle/apoptosis</td>
<td>↑</td>
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<tr>
<td>Secreted frizzled-related protein 4 (SFRP4)</td>
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<tr>
<td>Partitioning defective 3 (PARD3)</td>
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<tr>
<td>Intracellular signaling</td>
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<tr>
<td>Chondroitin sulfate proteoglycan 2 (CSPG2)</td>
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<tr>
<td>Immune/inflammatory response</td>
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<tr>
<td>Complement component 1 (C1QB)</td>
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<tr>
<td>Fc fragment of IgG (FCGR3B)</td>
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<tr>
<td>Serine protease inhibitor 4 (SERPINB4)</td>
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<tr>
<td>Phospholipase A2 (PLA2G4A)</td>
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<tr>
<td>Interleukin 1 receptor antagonist (IL1RM)</td>
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<tr>
<td>Cellular differentiation</td>
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<tr>
<td>Chondroitin sulfate proteoglycan 2 (CSPG2)</td>
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<tr>
<td>Cellular retinoic acid-binding protein 2 (CRABP2)</td>
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<tr>
<td>Transcription factor</td>
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<tr>
<td>Transcription elongation factor A (TCEA-2)</td>
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<tr>
<td>Angiogenesis</td>
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<tr>
<td>Extracellular matrix protein 1 (ECM1)</td>
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Abbreviation: OC/OP SCC, oral cavity/pharynx squamous cell carcinoma. *Arrow indicates directional change in gene expression of metastatic tumors with respect to both nonmetastatic tumors and normal mucosa samples.
The integrity of the ECM is maintained by various metalloproteinases, several of which have an association with tumor invasion, angiogenesis, and metastasis. Therefore, it may seem counterintuitive that we observed increased expression of TIMP-1, a metalloproteinase inhibitor, in the setting of metastatic disease. However, recent studies have reported a paradoxical role for TIMP-1. In addition to metalloproteinase inhibition, TIMP-1 rescues epithelial cells from apoptosis and mediates up-regulation of Bcl-X<sub>L</sub>, which is known to have antiapoptotic properties. Furthermore, an association between overexpression of Bcl-X<sub>L</sub> and HNSCC has been reported. Although a previous HNSCC study found an association between TIMP-1 and T stage but not metastatic nodal status, our study is consistent with colorectal and breast carcinoma findings associating TIMP-1 expression with metastasis.

Validation of gene microarray analysis is often achieved through quantitative reverse transcription polymerase chain reaction, and we have previously validated our oligonucleotide microarray methodology in this manner. However, application of quantitative polymerase chain reaction to the same tumor samples simply confirms that the microarray technique is accurate in detecting RNA. It does not provide additional information related to protein expression, nor does it confirm cellular origin. For this reason, we chose to investigate TIMP-1 using tissue microarray immunohistochemical analysis in order to confirm protein expression and to determine cellular origin using a larger, independent cohort. We demonstrated that TIMP-1 was expressed at the protein level and confirmed that expression of TIMP-1 was from HNSCC tumor cells as opposed to fibroblasts, stroma, or normal mucosa (P<.0001). As predicted by genomic profiling, patients with cervical lymph node metastasis had higher levels of TIMP-1 expression by immunohistochemical analysis. Although the difference was not statistically significant, a P value of .06 remains of interest because our sample size provided sufficient power to detect only large differences, and immunohistochemical analysis cannot provide the degree of quantitative measure generated using gene microarray technology.

CONCLUSIONS

Cervical lymph node metastasis is the most important prognostic marker for both tumor recurrence and overall survival. We identified a gene expression profile for regionally metastatic OC/OP SCC using oligonucleotide microarray analysis. Genes related to the extracellular matrix, cellular differentiation, intracellular signaling, adhesion, and immune response were identified within this profile of 57 genes. TIMP-1, COL11A1, and SERPINB2 were among the genes with the greatest degree of differential expression between metastatic and nonmetastatic tumors. Oral cavity/oropharynx SCC tumors, which have a propensity to metastasize, remain a therapeutic challenge. The ability to develop a gene expression profile for metastatic tumors is an important first step because improved patient survival requires early detection of aggressive tumors, as well as targeted therapeutic interventions.

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Corresponding author and reprints: Cecelia E. Schmalbach, MD, Department of Otolaryngology—Head and Neck Surgery, 1500 W Medical Center Dr, Room A520 MSRB-1, Ann Arbor, MI 48109-0656 (e-mail: cecelia@umich.edu).

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