Examination of Oral Cancer Biomarkers by Tissue Microarray Analysis

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Objective: To validate the DNA microarray results on a subset of genes that could potentially serve as biomarkers of oral squamous cell carcinoma (OSCC) by examining their expression with an alternate quantitative method and by assessing their protein levels.

Design: Based on DNA microarray data from our laboratory and data reported in the literature, we identified 6 potential biomarkers of OSCC to investigate further. We used quantitative real-time polymerase chain reaction to examine expression changes of CDH11, MMP3, SPARC, POSTN, TNC, and TGM3 in OSCC and histologically normal control tissues. We further examined validated markers at the protein level by immunohistochemical analysis of OSCC tissue microarray sections.

Results: Quantitative real-time polymerase chain reaction analysis revealed upregulation of CDH11, SPARC, POSTN, and TNC gene expression and decreased TGM3 expression in OSCC tissue compared with control tissue; MMP3 was not found to be differentially expressed. In tissue microarray immunohistochemical analyses, SPARC (secreted protein, acidic, rich in cysteine), periostin, and tenascin C exhibited increased protein expression in tumor tissue compared with control tissue, and their expression was primarily localized within tumor-associated stroma rather than tumor epithelium. Conversely, transglutaminase 3 protein expression was found only within keratinocytes in control tissue and was significantly downregulated in cancer cells.

Conclusions: Of 6 potential gene markers of OSCC, initially identified by DNA microarray analyses, differential expression of CDH11, SPARC, POSTN, TNC, and TGM3 were validated by quantitative real-time polymerase chain reaction. Differential expression and localization of proteins encoded by SPARC, POSTN, TNC, and TGM3 were clearly shown by tissue microarray immunohistochemical analysis.


HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) is the fifth most common cancer worldwide.¹ The American Cancer Society estimates that approximately 30 990 Americans were diagnosed as having and 7430 died of cancer of the oral cavity and pharynx in 2006.² Despite considerable advances in the treatment of HNSCC during the past 2 decades, overall disease outcomes have only modestly improved.² Local tumor recurrence affects approximately 60% of patients, and metastases develop in 15% to 25%.³ Less than 30% of patients with HNSCC experience 3 or more years of disease-free survival, and many suffer from impaired speech, swallowing, and/or breathing because of the sensitive location of HNSCC tumors within the upper aerodigestive tract.⁴ Of the various subgroups of HNSCC, oral squamous cell carcinoma (OSCC) is the most common, representing about 75% of all HNSCC cases.² High throughput investigations into the molecular characteristics of HNSCC have mainly used DNA microarray technology to search for gene expression profiles associated with disease and disease outcomes. The literature on DNA microarray profiling of HNSCC shows heterogeneity in the specific genes that were found to be upregulated or downregulated in HNSCC. After comparing results from multiple studies, Choi and Chen⁵ provided a list of genes commonly found to have dysregulated expression in HNSCC tumors. Only a handful of these gene expression alterations have been validated by alternate experimental methods, such as quantitative real-time polymerase chain re-
action (qRT-PCR) and Western blot, Northern blot, and immunohistochemical analyses. Even fewer have been examined for their correlation with disease severity and metastasis status.

Based on various criteria, we selected 6 genes to analyze further: CDH11 (OMIM 600023), MMP3 (OMIM 185250), SPARC (OMIM 182120), POSTN (OMIM 608777), TNC (OMIM 187380), and TGM3 (OMIM 600023). CDH11 encodes an integral membrane protein, cadherin-11, which mediates cell-cell adhesion and is thought to be involved in bone cell differentiation and bone formation. TGM3 encodes a secreted protease, matrix metalloproteinase 3, that degrades the major components for use in immunohistochemical analyses.

### METHODS

#### BIOMARKER SELECTION

For the present study, we selected as OSCC markers genes that (1) showed the highest z scores and greatest expression fold changes between cancerous and histologically normal tissues in our DNA microarray data, (2) have been shown to have significantly altered expression in OSCC when compared with noncancerous tissues in at least 4 other laboratories; and (3) had available antibodies against their encoded protein products for use in immunohistochemical analyses.

#### QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Differential gene expression of CDH11, MMP3, SPARC, POSTN, TNC, and TGM3 between control and cancerous tissue specimens was quantified by using SYBR Green I technology and melting-point dissociation curve analyses per manufacturer protocol (Applied Biosystems, Foster City, California). Total RNAs extracted from 6 histologically normal tissue samples and 6 tumor tissue samples (Table 1) were used as templates in qRT-PCR to generate complementary DNA. Each sample was divided into 5 wells for the qRT-PCR: 3 for the gene of interest and 2 for the endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The qRT-PCR analyses were performed on an ABI 5700 Sequence Detector (Applied Biosystems) using 10 ng of complementary DNA and gene-specific primers in 1× SYBR Green I PCR Master Mix in a 50-µL reaction. Cycling parameters were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. Primer sequences were designed using PE/ABI Primer Express software (Applied Biosystems), checked for specificity against the National Center for Biotechnology Information nucleotide database, and were as follows: CDH11 forward, 5-GCT ACG GCT TTT TGI CTA C-3; CDH11 reverse, 5-AGA ATG CAG CTG TCA CCC CTT-3; MMP3 forward, 5-GGC AAG ACA GCA AGG CAT AGA-3; MMP3 reverse, 5-TGG ATA GGC TGA GCA AAC TGC-3; SPARC forward, 5-CCG CCT TGT GGA CAT CCC TA-3; SPARC reverse, 5-GAG AGG ACT CAT CAG CTG CAT C-3; POSTN forward, 5-ACA ACG CAG CGC TAT TCT GAC-3; POSTN reverse, 5-ATC CA TGT GCC ACA AGC CTG-3; TNC forward, 5-AGA AAC TCA TCC GGC AGC AGC-3; TNC reverse, 5-CTT CCA GAT CCA CCC AAC ACT G-3; TGM3 forward, 5-GAC AAG GCC ATC ACA AAG CACA-3; and TGM3 reverse, 5-TCT TGC GGT AGA GCC AAG GCC-3. Melting-curve analyses were run immediately after cycling to verify specificity of the reactions. Quantification of the transcripts was determined by choosing a fluorescence threshold at which the amplification of the target gene was exponential in both samples of tumor tissue and histologically normal tissue. The PCR cycle number at which the amplification curve intercepted the threshold is termed the threshold cycle (Ct). The threshold cycle is inversely proportional to the copy number of the target template. Relative fold changes
were calculated by $2^{-\Delta \Delta Ct}$, where $\Delta \Delta Ct = (\text{average } Ct_j - \text{average } Ct_i, \text{gene } j) - (\text{average } Ct_l, \text{gene } j - \text{average } Ct_m, \text{GAPDH})$ tumor tissue - (average Ct, gene GAPDH) control tissue.

### IMMUNOHISTOCHEMICAL ANALYSIS

Genes that satisfied the following criteria were studied further by immunohistochemical analysis of OSCC tissue microarray sections: (1) gene expression in cancer tissues was significantly different than in normal tissues by each of the 2 different methods, qRT-PCR and GeneChip (Affymetrix, Santa Clara, California) analysis, and (2) the relative gene expressions determined by the 2 methods were significantly correlated with each other.

The SPARC immunohistochemical analysis was performed using mouse monoclonal anti-SPARC antibody (US Biological, Swampscott, Massachusetts) at a dilution of 1:2000 following antigen retrieval, consisting of 20 minutes of nonpressurized steam incubation in 10mM citrate buffer, pH 6.0. Periostin immunohistochemical analysis was performed using rabbit polyclonal antiperiostin antibody (BioVendor, Candler, North Carolina) at a dilution of 1:900. Tenascin C immunohistochemical analysis was performed using mouse monoclonal anti-tenascin C antibody (BioVendor) at a dilution of 1:50 following antigen retrieval, consisting of 2-5 minute microwave incubations in 10mM citrate buffer, pH 6.0. Transglutaminase-3 immunohistochemical analysis was performed using mouse monoclonal antibody (gift from Kiyotaka Hitomi, PhD, Nagoya University, Nagoya, Japan) raised against purified recombinant human T Gase-3, at a dilution of 1:1000. Tissue microarray sections incubated with antibody diluted alone, phosphate-buffered saline containing 1% bovine serum albumin.
CDH11, SPARC, POSTN, TNC, and TGM3 exhibited significant differences in expression between cancerous and normal tissue specimens by qRT-PCR (Table 3). There was a trend toward upregulation of MMP3 expression in cancerous tissue compared with histologically normal specimens, but this did not reach statistical significance.

Results of the linear regression analyses show good correlation between relative gene expression as determined by qRT-PCR and that previously determined by DNA microarray on the same specimens for MMP3, SPARC, POSTN, TNC, and TGM3 (Table 3). Correlation between qRT-PCR and gene microarray expression data for CDH11 did not reach statistical significance (r=0.45, P≤.14).

Representative tissue microarray cores stained for SPARC, peristin, tenascin C, and transglutaminase 3 are shown for both normal mucosa and primary OSCC tumors (Figure). Staining with antibodies to SPARC was predominantly localized to vessels, fibroblasts, and subsets of carcinoma cells. Virtually no epithelial cell staining was observed in normal mucosa (Figure, A). The SPARC immunohistochemical staining in epithelium, fibroblasts, and vessels was significantly higher in tumor specimens compared with normal control specimens (Figure, B, and Table 4). There were no significant differences with regard to epithelial SPARC expression among tumors of different TNM stage.

Staining with antibodies to periostin was predominantly associated with fibroblasts and the extracellular matrix. Expression was significantly higher in cancerous vs control specimens (Figure, C and D and Table 4). In 14 of 24 primary tumors (58%) and 4 of 17 cervical metastatic tumors (24%), epithelial cancer cells exhibited faint staining, whereas the remaining tumors had no epithelial cell staining. Of 14 tumors that exhibited epithelial staining, 13 (93%) were stage III or IV tumors, representing 69% of the 20 stage III and IV tumors examined. Only 1 (7%) was an early stage tumor (case No. 16, a recurrent T2N0M0), representing 1 of 4 stage I and II tumors examined. In addition, all 8 T4 tumors in our tissue microarray analysis exhibited epithelial periostin staining. Nonneoplastic epithelium was virtually negative for staining. Staining of carcinoma cells was, on average, higher in primary tumors com-

### Table 3. qRT-PCR Validation of Gene Microarray Data

<table>
<thead>
<tr>
<th>Gene</th>
<th>Method</th>
<th>Cancer:Control Ratio (P Value)</th>
<th>Linear Correlation, r (P Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDH11</td>
<td>Microarray</td>
<td>19.8 (.003)</td>
<td>0.45 (.14)</td>
</tr>
<tr>
<td>MMP3</td>
<td>Microarray</td>
<td>18.9 (.03)</td>
<td></td>
</tr>
<tr>
<td>SPARC</td>
<td>qRT-PCR</td>
<td>22.9 (.09)</td>
<td>0.97 (&lt;.001)</td>
</tr>
<tr>
<td>POSTN</td>
<td>Microarray</td>
<td>4.3 (.10)</td>
<td></td>
</tr>
<tr>
<td>TNC</td>
<td>Microarray</td>
<td>5.7 (.001)</td>
<td>0.91 (&lt;.001)</td>
</tr>
<tr>
<td>TGM3</td>
<td>Microarray</td>
<td>3.9 (.001)</td>
<td></td>
</tr>
</tbody>
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Abbreviation: qRT-PCR, quantitative real-time polymerase chain reaction.
pared with cervical lymph node metastases (Table 4). Average extracellular matrix staining of periostin also was greater in primary tumors compared with metastatic lymph nodes (Table 4).

Staining with antibodies to tenasin C was predominantly localized to the extracellular matrix (Figure, E and F). In normal mucosa, only the region of extracellular matrix immediately adjacent to the basal epithelium showed moderate staining. Tenasin C staining in the extracellular matrix was significantly higher in OSCC tumors compared with histologically normal mucosa (Table 4). Staining was occasionally associated with carcinoma cells, particularly in tumor regions directly adjacent to desmoplasic stroma.

Staining with antibodies to transglutaminase 3 localized only within epithelial cells; no stromal expression was observed (Figure, G and H). Within nonneoplastic epithelium, the suprabasal layers stained intensely, whereas the basal layer of epithelial cells did not exhibit staining. Staining in invasive carcinomas was patchy, and when present, was typically associated with areas of increased differentiation and keratinization. Many carcinomas had no staining. There were statistically significant differences in transglutaminase 3 immunoreactivity between different specimen groups; the highest expression was seen in nonneoplastic epithelium, with significantly lower expression in carcinoma in situ specimens, and stepwise significantly lower expression in primary tumors (Table 4).

We selected 6 potential biomarkers of OSCC for the present validation study by examining DNA microarray data from our laboratory as well as that published in the literature. The SPARC, POSTN, TNC, and TGM3 microarray expression differences were validated by qRT-PCR and immunohistochemical analysis of tissue microarray sections. The qRT-PCR results for MMP3 and CDH11 did not reach statistical significance. In contrast with another immunohistochemical study reporting high levels of periostin expression within oral carcinoma epithelium, we noted periostin staining to be localized primarily to the stroma and did not see robust staining within tumor cells themselves (Figure, D). The reason for this disparity is unclear. Nonoverlapping antibody epitopes may partially explain the disparity of periostin staining patterns. The antibody we used for periostin was raised in rabbits against recombinant human periostin containing 648 amino acid residues (corresponding to amino acids 22-669 of full-length periostin) with an N-terminal HisTag fusion (available at http://biovendor.com/pdf/RD181045050.pdf), whereas periostin immunohistochemical experiments by Siriwardena et al. used a polyclonal antibody generated by immunizing rabbits with a specific peptide (EGEPEFLIKETGEC) corresponding to amino acids 679-692 of full-length periostin.

Despite the strong stromal predominance of periostin expression, the percentage of OSCC tumor specimens that exhibited epithelial periostin in our study (38%) was comparable to the 69% reported by Siriwardena et al. Of the stage III and IV OSCC tumors, including 100% of T4 tumors we examined, 65% were positive for epithelial periostin immunostaining, compared with only 25% of the stage I and II tumors. These findings suggest that epithelial expression of periostin may be associated with a more aggressive tumor phenotype in OSCC. This is supported by other studies, which show that subsets of HNSCC cells expressing periostin, or cells engineered to overexpress periostin, exhibit enhanced tumor growth and invasiveness, and tumors that express periostin have a more invasive phenotype.
We found the proportion of metastatic lymph node tumors positive for epithelial peristin expression (24%) was less than half that of primary tumors (58%). However, each lymph node tumor specimen that exhibited staining was associated with a primary tumor that also had epithelial peristin expression, suggesting that presence of peristin in the epithelium of primary tumors may be necessary, but not sufficient, for its presence in metastatic tumors.

In laryngeal and OSCC, increased levels of tenascin C immunostaining have been found to correlate with malignancy and invasion. Abundant expression of tenascin C in our OSCC tissue microarray sections was localized primarily to the stroma, although some minor staining of tumor cells was also observed, particularly at tumor edges adjacent to desmoplastic stroma. This observation is consistent with reports in the literature showing tenascin C localization along the invasive fronts of carcinomas of the lung, liver, bladder, and skin.

Roepman et al and Schmalbach et al identified TGM3 to be significantly downregulated in metastatic HNSCC compared with both nonmetastatic tumors and normal epithelium. O’Donnell et al similarly found significant downregulation of TGM3 gene expression in metastatic primary OSCC tumors compared with nonmetastatic primary tumors. Our cross-sectional immunohistochemical data show that the levels of transglutaminase 3 protein expression were seen to decrease in a stepwise fashion from normal to premalignant to malignant specimens. This suggests that the loss of transglutaminase 3 activity might be associated with the progression of squamous cell carcinoma.

All of the upregulated gene markers we identified by reviewing gene microarray reports, validated by qRT-PCR, and subsequently studied with immunohistochemical analysis revealed protein expression to be localized primarily within the stroma and modestly or not at all within tumor cells. This finding illustrates an important point regarding the interpretation of gene microarray data based on the methods used for specimen processing. The methods used by different laboratories for tumor specimen processing vary significantly. Although some investigators isolated relatively homogeneous populations of tumor cells for microarray analysis via laser capture microdissection, others established arbitrary thresholds for minimum tumor cell content in surgical specimens, as assessed by histologic evaluation of adjacent tissue, before RNA extraction and microarray analysis. The latter method clearly results in varying amounts of stromal cells contributing to the final pool of extracted RNA, and thus the variability of the resultant microarray data. Notably, even laser capture microdissection does not ensure isolation of a purely homogeneous population of tumor cells, as varying degrees of leukocytosis and neovascularization within tumors exist and correlate with survival, tumor stage, metastases, and presence of extracapsular spread in HNSCC.

Upregulation of SPARC, POSTN, or TNC was not reported by any of the DNA microarray studies that examined expression of HNSCC cell lines or by others that used laser capture microdissection to isolate tumor cells from stroma. Presumably this is because of the relative absence of stromal cells within the analyzed specimens in these studies, although absence of 1 or more of these markers on the microarrays used by these studies may also contribute. These findings, together with the immunohistochemical data we report herein, suggest that upregulation of SPARC, POSTN, and TNC is caused by (1) upregulation within stromal cell populations vs carcinoma cells and/or (2) stroma-induced transcriptional upregulation of these markers in cancer cells. In any case, these observations underscore the importance in examining tumor and stroma in the pathogenesis of OSCC.

The “seed and soil” hypothesis of tumor-stromal interaction was originally proposed by Paget in 1889, but only recently have researchers examined how tumor microenvironments influence the growth and spread of cancers. Carcinoma-associated fibroblasts, extracellular matrix macromolecules, neovascularization, and inflammatory and immune cell infiltration within the stroma adjacent to tumors can have profound effects on tumor progression in breast, prostate, and skin carcinomas. The situation in OSCC is less well understood, but studies of carcinoma-associated fi-
broblasts, extracellular matrix turnover, and tumor cell motility have begun to delineate the role of desmoplastic stroma in OSCC carcinogenesis.43–45 Recently, Weber et al46 performed genome-wide analysis of loss of heterozygosity and allelic imbalance on specimens of tumor stroma and tumor epithelium isolated by laser capture microdissection from more than 120 patients with OSCC and a history of smoking. They discovered more than 40 hot spots of loss of heterozygosity/allelic imbalance within the stroma, nearly twice as many as they found in the epithelium, and subsequently identified 3 stroma-specific loci that were significantly associated with tumor size and cervical lymph node metastasis.60 These findings again highlight the importance of examining stromal as well as epithelial elements in OSCC and suggest that stromal alterations play a crucial part in facilitating OSCC invasion and metastasis.

CONCLUSIONS

Our observations indicate that significant changes in the expression of 4 genes, SPARC, POSTN, TNC, and TGM3, initially identified by gene microarray studies, are associated with similar changes in protein expression based on immunohistochemical analyses. The localization of SPARC, periostin, and tenascin C predominantly within the stroma of OSCC tumors supports the idea that stromal elements are important in OSCC pathogenesis.

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