

**1 Examination of Oral Cancer Biomarkers by Tissue Microarray Analysis**

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29 **ABSTRACT**

30 **Background.** Oral squamous cell carcinoma (OSCC) is a major healthcare problem worldwide.  
31 Efforts in our laboratory and others focusing on the molecular characterization of OSCC tumors  
32 with the use of DNA microarrays have yielded heterogeneous results. To validate the DNA  
33 microarray results on a subset of genes from these studies that could potentially serve as  
34 biomarkers of OSCC, we elected to examine their expression by an alternate quantitative method  
35 and by assessing their protein levels.

36 **Design.** Based on DNA microarray data from our lab and data reported in the literature, we  
37 identified six potential biomarkers of OSCC to investigate further. We employed quantitative,  
38 real-time polymerase chain reaction (qRT-PCR) to examine expression changes of *CDH11*,  
39 *MMP3*, *SPARC*, *POSTN*, *TNC*, *TGM3* in OSCC and normal control tissues. We further  
40 examined validated markers on the protein level by immunohistochemistry (IHC) analysis of  
41 OSCC tissue microarray (TMA) sections.

42 **Results.** qRT-PCR analysis revealed up-regulation of *CDH11*, *SPARC*, *POSTN*, and *TNC* gene  
43 expression, and decreased *TGM3* expression in OSCC compared to normal controls. *MMP3* was  
44 not found to be differentially expressed. In TMA IHC analyses, SPARC, periostin, and tenascin  
45 C exhibited increased protein expression in cancer compared to normal tissues, and their  
46 expression was primarily localized within tumor-associated stroma rather than tumor epithelium.  
47 Conversely, transglutaminase-3 protein expression was found only within keratinocytes in  
48 normal controls, and was significantly down-regulated in cancer cells.

49 **Conclusions.** Of six potential gene markers of OSCC, initially identified by DNA microarray  
50 analyses, differential expression of *CDH11*, *SPARC*, *POSTN*, *TNC*, and *TGM3* were validated by

51 qRT-PCR. Differential expression and localization of proteins encoded by *SPARC*, *POSTN*,  
52 *TNC*, and *TGM3* were clearly shown by TMA IHC.

53

#### 54 **Introduction**

55 Head and neck squamous cell carcinoma (HNSCC) is the 5<sup>th</sup> most common cancer  
56 worldwide.<sup>1</sup> The American Cancer Society estimates that approximately 30,990 Americans were  
57 diagnosed with and 7,430 died of cancer of the oral cavity and pharynx in 2006.<sup>2</sup> Despite  
58 considerable advances in the treatment of HNSCC over the past two decades, overall disease  
59 outcomes have only modestly improved.<sup>2</sup> Local tumor recurrence affects approximately 60% of  
60 patients and metastases develops in 15-25%.<sup>3</sup> Less than 30% of HNSCC patients experience  
61 three or more years of disease-free survival, and many suffer from impaired speech, swallowing,  
62 and/or breathing due to the sensitive location of HNSCC tumors within the upper aerodigestive  
63 tract.<sup>4</sup> Of the various subgroups of HNSCC, oral squamous cell carcinoma (OSCC) is the most  
64 common, representing about 75% of all HNSCC cases.<sup>2</sup> High throughput investigation into the  
65 molecular characteristics of HNSCC has mainly utilized DNA microarray technology to search  
66 for gene expression profiles associated with disease and disease outcomes. The literature on  
67 DNA microarray profiling of HNSCC shows heterogeneity in the specific genes that were found  
68 to be up- or down-regulated in HNSCC. After comparing results from multiple studies, we  
69 reported a list of genes commonly found to have dysregulated expression in HNSCC tumors.<sup>5</sup>  
70 Only a handful of these gene expression alterations have been validated by alternate  
71 experimental methodologies such as qRT-PCR, Western blot, Northern blot, and IHC. Even  
72 fewer have been examined for their correlation with disease severity and metastasis status.

73           Based on various selection criteria (see Materials & Methods), we selected six genes to  
74 analyze further: *CDH11*, *MMP3*, *SPARC*, *POSTN*, *TNC*, and *TGM3*. *CDH11* encodes an  
75 integral membrane protein, cadherin-11, which mediates cell-cell adhesion and thought to be  
76 involved in bone cell differentiation and bone formation.<sup>6</sup> *MMP3* encodes a secreted protease,  
77 matrix metalloproteinase-3, whose action is to degrade the major components of the extracellular  
78 matrix (ECM), and is thought to be associated with cervical lymph node metastases in HNSCC.<sup>7</sup>  
79 *SPARC* (secreted protein, acidic, rich in cysteine), encodes an ECM-associated protein, a.k.a.  
80 osteonectin, that inhibits cell-cycle progression, causes changes in cell shape, and influences  
81 ECM synthesis.<sup>8</sup> *SPARC* has also been found to be an independent prognostic marker for short  
82 disease-free interval and poor overall survival in HNSCC patients.<sup>9</sup> *POSTN* encodes the protein,  
83 periostin, which is a ligand for various integrins and as such, supports adhesion and migration of  
84 epithelial cells.<sup>10</sup> Periostin is thought to promote invasion and angiogenesis in OSCC.<sup>11,12</sup> *TNC*  
85 encodes an ECM protein, tenascin-C, that regulates cell adhesion, migration, and growth.<sup>13</sup>  
86 *TGM3* encodes transglutaminase-3, which crosslinks intracellular structural proteins and is  
87 important in cell envelope formation of the epidermis.<sup>14</sup> Transglutaminase-3 is expressed  
88 normally in terminally differentiated epithelial cells.<sup>15</sup> It has been shown to be down-regulated  
89 in esophageal squamous cell carcinoma<sup>16-18</sup> and in the progression of oral leukoplakia to  
90 OSCC.<sup>19</sup> *CDH11*, *MMP3*, *SPARC*, *POSTN*, and *TNC* have all been shown in gene microarray  
91 experiments to be significantly upregulated in cancer tissues compared to normal controls,  
92 whereas *TGM3* is significantly down-regulated.<sup>5</sup>

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## 96 **Materials & Methods**

97 *Biomarker Selection.* The criteria for choosing potential OSCC markers for the current study  
98 were: 1) genes from our own DNA microarray data that show the highest Z-scores and greatest  
99 expression fold changes between cancer and normal;<sup>20</sup> 2) these genes have been shown to have  
100 significantly altered expression in OSCC when compared with non-cancer tissues in at least four  
101 other laboratories, and 3) the genes had available antibodies against their encoded protein  
102 products for use in IHC analyses.

103  
104 *qRT-PCR.* Differential gene expression of *CDH11*, *MMP3*, *SPARC*, *POSTN*, *TNC*, and *TGM3*  
105 between normal and cancer specimens was quantified by using SYBR<sup>®</sup> Green I technology and  
106 melting-point dissociation curve analyses per manufacturer protocol (Applied Biosystems, Foster  
107 City, CA). Total RNA extracted from six normal tissue samples and six tumor tissue samples  
108 (Table 1) were used as templates in RT-PCR reactions to generate cDNA. Each sample was  
109 divided into five wells for the qRT-PCR reactions: three for the gene of interest and two for the  
110 endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). qRT-PCR analyses  
111 were performed on an ABI 5700 Sequence Detector using 10 ng of cDNA and gene specific  
112 primers in 1 × SYBR<sup>®</sup> Green I PCR Master Mix in a 50-μL reaction. Cycling parameters were  
113 50 °C for 2 minutes, 95 °C for 10 minutes, and 40 cycles at 95 °C for 15 seconds and at 60 °C  
114 for 1 minute. Primer sequences were designed using PE/ABI Primer Express<sup>®</sup> software, checked  
115 for specificity against the National Center for Biotechnology Information nucleotide data base,  
116 and were as follows: *CDH11* forward, 5-GCT CAA CCA GCA GAG ACA TTC C-3; *CDH11*  
117 reverse, 5-AGA ATG CAG CTG TCA CCC CTT-3; *MMP3* forward, 5-GGC AAG ACA GCA  
118 AGG CAT AGA-3; *MMP3* reverse, 5-TGG ATA GGC TGA GCA AAC TGC-3; *SPARC*

119 forward, 5-CGG CTT TGT GGA CAT CCC TA-3; *SPARC* reverse, 5-GGA AGG ACT CAT  
120 GAC CTG CAT C-3; *POSTN* forward, 5-ACA ACG CAG CGC TAT TCT GAC-3; *POSTN*  
121 reverse, 5-ATC CAA GTT GTC CCA AGC CTC-3; *TNC* forward, 5-AGA AAG TCA TCC  
122 GGC ACA AGC-3; *TNC* reverse, 5-ACT CCA GAT CCA CCG AAC ACT G-3; *TGM3* forward,  
123 5-GAC AAG CGC ATC ACA CAG ACA-3; *TGM3* reverse, 5-TCT TTC GTT AGA GCC AAG  
124 GCC-3. Melting-curve analyses were run immediately after cycling to verify specificity of the  
125 reactions. Quantification of the transcripts was determined by choosing a fluorescence threshold  
126 at which the amplification of the target gene was exponential in both tumor samples and normal  
127 samples. The PCR cycle number at which the amplification curve intercepted the threshold is  
128 termed the threshold cycle ( $C_T$ ). The threshold cycle is inversely proportional to the copy  
129 number of the target template. Relative fold changes were calculated by  $2^{-\Delta\Delta C_T}$ , where  $\Delta\Delta C_T =$   
130 [average  $C_T$ , gene  $j$  - average  $C_T$ , GAPDH] tumor tissue - [average  $C_T$ , gene  $j$  - average  $C_T$ ,  
131 GAPDH] normal tissue.

132

133 *TMA Construction.* TMA blocks (one master and one copy) were constructed with the use of an  
134 automated tissue arrayer per manufacturer protocol (Beecher Instruments, Sun Prairie, WI).  
135 Formalin-fixed, paraffin-embedded tissue specimens were obtained from the University of  
136 Washington, Department of Pathology under Institutional Review Board approval by the Human  
137 Subjects Division at the University of Washington and the Fred Hutchinson Cancer Research  
138 Center. Our TMA is comprised of 63 tissue specimens from 31 patients (Table 2). Patients  
139 ranged from 28 to 88 years ( $60 \pm 15$ ) in age and three quarters were male (74.2%). Tissues came  
140 from the oral cavity, oropharynx, and lymph node metastases. Twenty-four of the tissue  
141 specimens were from primary OSCC tumors, 16 were from normal tissue taken from cancer

142 patients, 17 were from cervical lymph node metastases, three were premalignant lesions, and  
143 three were from patients without cancer (normal patients). Of the 24 tumors, 4 were stage I/II  
144 and 20 were stage III/IV, 12 were T1/T2 and 12 were T3/T4, and 18 had associated cervical  
145 lymph node metastases while 6 were non-metastatic. Cores were arrayed in quadruplicate, with  
146 a diameter of 0.6 mm. TMA blocks were stored in a nitrogen chamber for antigen preservation.  
147 Sections were cut at 5  $\mu$ M for IHC analyses.

148

149 *Immunohistochemistry.* Genes that satisfied the following criteria were studied further by IHC  
150 analysis of OSCC TMA sections: 1) gene expression in cancer tissues had to be significantly  
151 different than in normal tissues by each of the two different methods, qRT-PCR and GeneChip<sup>®</sup>  
152 analysis, and 2) the relative gene expression determined by these two different methods had to be  
153 significantly correlated with one another.

154 SPARC IHC was performed using mouse monoclonal anti-SPARC antibody (US  
155 Biological, Swampscott, MA) at a dilution of 1:2000, following antigen retrieval consisting of 20  
156 minutes of non-pressurized steam incubation in 10 mM citrate buffer, pH 6.0. Periostin IHC was  
157 performed using rabbit polyclonal anti-periostin antibody (BioVendor, Candler, NC;  
158 <http://biovendor.com/pdf/RD181045050.pdf>) at a dilution of 1:900. Tenascin-C IHC was  
159 performed using mouse monoclonal anti-tenascin-C antibody (BioVendor, Candler, NC;  
160 <http://biovendor.com/pdf/RE11370C100.pdf>) at a dilution of 1:50 following antigen retrieval  
161 consisting of two, five-minute microwave incubations in 10 mM citrate buffer, pH 6.0.  
162 Transglutaminase-3 IHC was performed using mouse monoclonal antibody (a generous gift from  
163 Dr. Kiyotaka Hitomi, Nagoya University, Nagoya, Japan) raised against purified, recombinant  
164 human TGase-3,<sup>15</sup> at a dilution of 1:1000. TMA sections incubated with antibody diluent alone,

165 phosphate buffered saline (PBS) containing 1% bovine serum albumin, served as negative  
166 controls to confirm specificity of immunostaining for all markers.

167 Slides were deparaffinized by three changes of xylene, seven minutes each, and  
168 rehydrated by three changes of 100% ethanol x two minutes, two changes of 95% ethanol x two  
169 minutes, and one change of 70% ethanol x one minute. Endogenous peroxidase activity was  
170 blocked by incubation in 0.3% H<sub>2</sub>O<sub>2</sub> at room temperature for 10 minutes. Non-specific binding  
171 sites were subsequently blocked with 2% normal goat serum (Vector Laboratories, Burlingame,  
172 CA) in PBS, pH 7.4, at room temperature for 15 minutes. Slides were then washed and  
173 incubated with primary antibody. Washes consisted of a five-minute soak in PBS, pH 7.4,  
174 followed by 10 dips in PBS with 1% BSA and 0.01% Triton-X-100 (Sigma, St. Louis, MO), pH  
175 7.4, and a second five-minute wash in PBS, pH 7.4. TMA slides were then incubated with  
176 primary antibody at the above listed dilutions in PBS with 1% BSA, pH 7.4, at room temperature  
177 for one hour in a humidity chamber (Shandon Lipshaw, Inc, Pittsburgh, PA). Sections were then  
178 washed again as before, incubated with either biotinylated goat anti-mouse or biotinylated goat  
179 anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA), washed again, and  
180 subsequently incubated with ABC Elite reagent (Vector Laboratories, Burlingame, CA) per  
181 manufacturer protocol. Slides were then stained by incubation with 0.08% diaminobenzidine,  
182 0.01% FeCl<sub>3</sub> in 0.05 M Tris buffer, pH 8.0 (Sigma, St. Louis, MO) at 37°C for seven minutes.  
183 Sections were counterstained with Mayers Hematoxylin (Dako Cytomation, Carpinteria, CA),  
184 dehydrated, mounted with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI) and covered  
185 with a coverslip.

186

187 *Scoring of IHC results.* Images of the IHC-stained TMA sections were digitized using the  
188 BLISS Tracer imaging system and visualized with WebSlide Server software (Bacus  
189 Laboratories, Lombard, IL). Marker immunoreactivity was scored using a validated, modified  
190 H-score system<sup>21</sup> by a board-certified pathologist (C.D.J.), blinded to all characteristics of the  
191 cases and controls. IHC scores were determined by taking the product of the estimated staining  
192 intensity (0, 1+, 2+, 3+) and area of tissue (tumor or normal; epithelial or stromal) stained (0% =  
193 0, <25% = 1, 25-75% = 2, >75% = 3), giving a range of possible scores between 0 and 9. IHC  
194 scores for replicate cores were averaged to determine a composite score for each case.

195  
196 *Statistical Analysis.* qRT-PCR results were analyzed by an unpaired Student's t-test to compare  
197 gene expression between cancer and normal specimens. Relative gene expression values  
198 determined by qRT-PCR analyses were then compared with those previously determined by gene  
199 microarray analyses<sup>20</sup> by linear regression analysis. Pairwise comparisons of IHC scores were  
200 made between primary tumor tissue and normal mucosa, tumor tissue and lymph nodes, tumor  
201 tissue and CIS, and CIS tissue and normal mucosa, using unpaired Student's t-tests. Because of  
202 multiple comparisons, we felt that a threshold of  $P < .05$  was too low, and considered comparisons  
203 to be statistically significant only if  $P < .01$ . All statistical analyses were performed using Stata  
204 9.0 software (StataCorp, College Station, TX).

205

## 206 **Results**

207 *CDH11, SPARC, POSTN, TNC, and TGM3* exhibited significant differences in  
208 expression between cancer and normal specimens by qRT-PCR (Table 3). There was a trend

209 towards up-regulation of *MMP3* expression in cancer compared to normal specimens, but this  
210 did not reach statistical significance.

211 Results of the linear regression analyses show good correlation between relative gene  
212 expression as determined by qRT-PCR and that previously determined by DNA microarray<sup>20</sup> on  
213 the same specimens for *MMP3*, *SPARC*, *POSTN*, *TNC*, and *TGM3* (Table 3). Correlation  
214 between qRT-PCR and gene microarray expression data for *CDH11* did not reach statistical  
215 significance ( $r = 0.45$ ,  $p \leq 0.14$ ).

216 Representative TMA cores stained for SPARC, periostin, tenascin-C, and  
217 transglutaminase-3 are shown for both normal mucosa and primary OSCC tumors (Figure 1).  
218 Staining with antibodies to SPARC was predominantly localized to vessels, fibroblasts, and  
219 subsets of carcinoma cells. Virtually no epithelial cell staining was observed in normal mucosa  
220 (Figure 1A). SPARC IHC staining in epithelium, fibroblasts, and vessels was significantly  
221 higher in tumor specimens compared to normal controls (Figure 1B, Table 4). There were no  
222 significant differences with regard to epithelial SPARC expression among tumors of different  
223 TNM stage.

224 Staining with antibodies to periostin was predominantly associated with fibroblasts and  
225 the ECM. Expression was significantly higher in cancer versus normal controls (Figure 1C-D,  
226 Table 4). Epithelial cancer cells in over half (14/24, 58.3%) of the primary tumors and  
227 approximately one fourth (4/17, 23.5%) of the cervical metastatic tumors exhibited faint staining,  
228 whereas the remaining tumors were completely negative for epithelial cell staining. Of the  
229 tumors that were positive for epithelial staining, 13/14 (92.8%) were stage III/IV tumors  
230 representing 65% of the 20 stage III/IV tumors examined, whereas only one (7.2%) was an early  
231 stage tumor (Case 16, a recurrent T2N0M0), representing one of the four stage I/II tumors

232 examined. In addition, all 8 (100%) of the T4 tumors on our TMA were positive for epithelial  
233 periostin staining. Non-neoplastic epithelium was virtually negative for staining. Staining of  
234 carcinoma cells was, on average, higher in primary tumors compared to cervical lymph node  
235 metastases (Table 4). Average ECM staining of periostin also was greater in primary tumors  
236 compared to that within the metastatic lymph nodes (Table 4).

237         Staining with antibodies to tenascin-C was predominantly localized to the ECM (Figure  
238 1E-F). In normal mucosa, only the region of ECM immediately adjacent to the basal epithelium  
239 showed moderate staining. Tenascin-C staining in the ECM was significantly higher in OSCC  
240 tumors compared to normal mucosa (Table 4). Staining was occasionally associated with  
241 carcinoma cells, particularly in tumor regions directly adjacent to desmoplastic stroma.

242         Staining with antibodies to transglutaminase-3 localized only within epithelial cells; no  
243 stromal expression was observed (Figure 1G-H). Within non-neoplastic epithelium, the  
244 suprabasal layers stained intensely, while the basal layer of epithelial cells was negative for  
245 staining. Staining in invasive carcinomas was patchy, and when present, was typically  
246 associated with areas of increased differentiation and keratinization. Many carcinomas were  
247 completely negative for staining. There were statistically significant differences in  
248 transglutaminase-3 immunoreactivity between different specimen groups, such that the highest  
249 expression was seen in non-neoplastic epithelium, with significantly lower expression in CIS  
250 specimens, and stepwise significantly lower expression in primary tumors (Table 4).

251

## 252 **Discussion**

253         We selected six potential biomarkers of OSCC for the current validation study by  
254 examining DNA microarray data both from our laboratory, as well as that published in the

255 literature.<sup>5</sup> *SPARC*, *POSTN*, *TNC*, and *TGM3* microarray expression differences were validated  
256 by both qRT-PCR and IHC of TMA sections. The qRT-PCR results for *MMP3* and *CDH11* did  
257 not reach statistical significance. In contrast with another IHC study reporting high levels of  
258 periostin expression within oral carcinoma epithelium,<sup>12</sup> we noted periostin staining to be  
259 localized primarily to the stroma and did not see robust staining within tumor cells themselves  
260 (Figure 1D). The reason for this disparity in IHC staining is unclear. Non-overlapping antibody  
261 epitopes may partially explain the disparity of periostin IHC staining patterns. The antibody we  
262 used for periostin IHC was raised in rabbits against recombinant human periostin containing 648  
263 amino acid residues (corresponding to amino acids 22-669 of full-length periostin) with an N-  
264 terminal HisTag fusion (<http://biovendor.com/pdf/RD181045050.pdf>), whereas periostin IHC  
265 experiments by Siriwarden et al. utilized a polyclonal antibody generated by immunizing rabbits  
266 with a specific peptide (EGEPEFRLIKEGETC) corresponding to amino acids 679-692 of full-  
267 length periostin.<sup>12</sup>

268 Despite the strong stromal predominance of periostin expression we observed, the  
269 percentage of OSCC tumors positive for epithelial periostin in our study (58%) was comparable  
270 to that reported by Siriwarden et al.<sup>12</sup> (69%). A majority (65%) of the stage III/IV OSCC  
271 tumors, including 100% of T4 tumors we examined, were positive for epithelial periostin  
272 immunostaining, compared to only 25% of the stage I/II tumors. These findings suggest that  
273 epithelial expression of periostin may be associated with a more aggressive tumor phenotype in  
274 OSCC. This is supported by other studies, which show that subsets of HNSCC cells expressing  
275 periostin, or cells engineered to overexpress periostin, exhibit enhanced tumor growth and  
276 invasiveness, and tumors that express periostin have a more invasive phenotype.<sup>11,12</sup>

277 We found the proportion of metastatic lymph node tumors positive for epithelial periostin  
278 expression (23.5%) was less than half that of primary tumors (58.3%). However, each of the  
279 positively stained lymph node tumors was associated with a primary tumor that also had  
280 epithelial periostin expression, suggesting that presence of periostin in the epithelium of primary  
281 tumors may be necessary, but not sufficient, for its presence in metastatic tumors.

282 In oral and laryngeal squamous cell carcinoma, increased levels of tenascin-C  
283 immunostaining have been found to correlate with malignancy and invasion<sup>22-25</sup> Abundant  
284 expression of tenascin-C in our OSCC TMA sections was localized primarily to the stroma,  
285 although some minor staining of tumor cells was also observed, particularly at tumor edges  
286 adjacent to desmoplastic stroma. This observation is consistent with reports in the literature  
287 showing tenascin-C localization along the invasive fronts of carcinomas of the lung, liver,  
288 bladder, and skin.<sup>26,27</sup>

289 Roepman, et al.<sup>28</sup> and Schmalbach, et al.<sup>29</sup> identified *TGM3* to be significantly down-  
290 regulated in metastatic HNSCC compared to both non-metastatic tumors and normal epithelium.  
291 O'Donnell et al.<sup>30</sup> similarly found significant down-regulation of *TGM3* gene expression in  
292 metastatic primary OSCC tumors compared to non-metastatic primaries. Our cross-sectional  
293 IHC data show that the levels of transglutaminase-3 protein expression were seen to decrease in  
294 a stepwise fashion from normal to premalignant to malignant specimens. This suggests that the  
295 loss of transglutaminase-3 activity might be associated with the progression of squamous cell  
296 carcinoma.

297 All of the up-regulated gene markers we identified by reviewing gene microarray  
298 reports, validated by qRT-PCR, and subsequently studied with IHC revealed protein expression  
299 to be localized primarily within the stroma, and modestly or not at all within tumor cells. This

300 finding illustrates an important point regarding the interpretation of gene microarray data based  
301 on the methodology used for specimen processing. The methods employed by different  
302 laboratories for tumor specimen processing vary significantly.<sup>5</sup> While some investigators  
303 isolated relatively homogeneous populations of tumor cells for microarray analysis via laser  
304 capture microdissection (LCM), others established arbitrary thresholds for minimum tumor cell  
305 content in surgical specimens, as assessed by histologic evaluation of adjacent tissue, prior to  
306 RNA extraction and microarray analysis. The latter method clearly results in varying amounts of  
307 stromal cells contributing to the final pool of extracted RNA, and thus the variability of the  
308 resultant microarray data. Notably, even LCM does not ensure isolation of a purely  
309 homogeneous population of tumor cells, as varying degrees of leukocytosis and  
310 neovascularization within tumors exist and correlate with survival, tumor stage, metastases, and  
311 presence of extracapsular spread in HNSCC.<sup>31-33</sup>

312 Up-regulation of *SPARC*, *POSTN*, or *TNC* was not reported by any of the DNA  
313 microarray studies that examined expression of HNSCC cell lines<sup>34-36</sup> or by others that employed  
314 LCM to isolate tumor cells from stroma.<sup>37-40</sup> Presumably this is due to the relative absence of  
315 stromal cells within the analyzed specimens in these studies, although absence of one or more of  
316 these markers on the microarrays used by these studies may also contribute. These findings,  
317 together with the IHC data we report here, suggest that up-regulation of *SPARC*, *POSTN*, and  
318 *TNC* is due to 1) up-regulation within stromal cell populations vs carcinoma cells and/or 2)  
319 stroma-induced transcriptional upregulation of these markers in cancer cells. In any case, these  
320 observations underscore the importance in examining both tumor and stroma in the pathogenesis  
321 of OSCC.

322 The “seed and soil” hypothesis of tumor-stromal interaction was originally proposed by  
323 Paget in 1889, but only recently have researchers examined how tumor microenvironments  
324 influence the growth and spread of cancers. Carcinoma-associated fibroblasts (CAFs), ECM  
325 macromolecules, neovascularization, and inflammatory and immune cell infiltration within the  
326 stroma adjacent to tumors can have profound effects on tumor progression in breast, prostate,  
327 and skin carcinomas.<sup>41</sup> The situation in OSCC is less well understood, but studies of CAFs,  
328 ECM turnover and tumor cell motility have begun to delineate the role of desmoplastic stroma in  
329 OSCC carcinogenesis.<sup>42-44</sup> Recently, Weber et al.<sup>45</sup> performed genome-wide analysis of loss of  
330 heterozygosity (LOH) and allelic imbalance (AI) on LCM-isolated specimens of tumor stroma  
331 and tumor epithelium from over 120 OSCC patients with a history of smoking. They discovered  
332 over 40 hot spots of LOH/AI within the stroma, nearly twice as many as they found in the  
333 epithelium, and subsequently identified three stroma-specific loci that were significantly  
334 associated with tumor size and cervical lymph node metastasis.<sup>45</sup> These findings again highlight  
335 the importance of examining both stromal as well as epithelial elements in OSCC, and suggest  
336 that stromal alterations play a crucial part in facilitating OSCC invasion and metastasis.

337

### 338 **Conclusions**

339 Our observations indicate that significant changes in the expression of four genes,  
340 *SPARC*, *POSTN*, *TNC*, and *TGM3*, initially identified by gene microarray studies, are associated  
341 with similar changes in protein expression based on IHC analyses. The localization of *SPARC*,  
342 periostin, tenascin-C predominately within the stroma of OSCC tumors supports the idea that  
343 stromal elements are important in OSCC pathogenesis.

344

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**478 Figure 1 – IHC of OSCC TMA sections**

479 Representative tissue cores are shown. (A) SPARC IHC of normal mucosa – Occasional faint  
480 staining of vessels and fibroblasts was identified. Virtually no epithelial staining was seen. (B)  
481 SPARC IHC of a T4N2bM0 primary OSCC – Increased staining was observed, mostly within  
482 vessels and fibroblasts of the stroma, but also within some carcinoma cells. (C) Periostin IHC  
483 of normal mucosa – Focal faint-to-moderate vascular staining and very focal faint subepithelial  
484 stromal staining was identified. (D) Periostin IHC of T4N2bM0 primary OSCC – Strong  
485 staining localized primarily within the ECM and stromal fibroblasts. Many late stage tumors  
486 also exhibited modest staining within cancer epithelium, as in this case. (E) Tenascin-C IHC of  
487 normal mucosa – A narrow zone of faint-to-moderate stromal staining was identified beneath the  
488 basal layer of normal epithelium. (F) Tenascin-C IHC of T4N2bM0 primary OSCC – Strong  
489 staining was observed mostly within the carcinoma-associated stroma. (G) Transglutaminase-3  
490 IHC of normal mucosa – Moderate-to-strong staining was seen in suprabasal epithelium of all  
491 normal mucosa. (H) Transglutaminase-3 IHC of T4N2bM0 primary OSCC – Although some  
492 OSCC tumors exhibited faint epithelial staining, primarily in more differentiated and keratinized  
493 regions, many tumors did not stain at all, as in this case.

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502 **Table 1 – Characteristics of Specimens Used in qRT-PCR Analyses**

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<b>Specimen</b>	<b>Age</b>	<b>Sex</b>	<b>Diagnosis</b>	<b>Stage</b>	<b>Grade</b>
Normal	43	M	Obstructive sleep apnea	T0N0M0	N/A
Normal	37	M	Obstructive sleep apnea	T0N0M0	N/A
Normal	48	F	Obstructive sleep apnea	T0N0M0	N/A
Normal	48	M	Buccal mucosa from patient with lateral tongue SCCA	T2N0M0	N/A
Normal	54	F	Buccal mucosa from patient with lateral tongue SCCA	T2N0M0	N/A
Normal	64	M	Palate mucosa from patient with tonsil/palate SCCA	T2N2bM0	N/A
Cancer	48	M	Lateral tongue SCCA	T2N0M0	moderate
Cancer	81	F	Retromolar trigone SCCA	T4N2bM0	moderate-to-poor
Cancer	63	M	Tonsil/soft palate SCCA	T2N2bM0	moderate
Cancer	61	M	Retromolar trigone SCCA	T4N2bM0	moderate
Cancer	76	M	Gingivobuccal sulcus SCCA	T4N0M0	moderate
Cancer	54	F	Lateral tongue SCCA	T2N0M0	moderate

504 SCCA = squamous cell carcinoma

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518 **Table 2 – Characteristics of Tissue Microarray Specimens**  
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Case	Specimen(s)	Age	Sex	Diagnosis	Stage	Grade
1	NLn	43	M	Obstructive sleep apnea	T0N0M0	N/A
2	NLn	37	M	Obstructive sleep apnea	T0N0M0	N/A
3	NLn	48	F	Obstructive sleep apnea	T0N0M0	N/A
4	T, LN	51	F	Lateral tongue SCCA	T4N2bM0	well-to-moderate
5	NLc, T	48	M	Lateral tongue SCCA	T2N0M0	moderate
6	T, LN	81	F	Retromolar trigone SCCA	T4N2bM0	moderate-to-poor
7	NLc, T, LN	46	F	Lateral tongue SCCA	T2N2bM1	well
8	NLc, T, LN	63	M	Tonsil/soft palate SCCA	T2N2bM0	moderate
9	T, LN	61	M	Retromolar trigone SCCA	T4N2bM0	moderate
10	NLc, T	76	M	Gingivobuccal sulcus SCCA	T4N0M0	moderate
11	NLc, T, LN	54	M	Tonsil/soft palate SCCA	T4N2bM0	moderate
12	NLc, T, LN	77	M	Floor of mouth SCCA	T1N2bM0	well-to-moderate
13	NLc, T	54	F	Lateral tongue SCCA	T2N0M0	moderate
14	CIS, NLc	28	M	Lateral tongue leukoplakia	TisN0M0	CIS
15	CIS	83	F	Lower lip leukoplakia	TisN0M0	CIS
16	NLc, T	39	F	Recurrent floor of mouth SCCA	T2N0M0	well
17	T	51	M	Floor of mouth SCCA	T2N0M0	moderate
18	CIS	49	M	Floor of mouth leukoplakia	TisN0M0	CIS
19	NLc, T	50	M	Lateral tongue SCCA	T2N0M0	moderate
20	LN	57	M	Posterior tongue SCCA	T1N2bM0	not available
21	T, LN	88	F	Retromolar trigone SCCA	T4N2bM0	well
22	NLc, T, LN	61	M	Base of tongue SCCA	T3N2bM0	well-to-moderate
23	NLc, T	63	M	Lateral tongue SCCA	T1N2bM0	moderate-to-poor
24	T, LN	62	M	Recurrent lateral tongue SCCA	T2N2bM0	poor
25	NLc, T, LN	52	M	Anterior tongue SCCA	T3N1M0	well
26	NLc, T, LN	62	M	Lateral tongue SCCA	T2N2bM0	moderate
27	NLc, T, LN	62	M	Base of tongue SCCA	T3N2bM0	moderate
28	T, LN	70	M	Base of tongue SCCA	T3N2bM0	poor
29	T, LN	76	M	Lateral tongue SCCA	T4N2bM0	moderate
30	NLc, T, LN	85	M	Lateral tongue SCCA	T3N2bM0	moderate
31	T	81	M	Lateral tongue SCCA	T4N0M0	well

520 NLc = matched normal from cancer/CIS patients, NLn = unmatched normal from normal patients,

521 T = primary tumor, LN = lymph node metastasis, SCCA = squamous cell carcinoma

522 **Table 3 - qRT-PCR Validation of Gene Microarray Data**  
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<b>Gene</b>	<b>Method</b>	<b>CA:NML Ratio<sup>1</sup></b>	<b>Linear Correlation</b>
<b><i>CDH11</i></b>	microarray	19.8 (p=0.003)	r = 0.45, p ≤ 0.14
	qRT-PCR	18.9 (p=0.03)	
<b><i>MMP3</i></b>	microarray	22.9 (p=0.09)	r = 0.97, p ≤ 0.0004
	qRT-PCR	4.30 (p=0.10)	
<b><i>SPARC</i></b>	microarray	5.67 (p=0.001)	r = 0.91, p ≤ 0.0002
	qRT-PCR	3.90 (p=0.001)	
<b><i>POSTN</i></b>	microarray	18.8 (p=0.008)	r = 0.90, p ≤ 0.0003
	qRT-PCR	4.52 (p=0.009)	
<b><i>TNC</i></b>	microarray	7.80 (p=0.0003)	r = 0.90, p ≤ 0.0003
	qRT-PCR	6.67 (p<0.0001)	
<b><i>TGM3</i></b>	microarray	0.057 (p<0.0001)	r = 0.63, p ≤ 0.03
	qRT-PCR	0.068 (p=0.03)	

524 <sup>1</sup>CA:NML = cancer-to-normal  
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537 **Table 4 – Analysis of IHC Scores for SPARC, Periostin, Tenascin-C, and**  
 538 **Transglutaminase-3**

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	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>				
	<b>Primary</b>	<b>Normal</b>	<b>LN</b>	<b>CIS</b>	<b>A vs B</b>	<b>A vs C</b>	<b>A vs D</b>	<b>B vs D</b>
	<b>Tumor</b>		<b>Tumor</b>		<b>P-value</b>	<b>P-value</b>	<b>P-value</b>	<b>P-value</b>
TNC <sub>epithelium</sub>	1.45	0.07	1.13	1.00	<b>.0009</b>	.5720	.5215	.5215
TNC <sub>ECM</sub>	5.09	2.61	4.01	4.00	<b>.0084</b>	.3590	.0333	.0333
Periostin <sub>epithelium</sub>	1.28	0.45	0.38	0.83	.1057	.0181	.6214	.6214
Periostin <sub>ECM</sub>	5.76	3.38	3.34	4.00	<b>.0017</b>	<b>.0037</b>	.8087	.8087
Periostin <sub>fibroblast</sub>	5.48	3.31	3.84	3.25	<b>.0034</b>	.0373	.9714	.9714
SPARC <sub>epithelium</sub>	1.90	0.27	1.39	1.67	<b>.0002</b>	.4673	.3005	.3005
SPARC <sub>fibroblast</sub>	8.56	4.69	6.63	7.00	<b>.0000</b>	.0268	.0312	.0312
SPARC <sub>vessels</sub>	6.96	4.12	6.70	8.25	<b>.0000</b>	.7081	.0532	.0532
TGM3 <sub>epithelium</sub>	0.31	5.44	0.44	0.92	<b>.0000</b>	.6665	<b>.0000</b>	<b>.0000</b>

541 Columns A-D list IHC scores for the specified tissues. P-values for unpaired student t-tests are  
 542 listed in the remaining columns.

543 CIS = carcinoma in-situ, ECM = extracellular matrix, LN = lymph node, TGM3 =  
 544 transglutaminase-3, TNC = tenascin-C

