Examination of Oral Cancer Biomarkers by Tissue Microarray Analysis

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ABSTRACT

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

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

Results. qRT-PCR analysis revealed up-regulation of *CDH11, SPARC*, *POSTN*, and *TNC* gene expression, and decreased *TGM3* expression in OSCC compared to normal controls. *MMP3* was not found to be differentially expressed. In TMA IHC analyses, SPARC, periostin, and tenascin C exhibited increased protein expression in cancer compared to normal tissues, 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 normal controls, and was significantly down-regulated in cancer cells.

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

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

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the 5th most common cancer worldwide.[1] The American Cancer Society estimates that approximately 30,990 Americans were diagnosed with and 7,430 died of cancer of the oral cavity and pharynx in 2006.[2] Despite considerable advances in the treatment of HNSCC over the past two decades, overall disease outcomes have only modestly improved.[2] Local tumor recurrence affects approximately 60% of patients and metastases develops in 15-25%.[3] Less than 30% of HNSCC patients experience three or more years of disease-free survival, and many suffer from impaired speech, swallowing, and/or breathing due to 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 investigation into the molecular characteristics of HNSCC has mainly utilized 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 up- or down-regulated in HNSCC. After comparing results from multiple studies, we reported a list of genes commonly found to have dysregulated expression in HNSCC tumors.[5] Only a handful of these gene expression alterations have been validated by alternate experimental methodologies such as qRT-PCR, Western blot, Northern blot, and IHC. Even fewer have been examined for their correlation with disease severity and metastasis status.

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

Materials & Methods

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

qRT-PCR. Differential gene expression of *CDH11, MMP3, SPARC, POSTN, TNC,* and *TGM3* between normal and cancer specimens was quantified by using SYBR® Green I technology and melting-point dissociation curve analyses per manufacturer protocol (Applied Biosystems, Foster City, CA). Total RNA extracted from six normal tissue samples and six tumor tissue samples (Table 1) were used as templates in RT-PCR reactions to generate cDNA. Each sample was divided into five wells for the qRT-PCR reactions: three for the gene of interest and two for the endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). qRT-PCR analyses were performed on an ABI 5700 Sequence Detector using 10 ng of cDNA 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, checked for specificity against the National Center for Biotechnology Information nucleotide data base, and were as follows: *CDH11* forward, 5-GCT CAA CCA GCA GAG ACA TTC 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-CGG CTT TGT GGA CAT CCC TA-3; *SPARC* reverse, 5-GGA AGG ACT CAT GAC CTG CAT C-3; *POSTN* forward, 5-ACA ACG CAG CGC TAT TCT GAC-3; *POSTN* reverse, 5-ATC CAA GTT GTC CCA AGC CTC-3; *TNC* forward, 5-AGA AAG TCA TCC GGC ACA AGC-3; *TNC* reverse, 5-ACT CCA GAT CCA CCG AAC ACT G-3; *TGM3* forward, 5-GAC AAG CGC ATC ACA CAG ACA-3; *TGM3* reverse, 5-TCT TTC GTT 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 tumor samples and normal samples. The PCR cycle number at which the amplification curve intercepted the threshold is termed the threshold cycle (C_T). The threshold cycle is inversely proportional to the copy number of the target template. Relative fold changes were calculated by $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ = [average C_T, gene *j* - average C_T, GAPDH] tumor tissue - [average C_T, gene *j* - average C_T, GAPDH] normal tissue.

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

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

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

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

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

Slides were deparaffinized by three changes of xylene, seven minutes each, and rehydrated by three changes of 100% ethanol x two minutes, two changes of 95% ethanol x two minutes, and one change of 70% ethanol x one minute. Endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 at room temperature for 10 minutes. Non-specific binding sites were subsequently blocked with 2% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS, pH 7.4, at room temperature for 15 minutes. Slides were then washed and incubated with primary antibody. Washes consisted of a five-minute soak in PBS, pH 7.4, followed by 10 dips in PBS with 1% BSA and 0.01% Triton-X-100 (Sigma, St. Louis, MO), pH 7.4, and a second five-minute wash in PBS, pH 7.4. TMA slides were then incubated with primary antibody at the above listed dilutions in PBS with 1% BSA, pH 7.4, at room temperature for one hour in a humidity chamber (Shandon Lipshaw, Inc, Pittsburgh, PA). Sections were then washed again as before, incubated with either biotinylated goat anti-mouse or biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA), washed again, and subsequently incubated with ABC Elite reagent (Vector Laboratories, Burlingame, CA) per manufacturer protocol. Slides were then stained by incubation with 0.08% diaminobenzidine, 0.01% $FeCl_3$ in 0.05 M Tris buffer, pH 8.0 (Sigma, St. Louis, MO) at 37°C for seven minutes. Sections were counterstained with Mayers Hematoxylin (Dako Cytomation, Carpinteria, CA), dehydrated, mounted with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI) and covered with a coverslip.

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

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

Results

CDH11, *SPARC*, *POSTN*, *TNC*, and *TGM3* exhibited significant differences in 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[20] 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

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

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

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

Discussion

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

literature.[5] *SPARC, POSTN, TNC*, and *TGM3* microarray expression differences were validated by both qRT-PCR and IHC of TMA sections. The qRT-PCR results for *MMP3* and *CDH11* did not reach statistical significance. In contrast with another IHC study reporting high levels of periostin expression within oral carcinoma epithelium,[12] we noted periostin staining to be localized primarily to the stroma and did not see robust staining within tumor cells themselves (Figure 1D). The reason for this disparity in IHC staining is unclear. Non-overlapping antibody epitopes may partially explain the disparity of periostin IHC staining patterns. The antibody we used for periostin IHC 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 (http://biovendor.com/pdf/RD181045050.pdf), whereas periostin IHC experiments by Siriwarden et al. utilized a polyclonal antibody generated by immunizing rabbits with a specific peptide (EGEPEFRLIKEGETC) corresponding to amino acids 679-692 of full-length periostin.[12]

Despite the strong stromal predominance of periostin expression we observed, the percentage of OSCC tumors positive for epithelial periostin in our study (58%) was comparable to that reported by Siriwarenda et al.[12] (69%). A majority (65%) of the stage III/IV OSCC tumors, including 100% of T4 tumors we examined, were positive for epithelial periostin immunostaining, compared to only 25% of the stage I/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.[11,12]

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

In oral and laryngeal squamous cell carcinoma, increased levels of tenascin-C immunostaining have been found to correlate with malignancy and invasion[22-25] Abundant expression of tenascin-C in our OSCC TMA 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.[26,27]

Roepman, et al.[28] and Schmalbach, et al.[29] identified *TGM3* to be significantly down-regulated in metastatic HNSCC compared to both non-metastatic tumors and normal epithelium. O'Donnell et al.[30] similarly found significant down-regulation of *TGM3* gene expression in metastatic primary OSCC tumors compared to non-metastatic primaries. Our cross-sectional IHC 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 up-regulated gene markers we identified by reviewing gene microarray reports, validated by qRT-PCR, and subsequently studied with IHC 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 methodology used for specimen processing. The methods employed by different laboratories for tumor specimen processing vary significantly.[5] While some investigators isolated relatively homogeneous populations of tumor cells for microarray analysis via laser capture microdissection (LCM), others established arbitrary thresholds for minimum tumor cell content in surgical specimens, as assessed by histologic evaluation of adjacent tissue, prior to 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 LCM 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.[31-33]

Up-regulation of *SPARC*, *POSTN*, or *TNC* was not reported by any of the DNA microarray studies that examined expression of HNSCC cell lines[34-36] or by others that employed LCM to isolate tumor cells from stroma.[37-40] Presumably this is due to the relative absence of stromal cells within the analyzed specimens in these studies, although absence of one or more of these markers on the microarrays used by these studies may also contribute. These findings, together with the IHC data we report here, suggest that up-regulation of *SPARC*, *POSTN*, and *TNC* is due to 1) up-regulation 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 both 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 (CAFs), ECM 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.[41] The situation in OSCC is less well understood, but studies of CAFs, ECM turnover and tumor cell motility have begun to delineate the role of desmoplastic stroma in OSCC carcinogenesis.[42-44] Recently, Weber et al.[45] performed genome-wide analysis of loss of heterozygosity (LOH) and allelic imbalance (AI) on LCM-isolated specimens of tumor stroma and tumor epithelium from over 120 OSCC patients with a history of smoking. They discovered over 40 hot spots of LOH/AI within the stroma, nearly twice as many as they found in the epithelium, and subsequently identified three stroma-specific loci that were significantly associated with tumor size and cervical lymph node metastasis.[45] These findings again highlight the importance of examining both 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 four genes, *SPARC, POSTN, TNC*, and *TGM3*, initially identified by gene microarray studies, are associated with similar changes in protein expression based on IHC analyses. The localization of SPARC, periostin, tenascin-C predominately within the stroma of OSCC tumors supports the idea that stromal elements are important in OSCC pathogenesis.

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Figure 1 – IHC of OSCC TMA sections

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

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

Specimen	Age	Sex	Diagnosis	Stage	Grade
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

SCCA = squamous cell carcinoma

518 **Table 2 – Characteristics of Tissue Microarray Specimens**
519

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

Table 3 - qRT-PCR Validation of Gene Microarray Data

Gene	Method	CA:NML Ratio[1]	Linear Correlation
CDH11	microarray	19.8 (p=0.003)	r = 0.45, p ≤ 0.14
	qRT-PCR	18.9 (p=0.03)	
MMP3	microarray	22.9 (p=0.09)	r = 0.97, p ≤ 0.0004
	qRT-PCR	4.30 (p=0.10)	
SPARC	microarray	5.67 (p=0.001)	r = 0.91, p ≤ 0.0002
	qRT-PCR	3.90 (p=0.001)	
POSTN	microarray	18.8 (p=0.008)	r = 0.90, p ≤ 0.0003
	qRT-PCR	4.52 (p=0.009)	
TNC	microarray	7.80 (p=0.0003)	r = 0.90, p ≤ 0.0003
	qRT-PCR	6.67 (p<0.0001)	
TGM3	microarray	0.057 (p<0.0001)	r = 0.63, p ≤ 0.03
	qRT-PCR	0.068 (p=0.03)	

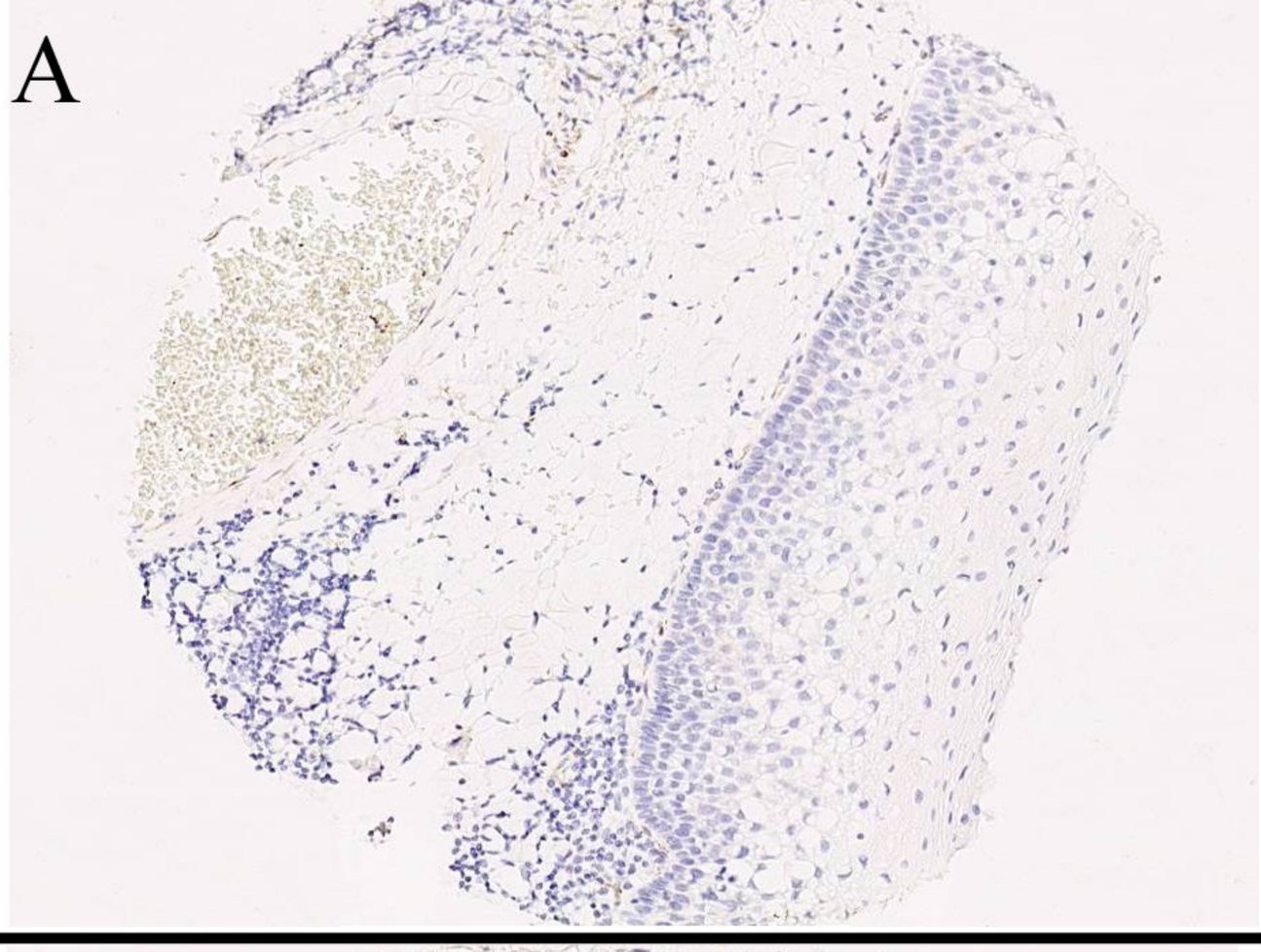
[1]CA:NML = cancer-to-normal

Table 4 – Analysis of IHC Scores for SPARC, Periostin, Tenascin-C, and Transglutaminase-3

	A Primary Tumor	B Normal	C LN Tumor	D CIS	A vs B P-value	A vs C P-value	A vs D P-value	B vs D P-value
$TNC_{epithelium}$	1.45	0.07	1.13	1.00	**.0009**	.5720	.5215	.5215
TNC_{ECM}	5.09	2.61	4.01	4.00	**.0084**	.3590	.0333	.0333
$Periostin_{epithelium}$	1.28	0.45	0.38	0.83	**.**1057	.0181	.6214	.6214
$Periostin_{ECM}$	5.76	3.38	3.34	4.00	**.0017**	**.0037**	.8087	.8087
$Periostin_{fibroblast}$	5.48	3.31	3.84	3.25	**.0034**	.0373	.9714	.9714
$SPARC_{epithelium}$	1.90	0.27	1.39	1.67	**.0002**	.4673	.3005	.3005
$SPARC_{fibroblast}$	8.56	4.69	6.63	7.00	**.0000**	.0268	.0312	.0312
$SPARC_{vessels}$	6.96	4.12	6.70	8.25	**.0000**	.7081	.0532	.0532
$TGM3_{epithelium}$	0.31	5.44	0.44	0.92	**.0000**	.6665	**.0000**	**.0000**

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

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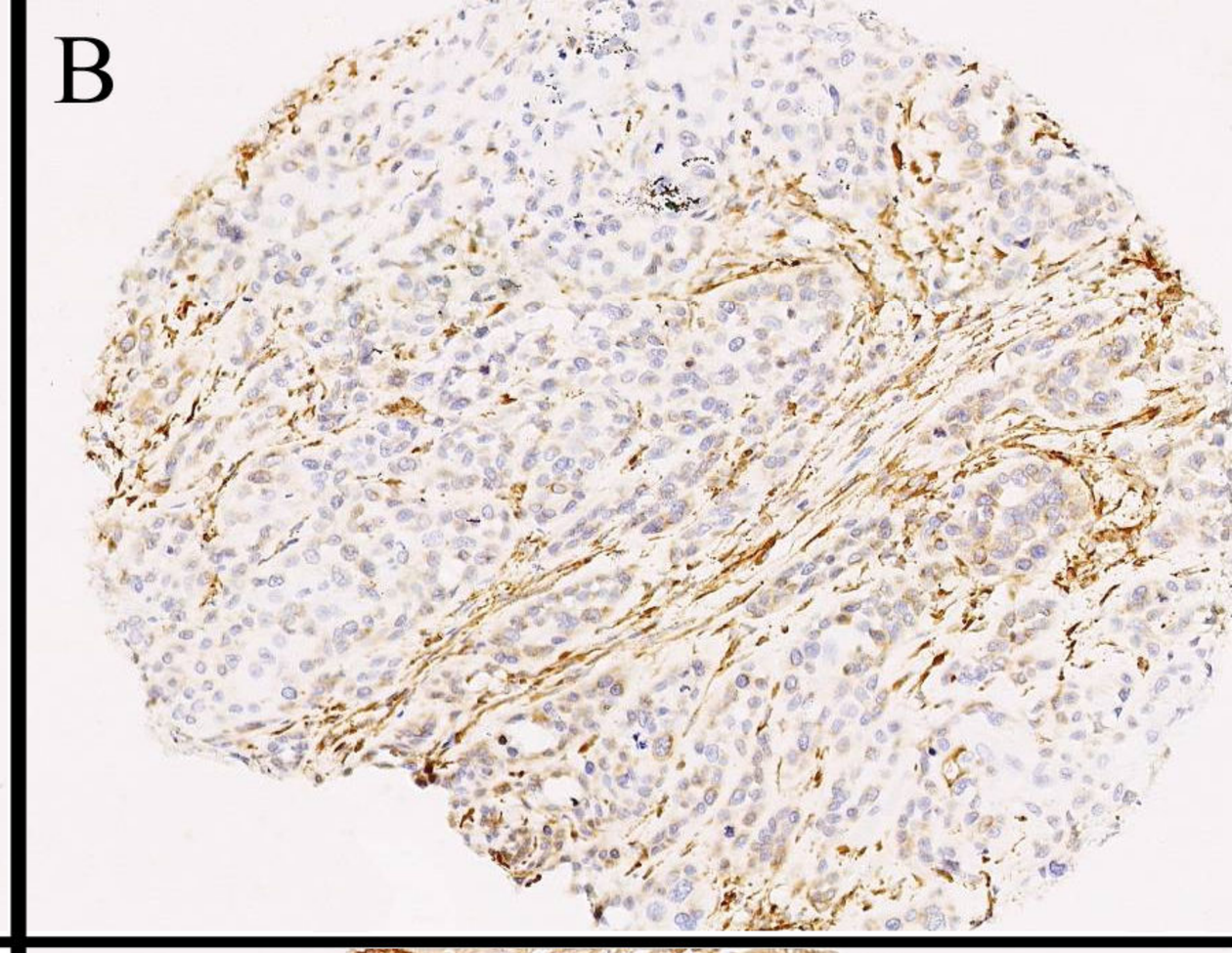
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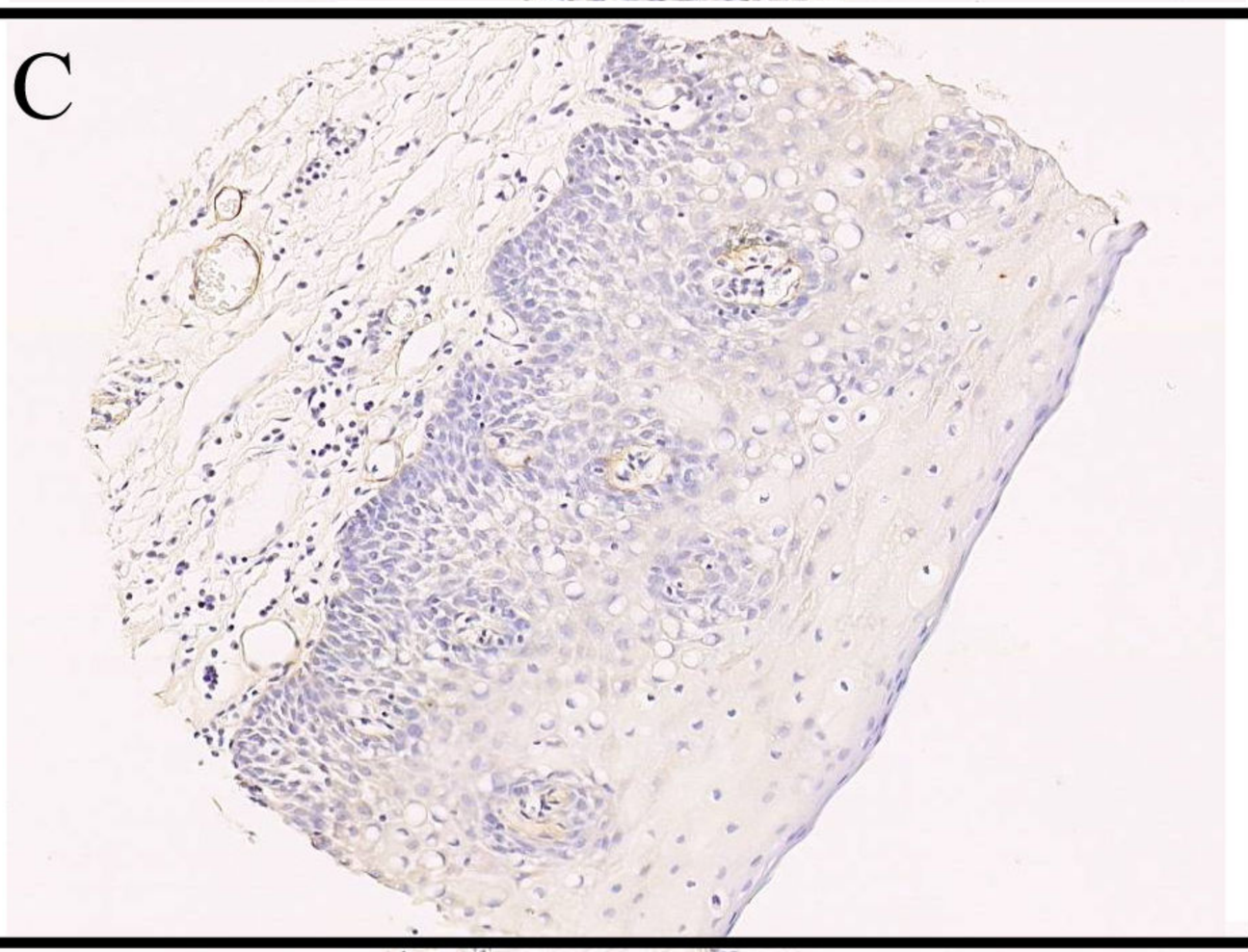
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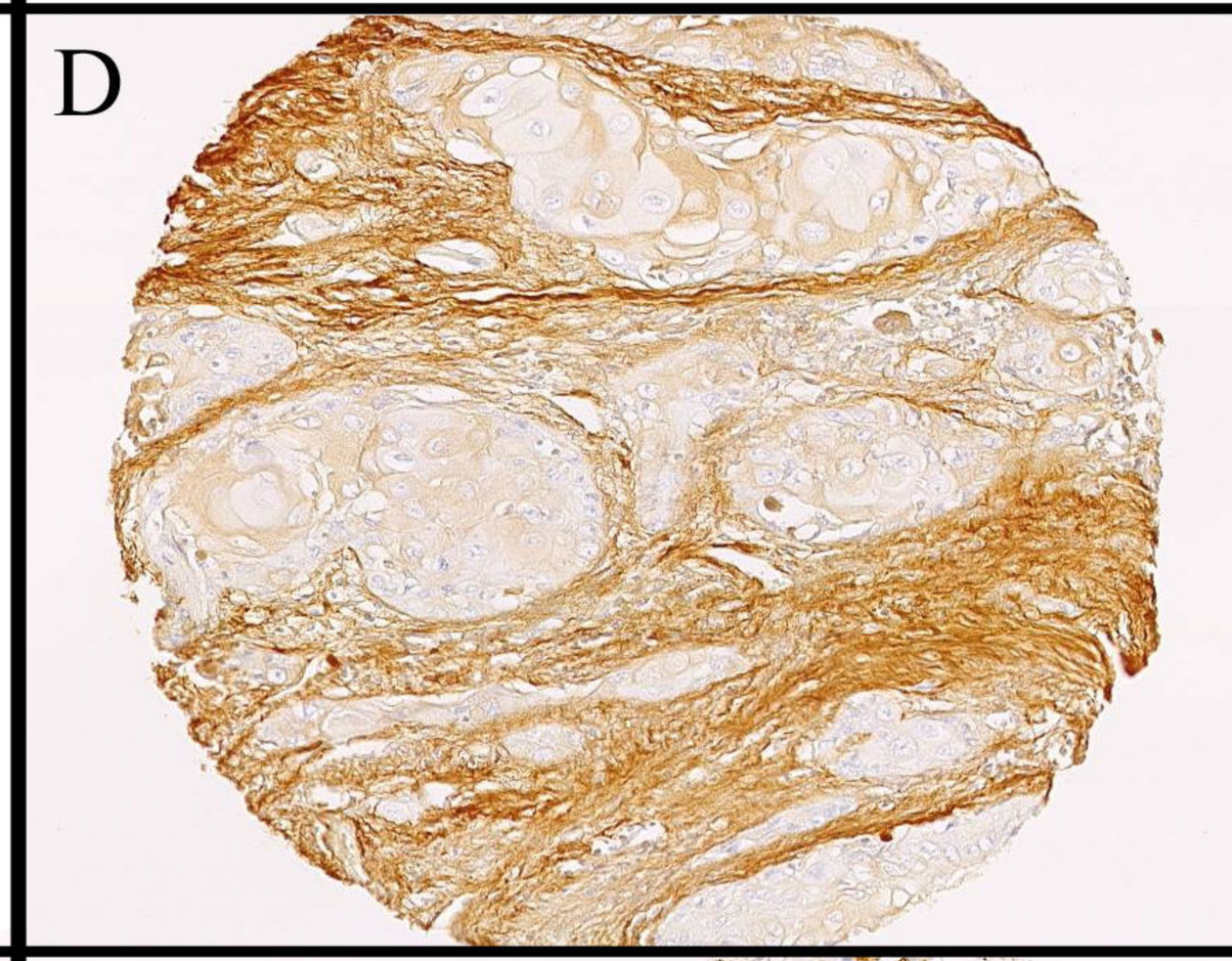
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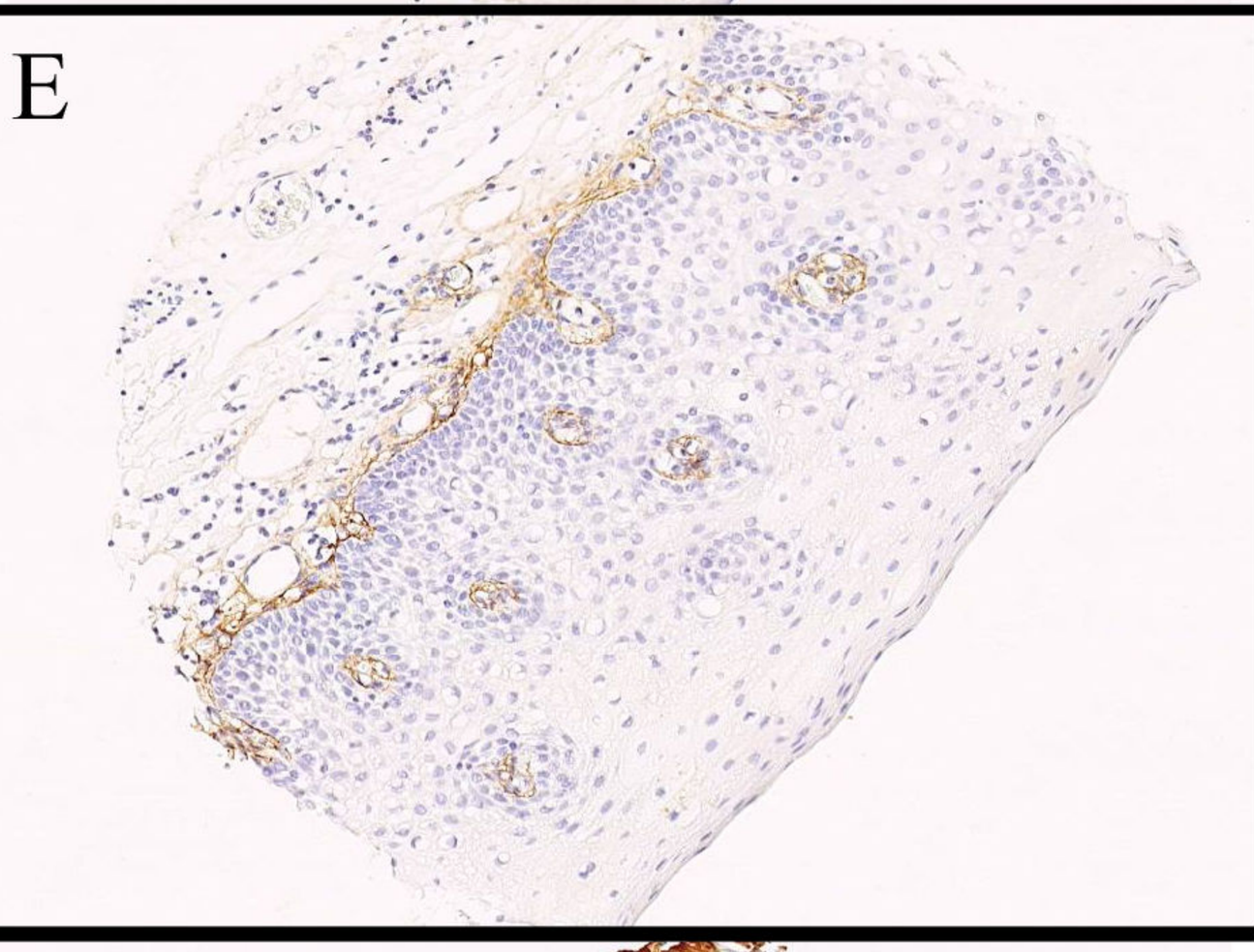
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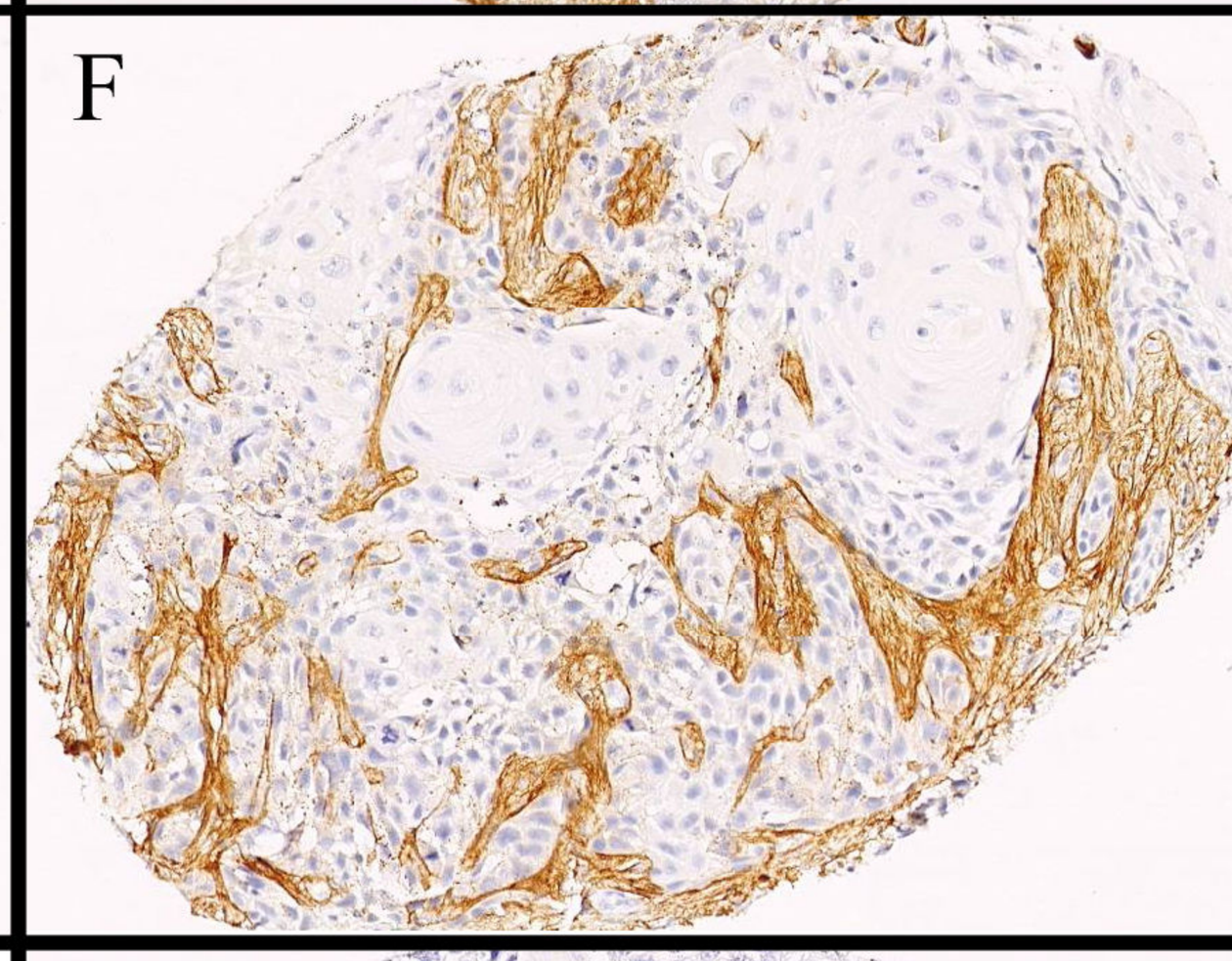
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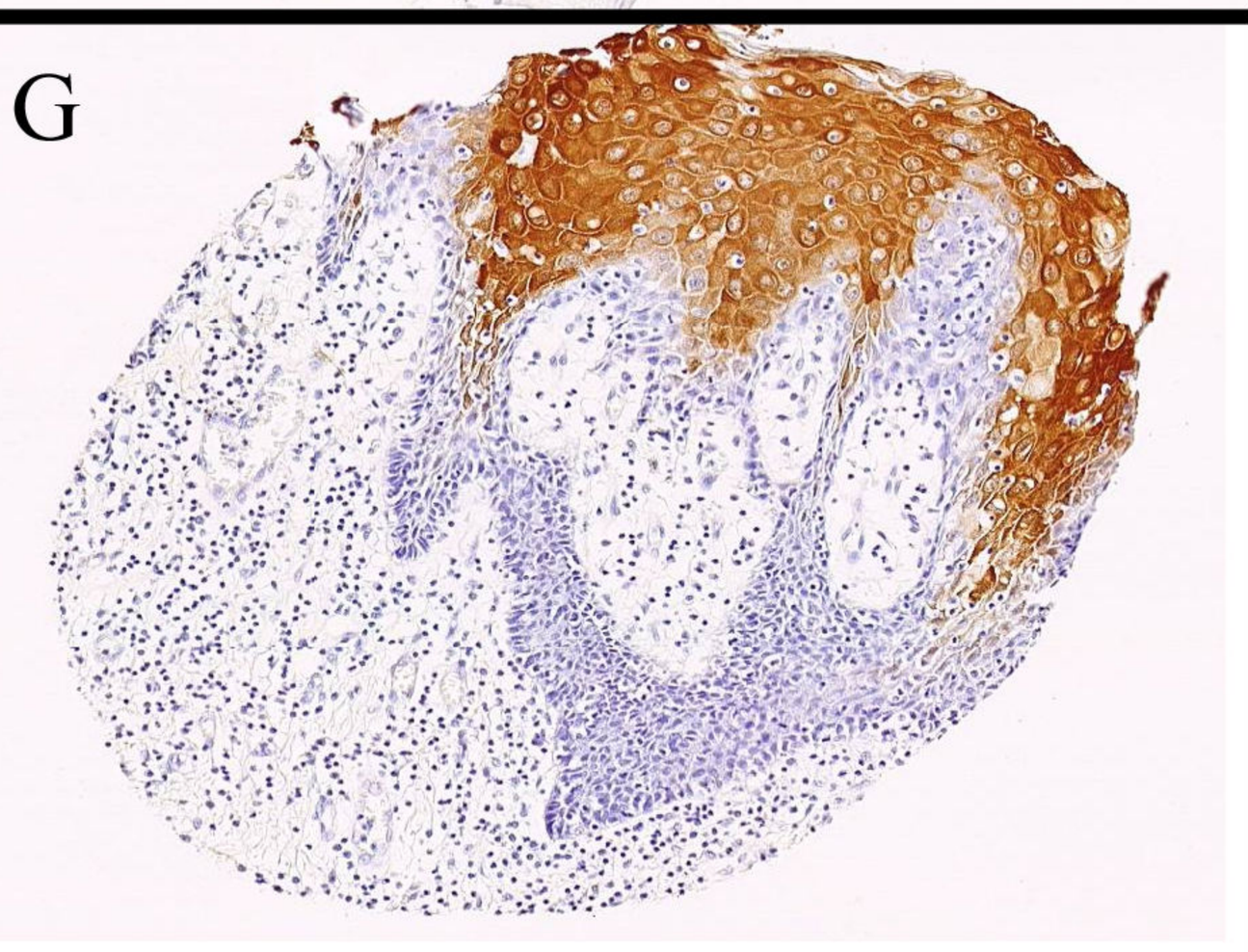
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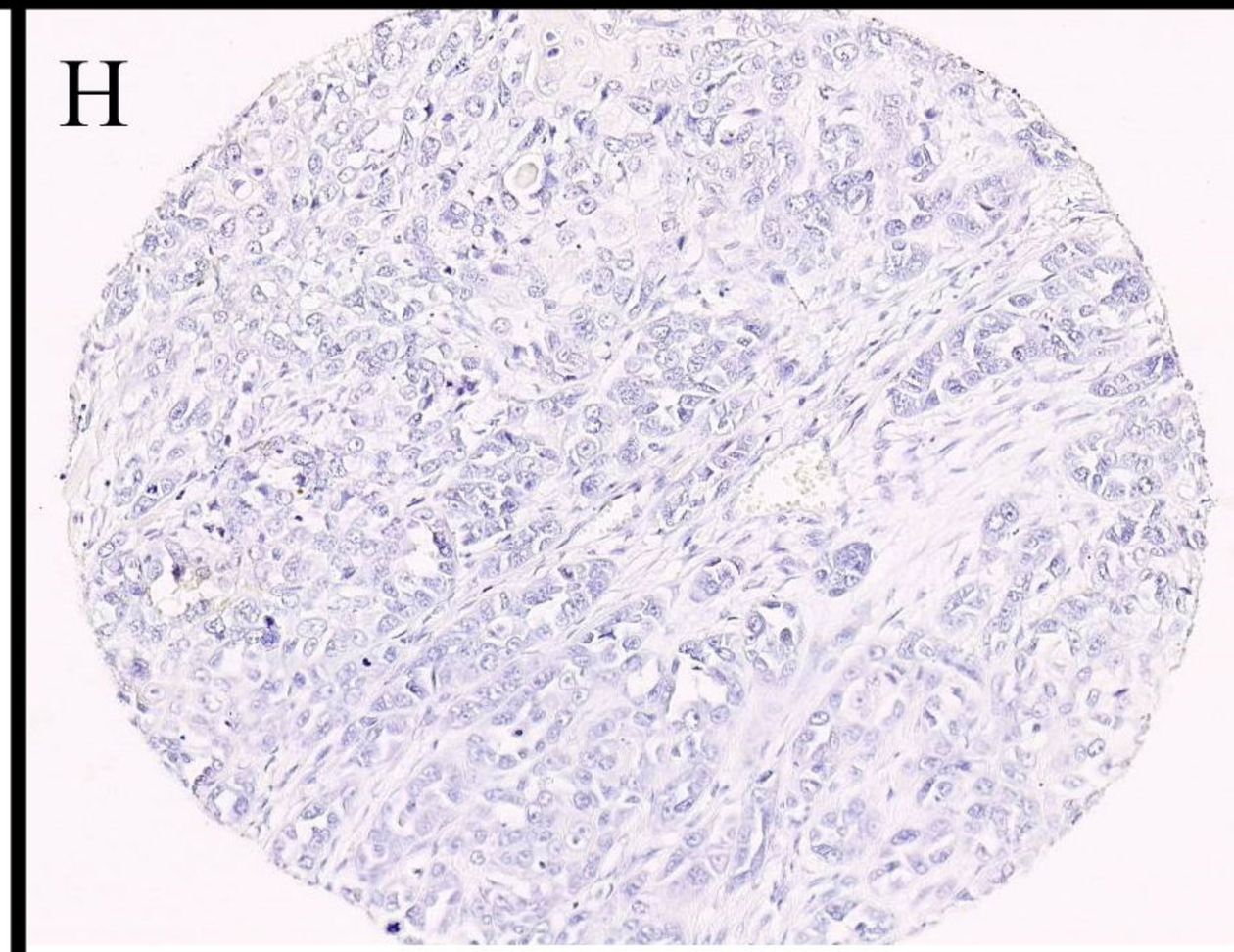
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