

Angiogenesis, Metastasis, and the Cellular Microenvironment

Urinary-Type Plasminogen Activator Receptor/α3β1 Integrin Signaling, Altered Gene Expression, and Oral Tumor Progression

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Abstract

Oral squamous cell carcinoma (OSCC) has 50% 5-year survival rate, highlighting our limited understanding of the molecular events that contribute to disease progression. Microarray analyses of primary oral tumors have identified urinary-type plasminogen activator (uPA) and its receptor (uPAR) as key genes associated with human OSCC progression. The uPAR functions as both a proteinase receptor and an integrin ligand, modifying proteolysis, migration, integrin signaling, and cellular transcription. In the current study, uPAR expression levels were modified in OSCC cells followed by analysis of tumor growth in an in vivo orthotopic xenograft model and by transcriptional profiling. Overexpression of uPAR resulted in more infiltrative and less differentiated tumors, with ill-defined borders, cytologic atypia, and enhanced vascularity. Analysis of serial sections of both murine experimental tumors and microarrayed human OSCC showed a statistically significant association between uPAR and α3 integrin colocalization in areas exhibiting extracellular signal-regulated kinase phosphorylation, suggesting that uPAR/α3 integrin interaction potentiates extracellular signal-regulated kinase signaling in vivo. This is supported by cDNA microarray analysis, which showed differential expression of 148 genes (113 upregulated and 35 downregulated). Validation of gene expression changes in human OSCC using immunohistochemistry and quantitative real-time PCR showed increased growth factors, proteinases/inhibitors, and matrix components in uPAR-overexpressing tumors. Together, these results support a model wherein increased uPAR expression promotes α3β1 integrin association, resulting in increased mitogen-activated protein kinase signaling and transcriptional activation, leading to the formation of more aggressive tongue tumors. This combined approach has efficacy to identify additional biomarkers and/or prognostic indicators associated with aggressive human OSCC. Mol Cancer Res; 8(2); 145–58. ©2010 AACR.

Introduction

Oral cavity cancer ranks among the top 10 most frequently diagnosed cancers worldwide, resulting in more than 200,000 deaths annually (1). Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity, with 30,000 new cases detected each year in the United States alone (2). Of these, 25% to 40% occur in the tongue [squamous cell carcinoma of the oral tongue (SCCOT); ref. 3]. In the United States, the incidence of oral tongue cancer has actually increased in the 20- to 44-year age group in the last 3 decades (4). However, the 5-year survival rate has remained at a low 50% for the past 20 years, highlighting our limited understanding of the molecular events that govern OSCC initiation, progression, and metastasis (5). Tongue tumors frequently remain asymptomatic until advanced stage due in part to anatomic location, and several studies suggest a poorer survival rate than other anatomic sites in the oral cavity (5, 6). As the high mortality from OSCC is attributed to regional and distant metastasis, a more detailed analysis of the molecular events that potentiate growth and dissemination is a necessary prerequisite to the development of novel early detection and treatment strategies (7).

Recent studies have used cDNA microarray analysis for genome-wide monitoring of genetic changes associated with primary oral tumors compared with normal oral mucosa or lymph node metastases (8, 9). These studies have identified the proteinase urinary-type plasminogen activator (uPA) as one of 25 genes that comprise an “OSCC gene signature” for molecular classification of oral tumors and
as a key candidate biomarker for prediction of poor disease outcome (8, 9). OSCC cell lines display enhanced uPA expression relative to cells derived from normal oral mucosa and exhibit increased uPA-dependent invasion in vitro (10-12). Explants of OSCC tissues also show higher levels of uPA relative to adjacent nonmalignant tissues (13, 14). Similarly, human OSCC tumors with high levels of uPA and its cell surface receptor (uPAR) are more invasive and exhibit enhanced lymph node metastasis (15-17) and more frequent tumor relapse (18). These data suggest the potential utility of uPA/uPAR as a prognostic indicator and/or therapeutic target. In addition to regulation of pericellular proteolysis, uPAR can also function as a lateral, nonmatrix ligand for $\alpha_3\beta_1$ integrin and thereby affect $\alpha_3\beta_1$ integrin function and integrin-dependent cellular signal transduction pathways (19, 20). We have previously reported a functional link between adhesion and proteolysis, showing that matrix engagement of the $\alpha_3\beta_1$ integrin exerts multifunctional control on the uPA system by inducing upregulated uPA activity together with redistribution of uPAR to sites of clustered integrins (19). Further mechanistic analyses showed that uPAR/$\alpha_3\beta_1$ integrin interaction initiates a Src/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK-dependent signaling pathway, leading to transcriptional activation of the uPA promoter (20). A functional requirement for uPAR/$\alpha_3\beta_1$ integrin interaction was confirmed using pharmacologic approaches, small interfering RNA (siRNA) knockdown of uPAR, and cells from $\alpha_3$ integrin knockout mice (20). The effect of uPAR regulation of integrin function in OSCC has not been explored in vivo.

As spontaneous OSCCs are very rare in domestic laboratory animals, orthotopic murine tumor models have utility for investigating cellular and molecular mechanisms in tumor progression and metastasis (21). Orthotopic implantation of cell lines or primary tumor explants into the tongue has been shown to result in both expansive and invasive growth (22, 23) and has shown efficacy in preclinical testing of potential therapeutics (24). To examine how uPAR modulation of $\alpha_3\beta_1$ integrin function may alter progression of SCCOT in vivo, the effect of modulated uPA/uPAR expression on SCCOT growth was evaluated in an orthotopic murine model (23). Changes in gene expression as a result of uPAR/integrin signaling were identified using cDNA microarray analysis and validated by quantitative real-time PCR (qPCR) and immunohistochemical analysis of murine tumors. The results support a model wherein uPAR/$\alpha_3\beta_1$ integrin binding leads to growth of more aggressive, invasive tumors with concomitant changes in gene expression that may contribute to SCCOT progression in vivo.

Materials and Methods

Cell Lines

SCC25 cells, originally derived from OSCC of the human tongue, were the generous gift of Dr. James Rhinewald (Brigham and Women’s Hospital, Harvard Institutes of Medicine, Boston, MA). SCC25 cells are well characterized with respect to molecular properties and display characteristics representative of those commonly found in human SCC25 (8). Cells were routinely maintained in DMEM/Ham’s F-12 1:1 medium containing 10% FCS and supplemented with 100 units/mL penicillin. A siRNA knockdown approach was used to generate cells with reduced levels of surface uPAR as previously described (20). The paired oligonucleotides indicated below were annealed and ligated to BstI-cut vector (psiroAH1neo from Inviogen) and transformed into HB101-competent cells. Target seq2 oligonucleotide 4A (5’-cctcaacggctacgatgaatctcagggattgag-3’) and target seq2 oligonucleotide 4B (5’-catcaacggctacgatgaatctcagggattgag-3’) DNA was isolated (QIAprep Spin Miniprep kit, Qiagen), and the identities of the clones were confirmed by restriction digestion and sequencing with primer OL381 (sequencing primer oligonucleotide OL381, 5’-ccctaacggcagacactct-3’). Selected clones were then grown in 500-mL cultures, and DNA isolations were done using a nuclease-free DNA isolation kit (Qiagen). SCC25 cells were transfected by electroporation using the human keratinocyte nucleofector kit and device (Amaza) following the recommended protocol. Briefly, the cells were cultured to ~65% confluence, trypsinized, and resuspended at a density of 500,000/100 μL nucleofector solution. DNA (1.5 μg in 5 μL) was added to each aliquot of cells and gently mixed, and each aliquot was electroporated. After 24-h growth under nonselective conditions, the medium was replaced with medium containing 850 μg/mL G418. During outgrowth, the cells were subcultured before reaching 70% confluence, and the selection medium was replaced every 2 d. Colonies were picked at the 100-cell stage and grown until they were numerous enough to test for knockdown of uPAR by flow cytometry. Loss of uPAR surface expression and purity of clonal cell lines was assessed by fluorescence-activated cell sorting (see below).

To generate cells that overexpress uPAR (SCC25-uPAR +), uPAR was cloned into the expression vector pcDNA 3.1(+). uPAR was cloned into the expression vector pcDNA 3.1(+) by real-time PCR with primer 1 (ggcaagctggttatttcaggtcgtcccagggctg) and primer 2 (taagctggttatttcaggtcgtcccagggctg). The DNA was gel purified using a QIAquick Gel Extraction kit (Qiagen), and the cDNA was cut with EcoRI and HindIII and ligated into EcoRI- and HindIII-cut vector. The DNA was used to transform HB101 host cells. Cells carrying the pcDNA 3.1-uPAR plasmid were grown in Luria-Bertani medium containing 100 μg/mL ampicillin, and plasmid DNA was isolated using the DNA Isolation kit (Qiagen). After the uPAR sequence was verified, the DNA was used as template with the following primer sets: PCR Rx 1A: T7 promoter primer + primer 3, cttgcatctcgcttcctgtgctgccaagacaggtcggagccta; Rx 1B: BGH reverse primer + primer 4, gactcaagagcagtagcagaagagctgtagctgtaag. The gel-purified cDNA products from Rxs 1A and 1B were used as template for the final PCR Rx 2 using T7 and BGH Rev primers. The resulting uPAR/flag cDNA was cut with EcoRI and HindIII and ligated into EcoRI- and HindIII-cut pcDNA 3.1(+) vector. The transformation and DNA purification were...
carried out as above, and the DNA was used to transfect SCC25 cells. The resulting clonal cell lines were routinely maintained in the medium described above supplemented with G418 and were assessed for expression of uPAR by fluorescence-activated cell sorting (see below). Changes in uPAR expression were also confirmed by qPCR (below). Alternatively, lentiviral transduction was used to generate UM-SCC1 cells (generous gift of Dr. Ernst Lengyel, University of Chicago, Chicago, IL) that overexpress uPAR (designated SCC1-uPAR+). Briefly, a retroviral uPAR expression vector was first created by subcloning into the EcoRI and SalI sites in pBabe-puro system (plasmid 1764 from Addgene, Inc.) with a full-length human uPAR from human cDNA clone SC319092 (Origene Technologies, Inc.). Retroviral particles were made by liposome-based transfection in HEK293 cell-derived retroviral packaging cell line, Phoenix amphotropic (originally created in Dr. Garry Nolan’s lab in Stanford University), and subclines expressing full-length uPAR or harboring empty retroviral vector with puromycin resistance gene only were correspondingly created by spinfection and puromycin selection (2.5 μg/mL). qPCR analysis showed a 46-fold increase in uPAR mRNA relative to parental UM-SCC1 cells with control viral vector.

Antibodies
For immunohistochemistry, the following antibodies were purchased: mouse anti-human cytokeratin clone AE1/AE3 (1:25 dilution; DAKO), mouse anti-human proliferating cell nuclear antigen clone PC10 (1:12.5 dilution; DAKO), mouse anti-human vimentin clone V9 (1:25 dilution; DAKO), mouse anti-human uPAR clone 3/B10 (1:25 dilution; American Diagnostica), mouse anti-human α3 integrin clone P1B5 (1:25 dilution; Chemicon), rabbit anti-human phosphorylated p44/42 MAPK [Thr202/Tyr204], phosphorylated ERK (pERK) 1/2; 1:25 dilution; Cell Signaling Technology], rabbit anti-human vascular endothelial growth factor-C (VEGF-C) clone H-190 (Santa Cruz Biotechnology), rabbit anti-human kallikrein-5 (1:25 dilution; DAKO), anti–plasminogen activator inhibitor-1 (PAI-1) (1:20 dilution; Santa Cruz Biotechnology), and anti–laminin-5–γ2 chain (1:20 dilution; Dakocytomation). Mouse anti-human E-cadherin clone HECID-1 (Zymed), mouse anti-human α3 integrin, and mouse anti-human uPAR clone 13.1 (American Diagnostica) were purchased for use in flow cytometric analyses.

Flow Cytometry
Surface expression of uPAR, α3 integrin, and E-cadherin was determined using flow cytometry as previously described (11, 20). Briefly, cells were trypsinized and 1.8 × 106 cells were incubated in 100 μL of medium containing specific antibodies against the respective protein for 45 min at room temperature. Antibodies were used at the following dilutions: anti-uPAR (1:100; American Diagnostica), anti–α3 integrin (1:100; Chemicon), and anti–E-cadherin (HECD-1; 1:100; Zymed). The cells were then washed twice with PBS and incubated with the corresponding FITC-conjugated secondary antibody (1:500 dilution; Molecular Probes) for 30 min in the dark at room temperature. The cells were then washed twice with PBS and resuspended in medium for fluorescence analysis on an Epics XL-MCL flow cytometer (Beckman Coulter). Control experiments contained only the appropriate secondary antibody.

Analysis of Invasion
Invasive activity was quantified using a Boyden chamber (8-μm pore size) coated with Matrigel (10 μg for 1 h at room temperature) as described earlier (20). The cells (2 × 105) were added to the upper chamber in 500 μL of serum-free medium containing the selection drug. Following 24 to 48 h of incubation at 37°C, the noninvasive cells were removed from the upper chamber with a cotton swab, the filters were fixed and stained with Diff-Quik stain, and the invading cells adherent to the underside of the filter were enumerated using an ocular micrometer and counting a minimum of 10 high-powered fields. The data are expressed as relative invasion over control (number of cells per field). Some experiments contained antibodies directed against uPAR (20 μg/mL; American Diagnostica), α3 integrin (20 μg/mL; Millipore), control IgG (20 μg/mL; Chemicon), the small-molecule MEK inhibitor PD98059 (10 μmol/L), or DMSO control, added at the time of cell seeding.

Orthotopic Sublingual Injections
Murine tongue tumors were generated as previously described (23). At least 8 mice per group were injected, and the experiment was repeated thrice over the course of 2 y for a total of 24 mice per group. Briefly, 6-wk-old female athymic nu/nu mice were purchased from Charles River and housed in a specific pathogen-free animal facility at the National Institute of Dental and Craniofacial Research (Bethesda, MD). All animal protocols were done with approval of the National Institute of Dental and Craniofacial Research Animal Care and Use Committee. Cells were cultured as described above, collected using trypsin, and resuspended in PBS. Mice were anesthetized using 2.5% isoflurane. A 1-mL syringe (Becton Dickinson) with a 25-gauge needle was used to inject 30 μL of cells (pooled clones of SCC25-uPAR-KD or SCC25-uPAR+; 6.25 × 106, in sterile PBS) into the lateral border of the tongue just anterior to the junction of the anterior 2/3 and posterior 1/3 of the tongue. The tongue of the anesthetized mouse was held with a small tooth forceps in the median raphe of the tongue while the cells were injected just under the mucosa of the tongue in an anteroposterior direction. Mice were examined every day for the development of tongue tumors and weight loss. At ~9 wk, mice were sacrificed using CO2 when they began to show adverse signs of disease, including weight loss. The tongue and cervical lymph nodes were dissected from each mouse (n = 24 for each group), fixed in freshly prepared 4% paraformaldehyde (4°C), paraffin embedded, sectioned (4 μm), and stained with H&E. For each tumor in each group, multiple sections were prepared and that with the greatest cross-sectional tumor area, as determined using ImageJ software, was used for histopathologic analysis. All tumors were analyzed by a
board-certified human pathologist (S.F.) and a veterinary pathologist (A.E.). Tumor invasiveness was subjectively graded on a scale of 1 to 5, based loosely on the number of distinct cross-sections of one tumor on one slide and which ranged from 1 to 50. Inflammation was also graded on a scale from 1 to 5, with 1 indicating absence of inflammation and 5 indicating marked diffuse inflammation. Tumors were placed in increasing order based on cross-sectional area, invasiveness (1-5), and inflammation (1-5), and an ANOVA on ranks was done for each criterion. The mitotic index was determined by counting a minimum of 1,000 cells per slide and scoring for the presence or absence of visible mitoses. Data were analyzed using a nonparametric ANOVA (Mann-Whitney U test; Systat 11.0, Systat, Inc.).

**Immunohistochemical Analysis**

Immunohistochemical analysis was done on paraformaldehyde-fixed, paraaffin-embedded sections (4 μm) from all murine tongue tumors in each group, and the experiment was repeated in triplicate, as indicated above (total n = 24 for each group; ref. 25). Images that most clearly represent the average group phenotype are shown. Quantitation of uPAR immunohistochemical staining shows staining of 50.2 ± 3.47% of cells in SCC25-uPAR-KD tumors and 71.1 ± 2.24% in SCC25-uPAR+ tumors (P < 0.005, ANOVA). Quantitation of staining for active ERK (nuclear pERK) was done by scoring a minimum of 10,000 cells per group from at least 85 high-powered fields. Analysis of human tongue tumors was done using microarrayed human tumor tissue (Biomax) containing 29 cases of tongue tumor of varying grade. Sections were deparaffinized with xylene and rehydrated in a series of ethanol washes. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 30 min. Antigen retrieval was enhanced by microwaving in 10 mmol/L sodium citrate (pH 6.0; or 0.01% anilin for 30 min). Antigen retrieval was enhanced by microvibration in 10 mmol/L sodium citrate (pH 6.0; or 0.01% anilin for 30 min). Antigen retrieval was enhanced by blocking for nonspecific binding was done in 10% normal horse serum in PBS for 20 min. Blocking for nonspecific binding was done in 10% normal donkey serum at room temperature for 1 h. Then, the primary antibodies goat anti-human uPAR from American Diagnostica at 1:10 dilution, rabbit anti-pERK1/2 (Thr202/Tyr204) from Cell Signaling at 1:25 dilution, and mouse anti–α3 integrin (P1B5) from Millipore at 1:25 dilution were coincubated in blocking solution at 4°C overnight. PBS containing 0.5% Tween 20 (PBS-T) was used to rinse slides for 5 min and repeated thrice. Fluorophore-conjugated secondary antibodies (donkey anti-goat IgG–Alexa Fluor 546, donkey anti-rabbit IgG–Alexa Fluor 488, and donkey anti-mouse IgG–Alexa Fluor 647, all from Invitrogen) were incubated together in a dilution of 1:400 at room temperature for 30 min. After appropriate wash in PBS-T and air drying, slides were mounted with ProLong Gold antifade medium containing 4′,6-diamidino-2-phenylindole (DAPI). All stained slides were examined with an Olympus IX-80 with DSU spinning disk confocal system equipped with Hamamatsu electron-multiplying charge-coupled device camera ImagEM and the following four sets of emission and excitation filters: DAPI, D350/50× and ET455/50 m (blue); Alexa Fluor 488, 490/20× and ET525/36 m (green); Alexa Fluor 546, 555/25× and ET605/52 m (red); and Alexa Fluor 647 (magenta), 645/30× and ET705/72 m. Image acquisition and normalization were all via the controlling software Slidebook 4.1 (Olympus). No additional image manipulations were done.

**cDNA Microarray**

All DNA microarray gene expression studies used human oligonucleotide arrays custom printed by a dedicated core facility within the Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center (Dr. David Kelly, Omaha, NE). Arrays were constructed from a set of 12,140 sense oligonucleotide (60-mers) probes designed for each human target gene by Compugen, Inc. and manufactured by Sigma-Genosys, Inc. Individual arrays contain 12,288 spot features, including 12,107 different genes, 28 replications of glyceraldehyde-3-phosphate dehydrogenase, and negative controls. In brief, ~40 μg of experimental and reference RNA samples (i.e., from SCC25-uPAR+ versus SCC25-uPAR-KD) were reverse transcribed with anchored oligo(dT) primer. cDNA was labeled with either Cy3 or Cy5 monofunctional NHS-ester (Amersham Pharmacia) and dye-swap experiments were done. Labeled cDNA in hybridization solution was applied to DNA microarrays and incubated at 42°C for 16 to 20 h. After hybridization, microarray slides were
Statistical Analysis of Microarray Data

Analysis of microarray gene expression data, accumulated from three independent experiments, was done using the limma package (26), available through the Bioconductor project (27) for use with R statistical software (28). Data quality was examined by looking for spatial effects across each microarray with image plots of raw log2 ratios and examining MA plots of the M values (log2 ratios) versus A values (average log2 intensities). Both views indicated no large-scale systematic effects indicative of technical problems with the arrays. Background correction was carried out using a normal plus exponential convolution model (27), and print-tip (within-array) loess normalization was used to reduce systematic dye-related bias in the intensity values (29). After preprocessing, the analysis of differential gene expression was based on moderated t statistics on the replicated log2 ratios for each gene. Statistical significance was assessed using an Empirical Bayes approach (26). Adjustment for multiple comparisons according to the false discovery rate method of Benjamini and Hochberg (30) was done, and genes with adjusted P values of <0.05 were selected as differentially expressed. Log2 ratios were transformed back to fold change values for interpretation purposes in this report (Supplementary Tables S1 and S2). Genes were annotated and biological processes were analyzed by the Database for Annotation, Visualization and Integrated Discovery6 (DAVID; Supplementary Table S3). The Functional Annotation Clustering Tool organizes redundant annotation terms into a clustered format, enabling the comprehensive pooling of all related genes associated with a functional annotation cluster consisting of many related terms (31).

qPCR Analysis

qPCR was used to monitor the changes in mRNA expression level in transfected SCC cells. Total RNA was extracted with Trizol reagent (Invitrogen). Reverse transcription was done with 10 μg of the total RNA from each specimen using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. The cDNA products were then diluted 1:10 and 5 μL of each were used for PCR templates. The following primer sequences were used at final concentrations of 167 nmol/L for each: VEGF-C, 5′-GTGCTTCTGATGATGAACTC-3′ (forward) and 5′-ATCTGTAGACGGACCTGAAAC-3′ (reverse); phosphoglycerate kinase (PGK), 5′-GGGCTGTACATCAGGAACTCACAATGCA-3′ (reverse); endogenous control gene keratin 13, 5′-AGCGTACCATGCA-3′ (forward) and 5′-AAGGTGAGATTATGGA-3′ (reverse); keratin 19, 5′-TGAGTGCATACGCCAATAT-3′ (forward) and 5′-GGGCTGTACATCAGGAACTCACAATGCA-3′ (reverse); and laminin-5-α2 chain, 5′-AGGCTGTCCACACCGAATGGG-3′ (forward) and 5′-GGGCTGTACATCAGGAACTCACAATGCA-3′ (reverse). DNA oligos were custom synthesized (Integrated DNA Technologies).

Real-time PCR was done with SYBR Green Master Mix (Applied Biosystems). PCR cycling conditions were 95°C for 10 min followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. All reactions were carried out on an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories). Each sample was in quadruplicate for each PCR measurement. Melting curves were checked to ensure specificity. Quantification of mRNA expression was calculated using the ΔΔCt method with the endogenous housekeeping gene PGK level as normalizer and control sample calibrator.

In control experiments, cells were serum starved in the presence of the Src inhibitor PP2 (10 μmol/L; Calbiochem), DMSO vehicle control, the uPAR/αβ3 blocking peptide α325 (160 μmol/L; Accurate Chemical & Scientific), or scrambled peptide control (160 μmol/L) for 3 h before incubation with microsphere-immobilized β1 integrin antibodies (β1 beads) for 3 h as previously described (20). RNA was extracted and qPCR was done using VEGF-C–specific primers as described above.

Results

Formation of SCCOT Tumors in Nude Mice

Genome-wide monitoring of genetic changes associated with OSCC primary tumors and lymph node metastases has identified the proteinase uPA and the cell-matrix adhesion molecule α3 integrin as key candidate biomarkers for the prediction of poor disease outcome (9, 11, 32, 33). To evaluate the potential contribution of uPAR/α3β1 integrin binding to tumor growth and invasion in vivo, cells with modified uPAR levels were generated for in vivo analysis. Pooled clones of SCC25 cells with enhanced (SCC25-uPAR+) or reduced (SCC25-uPAR-KD) levels of uPAR (Supplementary Fig. S1A) were evaluated using fluorescence-activated cell sorting for changes in expression of other cell surface markers. Neither α3 integrin nor E-cadherin levels were altered by modulation of uPAR expression, and no change in proliferation was observed in vitro (data not shown). However, uPAR overexpression resulted in a substantial increase in in vitro invasive activity (Supplementary Fig. S1B and C). These results also show the contribution of α3 integrin to invasion, as a 66% decrease in invasion was observed in the presence of α3 integrin function-blocking antibodies. Similarly, use of an antibody strategy to block uPAR decreased invasion by 70%. As was previously observed (Supplementary Fig. S1B), knockdown of uPAR expression using siRNA also decreased invasion substantially.

6 http://david.abcc.ncifcrf.gov
(90%). In addition, the contribution of ERK activity to the invasive process was shown using a MEK inhibitor (PD98059), resulting in a 54% inhibition of invasion. Together, these functional data implicate α3 integrin as well as uPAR and pERK in the invasive process.

To investigate the effect of uPAR modulation on tumor growth at the orthotopic site in vivo, pooled clones of SCC25-uPAR+ or SCC25-uPAR-KD cells were injected submucosally into the anterior tongue and mice were examined visually for the development of tongue tumors. Eight mice per group were injected and the experiment was repeated thrice; representative images are shown. Visual tongue tumors developed by 5 to 6 weeks, and tumors were allowed to progress for 9 weeks. No lymph node or lung metastases were observed in the time frame of this study. Examination of H&E and immunohistochemically stained sections shows neoplastic cells arranged in cysts, nests, and packets frequently extending between and through muscle bundles and surrounded by inflammatory infiltrate (Fig. 1A-F). Neoplastic cells are markedly pleomorphic but display all stages of development seen in keratinized stratified squamous epithelium with flattened to cuboidal basophilic cells and scant cytoplasm. The cells become increasingly eosinophilic and develop abundant cytoplasm toward the center. In tumors formed by SCC25-uPAR-KD cells, low-power evaluation shows multicentric well-circumscribed tumor nests with a pushing margin of invasion (data not shown). Higher-power examination (Fig. 1A, C, and E) reveals that all tumors formed from SCC25-uPAR-KD cells have features of a well-differentiated squamous cell carcinoma, including numerous aggregates of keratin (keratin pearls, arrowheads), some dystrophic calcification, low mitotic index, and minimal pleomorphism. Most tumor cells showed abundant eosinophilic cytoplasm and a lower nuclear to cytoplasmic ratio than that seen in the uPAR overexpressor group. Mitoses were present but infrequent.

FIGURE 1. Histologic and immunohistochemical examination of oral tongue tumors. Tumors generated from pooled clones of SCC25-uPAR-KD and SCC25-uPAR+ cells were (A and B) stained with H&E or immunostained with (C and D) anti-cytokeratin AE1/AE3 (1:25 dilution) or (E and F) anti-uPAR (1:25 dilution) followed by a biotinylated secondary antibody and detection of avidin-biotin with DAB chromogen and substrate as in Materials and Methods. Magnification, ×400. T, areas of tumor cells; M, host tongue muscle; arrowheads, focal keratinization ("keratin pearls" are highlighted); asterisk, desmoplastic host response.
Prominent desmoplasia, characterized by loose mesenchymal tissue, was also present (Fig. 1A, asterisk). No lymphovascular or perineural invasion was seen. In contrast, low-power examination of uPAR-overexpressing tumors (SCC25-uPAR+) reveals cords of tumor with ill-defined borders and high vascularity (data not shown). Higher-power evaluation shows that all tumors formed from SCC25-uPAR+ cells were moderately to poorly differentiated squamous cell carcinomas. These were poorly circumscribed and deeply, diffusely, and raggedly infiltrative with thin cords of tumor cells dissecting through skeletal muscle (Fig. 1B, D, and F). Tumors are composed of clusters of hyperchromatic cells with a high nuclear to cytoplasm ratio and cytologic atypia, with only focal keratin production (Fig. 1B, D, and F). Rare keratin pearls were seen. This is supported by qPCR analysis of keratin mRNA expression levels, showing loss of keratin 13 and keratin 19 expression in SCC25-uPAR+ relative to SCC25-uPAR-KD (Supplementary Fig. S2A). No quantitative differences in overall tumor area or degree of inflammation were apparent (Supplementary Fig. S2B). Furthermore, numerous foci of perineural invasion and focal vascular invasion were evident in tumors formed from SCC25-uPAR+ cells (Fig. 2A-D; Supplementary Fig. S3), and higher-magnification H&E images clearly show cords of tumor cells wrapping around nerves (Fig. 2B and C; Supplementary Fig. S3B) or invading small vessels (Fig. 2D; Supplementary Fig. S3A). Studies of human OSCC have identified tumors with diffuse spread, small cords, and vascular and perineural invasion as associated with a more aggressive tumor and a poor prognosis (34-36).

**Colocalization of uPAR, α3 Integrin, and pERK in Murine and Human SCCOT**

Lateral complex formation between uPAR and α3β1 integrin induces Src/MEK/ERK signaling and transcriptional activation in vitro (19, 20). To assess whether these molecular players are colocalized in vivo, uPAR, α3 integrin, and pERK staining was assessed in murine SCCOT tumors using both immunohistochemical analysis of serial sections and four-color immunofluorescence analysis of single tumor sections. Multiple areas of colocalization between uPAR and α3 integrin were evident in all SCC25-uPAR+ tumors in areas also exhibiting ERK phosphorylation (Fig. 3A), and this is supported by immunofluorescence analysis showing fluorescence overlap among uPAR, α3 integrin, and pERK (Fig. 3B, white areas). Furthermore, quantitation of ERK activation, by enumerating cells with positive nuclear pERK staining in a minimum of 10,000 cells per group, showed a highly significant difference between the groups ($P = 0.0001$), with 67.9 ± 15.0% of tumors generated from uPAR overexpressors exhibiting ERK activation, whereas only 14.1 ± 8.2% of tumors from uPAR knockdown cells exhibited activated ERK (Fig. 3C). Similar results were obtained from analysis of microarrayed human SCCOT serial sections, showing colocalization of uPAR, α3 integrin, and pERK (data not shown). In human oral tumors, the bivariate relationship between α3 integrin positivity and pERK was significantly positive, with 82.5% of the variance in the latter explained by variation in the former ($r = 0.909; P < 0.0001$). As uPAR and α3 integrin positivity are also significantly positively correlated ($r = 0.763; P < 0.0001$), a multiple regression was done to investigate if this interaction had a significant influence on pERK expression beyond the singular influence of α3 integrin. Such an analysis indicates a significant positive effect of the interaction between uPAR and α3 integrin on pERK ($P < 0.0001$). This multifactorial model explains 91.9% of the variance in pERK positivity, which is 9.4% greater than a more simple bivariate model where only the influence of α3 integrin on pERK positivity...
is considered. Thus, incorporation of the relationship between uPAR and α3 better models the variance in pERK staining positivity than does a model that omits uPAR/α3 integrin interaction.

**cDNA Microarray Analysis and Validation by qPCR and Immunohistochemistry**

To examine additional changes in gene expression that result from uPAR/α3β1 integrin interaction, gene expression profiles of SCC25-uPAR-KD and SCC25-uPAR+ cells were compared using cDNA microarray analysis. An array containing oligonucleotides corresponding to 12,500 known human genes was used to analyze changes in gene expression. Statistical analysis of triplicate microarray data yielded 148 genes that were >2-fold differentially expressed between SCC25-uPAR+ and SCC25-uPAR-KD (P < 0.05), including 35 downregulated and 113 upregulated genes (Supplementary Tables S1 and S2). Genes were annotated and biological processes were analyzed using the Functional Annotation Clustering Tool of DAVID, which organizes redundant annotation terms into a clustered format (31). This simplified annotation condenses the data.
into modules, enabling a focus on biological processes (Table 1). Several biological processes were altered in SCC25-uPAR+ cells, including developmental processes, inflammation, proliferation, and adhesion.

Several candidate genes with known relevance to oral cancer progression were validated using real-time PCR and immunohistochemical analysis of murine tumors generated from orthotopic injection of SCC25-uPAR+ or SCC25-uPAR-KD cells. For example, a significant increase in expression of VEGF-C is shown in SCC25-uPAR+ cells (Fig. 4A), consistent with the enhanced vascularity observed in SCC25-uPAR+ tumors (Fig. 2). This is supported by qPCR analysis of VEGF-C mRNA expression levels, showing a 5-fold increase in SCC25-uPAR+ relative to SCC25-uPAR-KD cells (Fig. 4A). Similarly, qPCR analysis of a distinct uPAR-overexpressing cell line, SCC1-uPAR+, Table 1. Functional annotation clusters identified by the David functional annotation clustering tool

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NOTE: Genes from Supplementary Tables S1 and S2 were analyzed using the DAVID Functional Annotation Clustering Tool (http://david.abcc.ncifcrf.gov). The top 20 annotation clusters are shown. Abbreviation: EGF, epidermal growth factor.
exhibited a similar 2.9-fold increase in VEGF-C mRNA relative to vector controls (data not shown). As we have previously shown that uPAR/α3β1 integrin interaction initiates a Src/MEK/ERK-dependent signaling pathway resulting in transcriptional activation (20), control experiments were done to evaluate the role of uPAR/α3β1 integrin interaction in VEGF-C induction. Cells treated with microsphere-immobilized integrin antibodies in the presence of the Src inhibitor PP2 showed a 4.4-fold reduction in VEGF-C mRNA relative to vehicle (DMSO)–treated controls. Similar results were obtained by preincubation with peptide α325, previously shown to block uPAR/α3β1 binding (20, 37), leading to a 2.3-fold decrease in VEGF-C mRNA relative to cells treated with a scrambled peptide control. This result is supported by further quantitation of serial tumor sections stained for active ERK (nuclear pERK) or VEGF-C, which showed that 66% of cells with strong positive nuclear pERK also exhibited strong positive VEGF-C staining (data not shown).

FIGURE 4. Validation of cDNA microarray results using immunohistochemical analysis of murine tongue tumors and qPCR. Representative sections from SCC25-uPAR+ or SCC25-uPAR-KD tumors were immunostained with (A) anti–VEGF-C (1:20 dilution), (B) anti–kallikrein-5 (1:25 dilution), (C) anti–PAI-1 (1:20 dilution), or (D) anti–laminin-γ2 chain (1:20 dilution) followed by biotinylated secondary antibody as in Materials and Methods. Magnification, ×100. Corresponding qPCR analyses of gene expression in SCC25-uPAR+ and SCC25-uPAR-KD cell lines (left) and quantitation of immunohistochemical staining in murine tumors (right) are shown in the bar graphs. For qPCR, relative expression levels were normalized to housekeeping gene PGK. Each bar depicts the mean of replicate values expressed as fold difference in mRNA level relative to SCC25-uPAR-KD cells (designated as 1; gray columns). Black columns, SCC25-uPAR+; gray columns, SCC25-uPAR-KD. For quantitation of tumor staining, 2,500 to 6,000 cells from 10 tumor sections were scored and results are presented as relative staining (% of total cell number scored) *, P < 0.05.
In addition to uPA, other proteinases associated with tumor progression and/or invasion were also elevated in SCC25-uPAR+ cells. For example, the serine proteinase kallikrein-5 is overexpressed in SCC25-uPAR+ tumors (Fig. 4B), whereas the related enzyme kallikrein-3 (also known as prostate specific antigen) was not detected (25). Kallikreins have been widely implicated as cancer biomarkers as well as potential therapeutic targets in several malignancies (38, 39). The serpin PAI-1 was also elevated in SCC25-uPAR+ cells and tumors (Fig. 4C). In addition to blocking uPA activity, PAI-1 is known to stimulate tumor cell motility and survival (40-42). Expression of several extracellular matrix proteins was upregulated in SCC25-uPAR+ cells, including LAMC2, encoding the γ2 chain of laminin-332 (also known as laminin-5; Fig. 4D). LAMC2 was increased 2.7-fold in SCC25-uPAR+ cells and 1.9-fold in SCC1-uPAR+ cells. This epithelial basement membrane component has been implicated as a promoter of tumor invasion and cell survival (43, 44).

Discussion

Squamous cell carcinoma of the oral cavity is one of the most common cancers worldwide, with a poor 5-year survival rate of ∼50% due to local recurrence, regional or distant metastasis, and/or second primary tumors (5, 45). Late presentation combined with the lack of early detection markers and poor chemotherapeutic response leads to poor outcome of oral cancer patients. Thus, a more detailed understanding of factors that regulate tumor progression and metastasis may result in advances in diagnostics or therapeutics. Approximately 25% to 40% of oral cancers occur on the tongue (3) and increased rates of tongue squamous cell carcinoma have been observed in young adults (<45 years old) in both the United States and Europe (4). Patients with tongue cancer have poorer survival rates relative to patients with tumors on other anatomic subsites in the oral cavity (3, 6, 46). This poor prognosis has been attributed to multiple factors, including lack of early detection due to the relative inaccessibility of the tumor to inspection, as well as anatomic factors, including proximity to bone and the rich lymphatic network of the tongue (3, 5).

Numerous studies have used cDNA microarray analysis to profile patterns of gene expression in primary oral tumors relative to metastases or normal oral mucosa. Interestingly, principal components analysis of expression profiling data showed a distinct separation of tongue from nontongue specimens, indicative of site-specific gene expression in the oral cavity (8). Enhanced expression of uPA was consistently observed in many studies (8, 47-49), and uPA was listed as one of 25 genes that comprise an OSCC gene signature to distinguish tumor versus nontongue specimens (8). Similarly, in vivo selection of cell lines for enhanced metastatic propensity in mice, coupled with cDNA microarray analysis, identified uPAR as a key metastasis-related gene (50). This is consistent with immunohistochemical analysis of primary oral cancers, showing a correlation between uPA or uPAR expression and both cancer invasion and regional lymph node metastasis (15). Immunohistochemical localization of uPAR at the invasive front of OSCC lesions has also been reported, suggesting a role for uPAR in early cancer invasion (16). This is supported by results of the current study, showing the development of poorly differentiated, aggressive infiltrative lesions with perineural and/or vascular invasion from uPAR-overexpressing cells. These features are well associated with aggressive clinical behavior and poor prognosis in humans (34-36). In contrast, tumors seen in the uPAR-KD group showed histologic features associated with less aggressive tumors, including pushing borders of invasion and absence of perineural or vascular invasion (34-36). Although loss of uPAR expression has been previously associated with tumor dormancy (51), no evidence for dormancy was observed in the current study.

Interaction of uPAR with transmembrane integrins regulates cellular adhesion, invasion, and motility (19, 51-53), but transcripitional activation downstream of αβ3 integrin/uPAR complex formation has not been extensively evaluated. Association of uPAR with αβ3 integrin is via the α3 subunit β3-propeller, in a region distinct from the binding matrix site (37). Binding of uPAR and αβ3 integrin results in activation of a Src/MEK/ERK-dependent signaling pathway, leading to transcriptional upregulation of uPA expression (20). Complex formation between αβ3 integrin and uPAR is necessary for transcriptional activation, as MAPK signaling is substantially attenuated by several blocking strategies that inhibit uPAR interaction with αβ3 integrin (20). Furthermore, αβ3 integrin engagement in cells in which uPAR levels were downregulated with siRNA failed to activate MAPK signaling and uPA transcription (20). Data from the current study support these results and show statistically significant colocalization of uPAR and α3 integrin in foci also exhibiting ERK activation in both murine and human tongue tumors, supporting the hypothesis that uPAR/α3β1 association may also regulate gene expression in vivo.

Altered transcriptional profiles of SCC25-uPAR+ cells are also apparent from cDNA microarray analysis as well as immunohistochemical analysis of murine tumors. Many of the genes transcriptionally activated in uPAR-overexpressing cells, including VEGF-C, kallikrein-5, PAI-1, and laminin-32 chain, have been previously implicated in OSCC progression. For example, VEGF-C is a major modulator of lymphatic vessel density and microvessel density and is elevated in human oral tumors, and its expression correlates with enhanced regional lymph node metastases of tongue tumors (54-58). Although staining for enhanced lymphatic endothelium was negative in the current study, it should be noted that lymph node metastases are also not present in our model, suggesting that upregulation of VEGF-C may be a precursor to altered lymphangiogenesis and metastasis. As a recent preclinical study has shown that antivascular therapy targeting tumor microvessels with a small-molecule inhibitor of VEGF receptor-2 blocks tumor growth in vivo, these data suggest...
that vascular targeting of lymphatic and angiogenic vessels may represent an effective therapeutic strategy for OSCC. Kallikrein-5 is a member of the human tissue kallikrein family of serine proteases. Although the physiologic roles of many kallikreins are not clearly defined, aberrant kallikrein expression patterns have been reported in many carcinomas and multiple kallikreins have been proposed as biomarkers in several malignancies (38, 39, 59). Kallikrein activity has been linked to malignant behavior at multiple stages of tumor progression, including proliferation, invasion, metastasis, and angiogenesis (38, 39, 59, 60). Further, kallikrein-5 as well as the related proteases kallikrein-7, kallikrein-8, and kallikrein-10 are abundantly expressed in human OSCC and may be implicated in malignant progression (25). PAI-1 is a serpin that blocks the activity of uPA but also regulates adhesion and motility by virtue of its ability to bind vitronectin. Somewhat paradoxically, PAI-1 levels are highly elevated in many tumor types and correlate with poor prognosis (42). Recent studies indicate that PAI-1 transcription is initiated in wounded keratinocytes as a component of the wound repair program (40). PAI-1 stimulates adhesion and migration and blocks anoikis, suggesting a role as a prosurvival, promigratory factor. Elevated PAI-1 correlates with poor outcome (relapse-free survival) in patients with oral cavity cancer (18) and is a biomarker for early oncogenesis and invasion (16, 61). The extracellular matrix component laminin-γ2 chain also correlates with early invasive events in OSCC (16). Laminin-γ2 is a subunit of laminin-332 (also known as laminin-5), a major basement membrane laminin isoform (44). Laminin-332 has been implicated in promotion of tumor cell adhesion, migration, and invasion (44) as well as in activating phosphoinositide 3-kinase and RAC1 to induce prosurvival pathways (43). Laminin-γ2 staining identified dysplastic epithelia in oral brush biopsies, suggesting the utility of evaluating laminin-γ2 expression as an initial diagnostic step (62). This is supported by studies showing that laminin-γ2 expression may be an effective biomarker for early detection of invasive OSCC (63, 64). Aside from these validated examples, the major-ity of the gene products listed in Table 1 have not been previously associated with aggressive OSCC, indicating the potential efficacy of this strategy for identification of novel OSCC-related genes.

In summary, orthotopic murine models of OSCC have shown efficacy for analysis of factors that modulate tumor progression and metastasis as well as for preclinical testing of potential therapeutics (23–25, 45, 65, 66). The current results together with available clinical data support a role for uPAR/α5β1 in progression of tongue tumors, indicating that inhibition of uPAR/α5β1 integrin interaction may represent a novel therapeutic approach to block Src/MEK/ERK signaling and the downstream functional consequences to OSCC progression. Furthermore, our results provide proof of concept that cDNA microarray analysis together with examination of orthotopic murine tumors can identify factors associated with aggressive human OSCC. It thereby follows that this approach may also provide a strategy for biomarker discovery to reveal novel diagnostic or prognostic indicators. As the vast majority of OSCC patients succumb due to complications of metastasis, identification of additional markers of aggressive disease is necessary to improve patient survival.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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