The Role of Novel Oncogenes Squamous Cell Carcinoma-related Oncogene and Phosphatidylinositol 3-Kinase p110α in Squamous Cell Carcinoma of the Oral Tongue


ABSTRACT

Purpose: Amplification at chromosome 3q26.3 is a common and crucial event in head and neck squamous cell carcinoma (HNSCC), impacting significantly on tumor progression and clinical outcome. Two novel oncogenes, namely squamous cell carcinoma (SCC)-related oncogene (SCCRO) and PIK3CA (gene encoding phosphatidylinositol-3 kinase catalytic α-polypeptide), have been identified as targets of 3q26.3 amplification. This study aimed to delineate the role of SCCRO and PIK3CA in the pathogenesis of oral tongue SCC.

Experimental Design: The association between gene copy number for SCCRO and PIK3CA measured by fluorescence in situ hybridization and level of mRNA expression quantitated by real-time reverse transcription-PCR was assessed in a panel of human HNSCC cell lines. In addition, gene expression in 49 consecutive primary SCCs of the oral tongue was determined and correlated with clinicopathological characteristics and outcome.

Results: The mRNA level of SCCRO and PIK3CA was significantly correlated to the gene copy number in nine HNSCC cell lines. In addition, the expression level of SCCRO and PIK3CA was significantly greater in malignant tissues compared with those in histologically normal mucosa (2.17- and 2.46-fold, respectively; \(P < 0.001\)). Matched tumor normal control analysis revealed that 24.5 and 69.4% of patients expressed high levels of SCCRO and PIK3CA, respectively. Univariate analyses demonstrated that SCCRO overexpression correlated with nodal metastases (\(P = 0.05\)) and advanced stage (\(P = 0.02\)), whereas PIK3CA overexpression was associated with vascular invasion (\(P = 0.04\)). Only SCCRO overexpression was associated with disease-specific (\(P = 0.04\)) and overall survival (\(P = 0.02\)). Furthermore, SCCRO overexpression remained an independent predictor for cervical nodal metastasis on multivariate regression analysis (\(\chi^2\) likelihood ratio = 4.38; \(P = 0.04\)).

Conclusions: Although both SCCRO and PIK3CA may play a role in the pathogenesis of oral tongue SCC through amplification at 3q26, SCCRO appears to be a significant predictor of regional metastasis and may be a marker for tumor aggressiveness and clinical outcome.

INTRODUCTION

Cancer of the oral cavity is a subset of HNSCC that ranks 11th in frequency and 13th in cancer-specific mortality worldwide (1). Despite several diagnostic and therapeutic advances, the overall incidence and mortality associated with oral cancer are rising, with current estimates of age-standardized incidence and mortality being 6.6/100,000 and 3.1/100,000 in males, 2.9/100,000 and 1.4/100,000 in females, respectively (1, 2). Within the oral cavity, SCCs of the oral tongue are characterized by a particularly aggressive clinical course, hallmarked by a propensity for local invasion and regional nodal metastasis (3). However, there is significant heterogeneity in the clinical behavior of these tumors that is not predictable by established clinicopathological variables. Delineation of markers identifying patients with biologically aggressive tumors (and hence poor prognosis) would provide a valuable opportunity for therapeutic targeting.

The genetic mechanisms underlying the development and progression of HNSCC are among the most complex of human tumors (4). Gene amplification is one of the essential mechanisms of oncogene activation in these cancers. Several recurrent DNA copy number increases have been identified in HNSCC by karyotypic analyses, such as double minute or homogeneously staining regions, and are considered to harbor putative oncogenes. Comparative genomic hybridization analyses have identified 3q as the most common site for gains and amplification in HNSCC (5–7), with a minimal common region at 3q26–27...
(7–10). We have shown that amplification of 3q26.3 is associated with transition to invasive cancer and is negatively correlated with clinical outcome (11). Accordingly, genes that drive selection for 3q26 amplification may play an important role in the pathogenesis and prognostication of HNSCC (7, 12).

To identify the genes involved, we used a positional cloning strategy to show two independent amplification peaks at the 3q26.3 locus and isolate candidate genes within them, namely SCCRO and PIK3CA (13, 14). Sequence annotation of SCCRO reveals a basic helix-loop-helix-leucine zipper motif, suggesting a potential role as a transcription factor. Overexpression of SCCRO in NIH-3T3 fibroblasts led to characteristics of transformed phenotype, such as serum-independent proliferation, colony formation, and anchorage-independent growth in soft agar assay (14). In addition, the SCCRO-transfected cells were capable of forming undifferentiated invasive tumor and developed associated regional lymph node metastasis in nude mice. PIK3CA encodes the p110α catalytic subunit of class I phosphatidylinositol 3'-kinases (15) and has established oncogenic activity. Phosphatidylinositol 3'-kinases constitute a family of lipid kinases that play a central role in many cellular functions associated with malignant behavior, including control of cell cycle progression, invasion, and survival (reviewed in Ref. 16). PIK3CA has been shown to be amplified and overexpressed in ovarian cancer (17) and cervical cancer (18). Evidence from recent studies suggests that PIK3CA is a strong candidate oncogene in upper aerodigestive tract malignancies (13). In addition, amplification of the PIK3CA locus was shown to be a potential marker for clinical outcome of 21 early stage HNSCC from multiple anatomical sites (12).

The present study aimed to determine the contribution of the candidate oncogenes (SCCRO and PIK3CA) at 3q26.3 in the pathogenesis of oral tongue SCC. We showed good correlation between amplification and expression of both genes in HNSCC cell lines, suggesting that both may play a role in the pathogenesis of these tumors. We used a real-time RT-PCR assay to quantify SCCRO and PIK3CA mRNA expression in a series of 49 consecutive patients with SCC of the oral tongue, showing that only SCCRO correlates with established clinicopathological predictor variables (depth of invasion, nodal metastasis) and outcome.

### MATERIALS AND METHODS

**Cell Lines.** Nine human cell lines derived from HNSCC (584, MDA686, MDA886, MDA922, MDA1186, MDA1386, MDA1586, MDA1986, and MSKQLL2) were used in the present study. The origins and cytogenetic characteristics of these cell lines were described previously (7). The cells were grown to 80% confluence at 37°C in a humidified atmosphere of 5% CO2 and 95% air in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin.

**Patient Materials.** Fresh tissue samples were sequentially obtained from 49 patients undergoing therapeutic surgical resection for SCC of the oral tongue at the Head and Neck Service, Memorial Sloan-Kettering Cancer Center, from January 28, 1998 to January 4, 2002 following guidelines established by the Institutional Review Board and after obtaining informed consent. All patients had no detectable metastases to distant organs at presentation. Tumors were staged according to the American Joint Committee on Cancer/Union International Contre Cancer Tumor-Node-Metastasis classification fifth edition (19). Adjuvant treatment was provided after surgery in appropriate cases following the center’s protocol. In each case, the portion of tumor was resected near the advancing edge of the tumor, avoiding its necrotic center, and immediately snap frozen and stored in liquid nitrogen until use. The adjacent tissues were submitted for histopathological study to confirm the presence of ≥80% cancer cells in the procured sample. Histologically normal mucosa of the upper aerodigestive tract, resected 5 cm distant from the tumor area, was obtained in all cases and used as controls. “Node-positive cases” in this study included cases in which positive cervical nodes were identified based on a histological diagnosis after a neck dissection. Patients who experienced no metastasis for ≥12 months postoperatively are scored as node-negative cases. The clinical and pathological characteristics of patients are summarized in Table 1. The median follow-up period for living patients was 8.8 months (range: 0.3–49.5 months). Forty-one patients (83.7%) were alive, whereas 7 patients (14.3%) died from disease, and 1 patient (2.0%) died from unknown causes.

**DNA Preparation and FISH.** Genomic DNA from each sample was extracted with a commercial kit (Qiagen, Santa Clarita, CA) following the manufacturer’s instructions. FISH analysis was performed as described previously (7, 13).

**RNA Preparation and Real-time RT-PCR.** Total RNA was extracted from cell lines or snap-frozen tissue samples with TRIzol reagent (Life Technologies, Inc., Grand Island, NY) following the manufacturer’s protocol and repurified by the RNAeasy Mini-spin column (Qiagen). RNA samples were treated before analysis with DNase I to eliminate residual genomic DNA. Two μg of total RNA were reversed transcribed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) in the presence of random primers (Roche, Mannheim, Germany). cDNA was used as a template for real-time RT-PCR with a TaqMan assay (Applied Biosystems, Foster City, CA). Primers and TaqMan probes were specific to the exonic regions of SCCRO and PIK3CA, with the following sequences: SCCRO forward, 5′-GTTGAGTTTGTGCTACCCATCG-3′; SCCRO reverse, 5′-ATGAGGCTGGTCACTTTTGG-3′; SCCRO probe, 5′-6FAM-CCTGCTGCTGTCCTGAGTGTC-TAMRA-3′; PIK3CA forward, 5′-CTCCCGTCTGATCAGGTGAC-3′; PIK3CA reverse, 5′-ATGGTCTCCAGCAGATCTG-3′; PIK3CA probe, 5′-6FAM-CATTTTTGACAGACAGAGTGATGTAT-TAMRA-3′. The expression of 18S ribosomal RNA was used as an internal control. RT-PCR was performed using the ABI 7500 Real-Time PCR System (Applied Biosystems) and the SYBR Green PCR Master Mix (Applied Biosystems). The expression of both genes was normalized to 18S levels and quantified by the 2–ΔΔCt method.

### Table 1  Clinicopathological features of 49 patients with oral tongue SCC

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>49 (100)</td>
</tr>
<tr>
<td>Median age (years)</td>
<td>59 (range, 35–97 years)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
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<tr>
<td>Males</td>
<td>26 (53)</td>
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<tr>
<td>Females</td>
<td>23 (47)</td>
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<tr>
<td>Histological grading</td>
<td></td>
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<tr>
<td>Well differentiated (G1)</td>
<td>9 (18)</td>
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<tr>
<td>Moderately differentiated (G2)</td>
<td>32 (65)</td>
</tr>
<tr>
<td>Poorly differentiated (G3)</td>
<td>8 (16)</td>
</tr>
<tr>
<td>T classification (pathological)</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>16 (33)</td>
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<tr>
<td>T2</td>
<td>21 (43)</td>
</tr>
<tr>
<td>T3</td>
<td>8 (16)</td>
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<tr>
<td>T4</td>
<td>4 (8)</td>
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<tr>
<td>Lymph node involvement</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>25 (51)</td>
</tr>
<tr>
<td>Positive</td>
<td>24 (49)</td>
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<tr>
<td>Pathological staging</td>
<td></td>
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<tr>
<td>I</td>
<td>11 (22)</td>
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<tr>
<td>II</td>
<td>12 (25)</td>
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<tr>
<td>III</td>
<td>7 (14)</td>
</tr>
<tr>
<td>IV</td>
<td>19 (39)</td>
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</tbody>
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with MultiScribe Reverse Transcriptase (Applied Biosystems, Inc., Foster City, CA). Highly purified salt-free, gene-specific primers were designed using the Primer3 program \(^5\) and purchased from Operon Technologies (Alameda, CA). Sequences of PCR primer set for SCCRO (in 5'-3' direction) were as follows: forward-TCTGTGATGACCTGGCACTC, reverse-TGTCTGGAGAACCTGCACGT. Sequences of PCR primer sets for PIK3CA and 18S rRNA were described previously \((12, 20)\). Specificity of the RT-PCR amplification products was documented with a 4% high-resolution NuSieve agarose gel electrophoresis and resulted in a single product with the desired length. Quantification of transcripts was performed by the ICycler Detection System (Bio-Rad Laboratories, Hercules, CA) using SYBR green detection. The PCR reaction included the 2 × SYBR Green PCR Master Mix (Applied Biosystems) mixed with primers and cDNA template (80 ng). Preliminary experiments were performed with each primer pair and serial dilutions (80, 8, 0.8, 0.08, or 0.008 ng) of reverse transcribed total RNAs from a head and neck carcinoma cell line MSK1186 to determine the annealing temperature that yielded the greatest amount of specific product with melting temperature (Tm) and to calculate the real-time PCR efficiency \((21)\). The following ICycler run protocol was used: denaturation program (95°C for 10 min) and amplification and quantification program repeated 30–50 cycles (95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and specified acquisition temperature for 15 s). Amplification was followed by melting curve analysis \((22)\). The acquisition temperature was set 1°C–2°C below the Tm of the specific PCR product. The relative quantification of a target gene in comparison with a reference (18S rRNA) was performed as described \((20, 21)\). Reverse transcribed total RNAs (80 ng) from MSK1186 cells were included in each experiment as a control standard for interexperimental variation, and the amount of mRNA expression in each sample was quantified in relation to a control standard. A negative control without cDNA template was run with every assay to assess the overall specificity. Similar PCR reactions were also performed on nonreverse transcribed RNA specimens to rule out residual genomic DNA contamination. Unless otherwise stated, each assay included duplicate reactions for each sample and was repeated twice.

**Statistical Analysis.** All correlation and outcome analysis was performed using the JMP statistical software package version 4.0.0 (SAS Institute, Inc., Cary, NC). Results were expressed as mean ± SE. For comparison between the levels of mRNA expression between groups, the unpaired t test was used. Correlations between the mRNA levels were computed using the two-tailed Spearman nonparametric correlation. The Pearson \(\chi^2\) test with Fisher’s exact test was used to assess the association of gene expression and clinicopathological parameters. Survival was measured in months from the date of surgery to the date of relapse/death or last follow-up. Survival curves and median survival times were calculated by the Kaplan-Meier method, and differences in survivor distributions were calculated by the Log-rank test. Multivariate logistic regression analysis was performed to determine predictive values of gene expression and clinicopathological characteristics on cervical nodal metastasis. Cox proportional hazards modeling was performed to identify factors with significant influence on survival while controlling for confounding variables. A \(P\) of ≤0.05 was considered statistically significant, and all tests were two sided. Risk ratio were estimated for each covariate.

## RESULTS AND DISCUSSION

### Expression of SCCRO and PIK3CA in Human HNSCC

Lines Correlates with Gene Copy Numbers Determined by FISH. Identification of gene(s) driving selection for chromosomal amplification is rarely unambiguous. As an example, the presence and clinical significance of 11q13 amplification in HNSCC are well established. However, even with a potent oncogene present at this locus (cyclin D1), several other candidate genes have been suggested to be targets driving selection for 11q13 amplification. Similarly, the amplification at 3q26.3 has several potential gene targets. In our previous work, we showed that two amplification peaks exist within the 3q26.3 amplification and identified SCCRO and PIK3CA as genes contained with each of these peaks \((13, 14)\). Both genes have a significant oncogenic potential, making them excellent candidates as oncogenes in HNSCC.

To assess the relationship between amplification and expression of the candidate oncogenes, we performed FISH analysis using BAC clone 202B22 (for SCCRO) or BAC clone 836M7 (for PIK3CA) as a probe to assess DNA sequence copy number in interphase nuclei from 9 cell lines established from head and neck cancers. Increased SCCRO and PIK3CA copy numbers (>5) were found in 6 and 4 of 9 cell lines, respectively \((Table 2)\). We used quantitative real-time RT-PCR to measure SCCRO and PIK3CA transcription levels in 9 HNSCC cell lines \((Table 2)\). The MDA1186 cells expressed the highest levels of both SCCRO \((7.25 ± 0.39)\) and PIK3CA \((19.85 ± 1.23)\). We found a significant correlation between increased copy number and the corresponding mRNA expression of both SCCRO \((\text{Spearman} \ r = 0.68; \ P = 0.05)\) and PIK3CA \((\text{Spearman} \ r = 0.89; \ P = 0.002)\). Thus, the increase in transcription level in HNSCC cell lines likely resulted from the SCCRO and PIK3CA gene copy number increase. There was no correlation between SCCRO and PIK3CA expression \((P = 0.09)\). These data strongly suggest that both SCCRO and PIK3CA may play a role in driving selection for 3q26 amplification in HNSCC by different regulatory mechanisms.

### Expression of SCCRO and PIK3CA in Tongue Carcinomas and Normal Tongue Tissues.

To determine the status of gene expression in primary tongue SCC, 49 matched pairs of primary tumor tissues and histologically normal adjacent mucosa were examined for mRNA levels of SCCRO and PIK3CA. The mean mRNA expression of SCCRO was significantly greater in malignant tissues in comparison with the mRNA levels in histologically normal mucosa \((13640 ± 6277 \text{ pg} \ versus 315.1 \text{ pg} \ P < 0.0001; \ Fig. 1)\). The mean expression level of PIK3CA was also significantly higher in tumor tissues compared with that of normal tissues \((981.3 ± 315.1 \text{ pg} \ versus 398.9 ± 164.6 \text{ pg} \ P < 0.0001; \ Fig. 1)\). We defined the cutoff value for differentiation between normal and overexpression of

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\(^5\) Internet address: http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi.
SCCRO and PIK3CA mRNA as ≥2-fold increase in tumor mRNA expression relative to matched normal tongue controls. Using this criterion, 24.5% of patients (12 of 49) expressed high levels of SCCRO, 69.4% (34 of 49) expressed high levels of PIK3CA, and 14.3% (7 of 49) coexpressed high levels of SCCRO and PIK3CA. There was no direct correlation observed between the expression of mRNAs encoding SCCRO and PIK3CA (P = 0.53).

**Correlation of SCCRO and PIK3CA Expression with Clinicopathological Characteristics.** We correlated expression of SCCRO and PIK3CA in 49 primary tumors to clinicopathological variables to determine their interrelationship (Table 3). Statistically significant correlations were identified between SCCRO overexpression and several established prognostic variables, including tumor thickness >10 mm (odds ratio = 4.17; P = 0.05), presence of nodal metastasis (odds ratio = 4.4; P = 0.05), and advanced pathological stages (odds ratio = 6.25; P = 0.02). Conversely, PIK3CA overexpression only correlated with less relevant prognostic variables, including female gender (odds ratio = 5.7; P = 0.02), histological grade (odds ratio = 5.2; P = 0.05), and presence of vascular invasion (odds ratio = 11.6; P = 0.04).

**Correlation of Clinicopathological Characteristics and SCCRO and PIK3CA Expression with Nodal Metastasis.** The presence of nodal metastasis is the single most important predictor of outcome in HNSCC. SCCRO overexpression was significantly correlated with cervical nodal metastases (odds ratio = 4.4; P = 0.05; Table 4). However, cervical metastasis did not correlate with age, gender, smoking, alcohol, grade, tumor thickness, perineural or perivascular invasion, T classification, and levels of PIK3CA (Table 4). Parameters that had P-values ≤ 0.5 were selected for multivariate regression analysis. SCCRO overexpression was the only characteristic to retain independent correlation with nodal metastasis (χ² likelihood ratio = 4.38; P = 0.04; Table 5).

The observed association between SCCRO and tumor thickness, a marker for invasiveness (23), suggests that SCCRO may play a role in tumor invasion. This association is confirmed by work showing that SCCRO plays a critical role in matrix metalloproteinase-2 regulation and secretion (14). In line with the increased invasiveness, SCCRO overexpression also correlated with the presence of nodal metastasis. Because the presence of neck nodal metastasis is by far the most decisive influence on prognosis in patients with HNSCC (24), its relationship with SCCRO is of interest. Among various biological and clinicopathological parameters examined, SCCRO was the only factor that demonstrated a significant correlation with nodal metastasis (Table 5). However, the molecular mechanisms underlying the association between SCCRO and lymphatic metastasis remain to be elucidated. Recently, analysis of downstream targets of SCCRO by oligonucleotide microarrays revealed up-regulation of...
several genes linked to malignant progression, i.e., transforming growth factor-β1, insulin-like growth factor-1, and vascular endothelial growth factor.\textsuperscript{6} The release of major angiogenic factors consequent to SCCRO expression, may lead to a permissive environment for tumor cell invasion and metastasis.

### Relationships with Survival
As shown in Table 6, poorly differentiated histological grade, tumor thickness >10 mm, T stage III and IV, positive nodal status, advanced pathological stage, and SCCRO overexpression were significantly associated with a poorer overall survival on univariate analysis (\(P = 0.02, 0.02, 0.002, 0.002, 0.002, \text{ and } 0.02\), respectively; Table 6). Furthermore, tumor thickness, T stage, N status, overall stage, and SCCRO status demonstrated statistically significant correlation with worse disease-specific and relapse-free survival (Table 6). Multivariate analysis was not possible because of the small number of events.

Nonetheless, this is the first report demonstrating a prognostic value of SCCRO in oral tongue cancers. In the present study, PIK3CA expression status did not show predictive value.

**Conclusion.** Although SCCRO and PIK3CA reside in a closely related chromosomal region at 3q26.3, our study shows divergent patterns of expression (and hence regulation) in oral tongue cancerous tissues. SCCRO, but not PIK3CA, seems to play a more prominent role in disease progression of oral tongue carcinoma. The up-regulation of SCCRO in lymph node-positive and hence poor prognosis patients suggests that the evaluation of SCCRO levels in tumor tissues at the time of presentation may allow the identification of a subset of oral tongue carcinoma patients who are more susceptible to metastatic spread via lymphatic pathway. Additional studies are needed to prospectively correlate SCCRO expression with the development of nodal and assess its role in reoperative planning.

The present study confirms that both SCCRO and PIK3CA may be targets of 3q amplification in HNSCC and play a role in pathogenesis of oral tongue SCC. However, only SCCRO overexpression was associated with aggressive clinical behavior, including nodal metastasis and poorer survival outcome. Furthermore, only SCCRO showed significant predictive value for nodal metastasis. Accordingly, SCCRO may be a prognostic factor and provide a basis for the development of novel anticancer strategies.

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We thank Nancy Bennett for excellent editorial assistance with this manuscript. We dedicate this manuscript to the memory of Dr. P. G. Reddy, who passed away unexpectedly during the completion of this work.

**REFERENCES**


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