Molecular-cytogenetic characterization of head and neck cancer: Identification of novel prognostic factors and gene targets for therapy [double dissertation 2]
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Squamous Cell Carcinoma Related Oncogene (SCCRO) Regulates Angiogenesis Through Vascular Endothelial Growth Factor-A

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Abstract

Background: Squamous cell carcinoma related oncogene (SCCRO) is a novel oncogene identified by positional cloning of a recurrent amplification at 3q26.3.[1,2] It is overexpressed in 39.8% of lung, head and neck, cervical, and ovarian carcinomas.[3] SCCRO imparts an aggressive phenotype to affected cancers, which may be related to increased angiogenesis due to SCCRO expression. Our previous work has demonstrated a link between SCCRO and vascular endothelial growth factor-A (VEGF-A) expression in vitro, suggesting a mechanism for SCCRO-induced angiogenesis. The present study aims to confirm and validate this link between SCCRO and VEGF-A expression in an ex vivo human tumor cohort.

Methods: Fresh tissue was collected at Memorial Sloan-Kettering Cancer Center from 34 patients undergoing primary resection of lung squamous cell carcinomas. RNA was extracted from this tissue, reverse-transcribed, and real-time PCR was carried out using a BioRad iQ iCycler with SYBR green fluorophore. Microvessel counting was performed on the tumor specimens using CD34 immunohistochemistry.

Results: The expression of both SCCRO and VEGF-A mRNA varies widely in both tumor and normal tissue. SCCRO and VEGF-A co-expression was significantly correlated (R2=0.63; p<0.032). Microvessel counts were not associated with expression of SCCRO or VEGF-A, and failed to significantly predict survival. VEGF-A expression in this patient group is a predictor of overall survival (p<0.032).

Conclusions: VEGF-A expression correlates with SCCRO expression in these primary human lung squamous cell carcinomas and is a predictor of clinical behavior. This data supports the association of SCRRO and VEGF-A in the induction of angiogenesis.

Amplification of 3q26-27 is a common and crucial event in many squamous cell carcinomas, including those of the upper aerodigestive tract.[1;2;4-6] Our previous work has shown that the SCCRO gene is amplified in 56% of head and neck squamous cell carcinomas compared with 3% of normal mucosa, and gene overexpression correlates with overall and disease-specific survival.[3]

Induction of neovascularization is one mechanism by which tumor cells attain their growth advantage.[7] Moreover, angiogenesis is often quoted as a sine qua non of tumor growth and progression for clinically important disease or metastases.[8;9] The use of anti-angiogenic agents has long been suggested as a potential therapeutic tumor target.[10] and considerable recent work has focused on the use of anti-angiogenesis as an adjunct to chemo-radiotherapy.[11]

Our previous work has investigated the link between SCCRO and angiogenesis.[Talbot et al, unpublished data] Our in vitro experiments confirmed that SCCRO is a potent inducer of angiogenesis, showing transformation of HUVEC cells into vascular structures in a co-culture assay with SCCRO-transfected 3T3-cells, but not in control cells. In addition, in vivo experiments demonstrated significant neovascularization and microvessel formation in vivo in tumors resulting from NIH-3T3 cells stably transfected with SCCRO. Screening of SCCRO-transfected cell lines with Affymetrix oligonucleotide microarray (Affymetrix HG_U95Av2) revealed a core group
of nuclear proteins, cytokines, growth factors, receptors, signaling molecules, enzymes, transporters, and ion channels with greater than 2-fold up-regulation relative to vector transfected controls.[1] The only prominent angiogenic factor in the genes dysregulated by SCCRO was vascular endothelial growth factor (VEGF-A), showing on average 7.1-fold up-regulation in two stably-transfected NIH-3T3 clones. The temporal relationship between SCCRO and VEGF-A mRNA and protein expression was confirmed using a tetracycline-inducible (Tet-off) SCCRO-3T3 cell line showing the induction of VEGF-A mRNA and protein expression with the induction of SCCRO by real-time RT-PCR and western blot analyses. The angiogenic effects of VEGF-A are induced by secretion of the protein and subsequent activation of stromal cells. In concordance, we showed the soluble, secreted fraction of VEGF-A protein in higher levels ($p<0.009$) in the cell conditioned media of SCCRO-transfected cells and confirmed that it was secreted by these cells using ELISA assay.

Vascular endothelial growth factor (VEGF-A) is one protein of the VEGF family (known as VEGF-A through E). In humans it is expressed as numerous splice variants of which the 121, 165, 189, and 206 amino acid variants are most common; with the smaller of these being more soluble due to loss of the exon 6 heparin-binding domain.[12-14] The mouse VEGF gene contains a frame-shift mutation and translates into 120, 164, and 188 amino acid splice variants.[15] VEGF-A is known to act on three tyrosine kinase receptors (VEGFR1-R3 also known as flt1, KDR/flk1, and flt4), but the exact pathways of VEGF action are poorly characterized. Importantly, however, increased VEGF-A expression has repeatedly been shown to be related to increased lymph node metastases, poorer treatment response, and poorer survival in squamous cell carcinoma patients.[16-19] The present paper investigates the link between SCCRO and VEGF-A expression in primary human squamous cell carcinoma samples and determines its effects on outcome.

**Experimental Procedures**

**Patients**

The patient cohort consists of 34 previously-untreated patients receiving surgical resection of primary lung squamous cell carcinomas at Memorial Sloan-Kettering Cancer Center between February

![Graph](image_url)

*Fig. 1. SCCRO mRNA fold change relative to normal in 34 patients. A change of 2-fold or greater was considered SCCRO up-regulation (n=18).*
27, 1990 and July 22, 1997. Tumor and matched normal tissue were collected from each patient according to Institutional Review Board guidelines and after obtaining informed consent. Patients were excluded if they had received prior treatment. Post-operatively, patients received adjuvant treatment as determined by Center protocols. Representative samples of normal and tumor tissue from the advancing tumor edge were was snap frozen in liquid nitrogen at the time of resection and stored at -80°C until use. All tumors were re-examined by a single pathologist (RG) on routine paraffin sections to confirm the diagnosis and grade of squamous cell carcinoma. The median age of the group at presentation was 69 years. The median follow-up of survivors was 83 months.

RNA Preparation and Reverse-Transcription

Total RNA was extracted from all tumor and normal samples simultaneously. 100μg of tissue was used and RNA was extracted using TRIzol® reagent (Invitrogen, Life Technologies) following manufacturer's protocol. Samples were further purified using RNeasy Mini Kits (Qiagen). DNase I was added to remove remaining genomic DNA. Two micrograms of total RNA was reverse-transcribed using Taqman® Reverse Transcriptase Reagents (Applied Biosystems) and following manufacturer's protocol.

Real-Time PCR

Primers were designed using Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and Qiagen Operon Toolkit (http://oligos.qiagen.com/oligos/toolkit.php), according to parameters outlined in the BioRad iCycler Manual. Specificity of primers was confirmed by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/). SCCRO PCR primers used were as follows: forward 5'-TCTGTGATGACCTGGACATGCCTG-3', reverse 5'-TGTGATGGAGAACACTCGACTG-3'. VEGF-A PCR primers used were designed from exon 3 as published in GenBank as follows: forward 5'-CCAATCGAGACCCTGGTG-3', reverse 5'-CACACAGGGATGGCTTGAAGA-3'. 18s rRNA was used as a reference for normalization as previously described,[20;21] and relative quantification was analyzed using iCycler iQ Optical System Software Version 3.0a (BioRad Laboratories, Ltd). Samples negative for 18S rRNA were excluded from analysis as RNA was assumed to be of too poor a quality for further analysis.

Real-time PCR was performed
using a BioRad iQ iCycler Detection System (BioRad Laboratories, Ltd) with SYBR green fluorophore. Reactions were performed in a total volume of 20μL - including 2x SYBR Green PCR Master Mix (Applied Biosystems), 5μL of each primer at 5μM concentration, and 2μL of the previously reverse-transcribed cDNA template.

Protocols for each primer set were optimized using five serial 10x dilutions of template cDNA obtained from human head and neck squamous cell carcinoma cell line MSK1186. The protocols used are as follows: denaturation (95°C for 10 minutes), amplification repeated 30-50 times (95°C for 30 seconds, specific annealing temperature for 30 seconds, 72°C for 30 seconds, and acquisition temperature for 15 seconds). For SCCRO, 50 cycles were used with a 55°C annealing temperature; while for VEGF-A 50 cycles were used with a 64°C annealing temperature. The 18S rRNA reference required 30 cycles with a 55°C annealing temperature. A melt curve analysis was performed following every run to ensure a single amplified product for every reaction. PCR fluorophore acquisition temperatures were set at 1°C below the melt curve peak.

All reactions were carried out in at least duplicate for every patient sample. The same reference standard dilution series (described above) was repeated on every experimental plate and semi-quantification of both the gene of interest and the 18S rRNA normalizations are based on this.

![Fig. 3. Bivariate fit of VEGF-A mRNA expression against SCCRO mRNA expression (Spearman's rank correlation, R²=0.63). Scales are fold-change x80000, normalized to 18S rRNA.](image)

![Fig. 4. Kaplan-Meier survival plot of squamous cell carcinoma patients grouped by VEGF-A mRNA expression (Log-rank, p<0.032).](image)
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Duplicate negative controls (no template cDNA) were also run with every experimental plate to assess specificity and indicate potential contamination.

Microvessel Density Assessment

For each of the 34 patients, original tumor and normal paraffin-embedded tissue blocks were obtained. These were reviewed by a single pathologist (RG) and 5µm sections cut from each. Slides were subjected to immunoperoxidase staining using the streptavidin-biotin method. Briefly, sections were submitted to antigen retrieval by microwave treatment for 30 minutes in 0.01M citrate buffer at pH 6.0. Primary mouse monoclonal antibody against human CD34 was used at a 1:2000 dilution (Beckmann Coulter, Brea, CA). Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain. Microvessel density was determined using an Olympus BX40F U-DO microscope was used at 400x magnification with 2 observers (RG and ST) according to standard published protocols.[22]

Statistical Analysis

Data were analyzed using the JMP 4.0 Statistical Package (SAS Institute). For comparison of VEGF-A levels in SCCRO positive or negative tumors an unpaired, two-tailed t-test was used. For the correlation between SCCRO and VEGF-A mRNA, Spearman's rank correlation was used. Survival data are based on the Kaplan-Meier method and differences were calculated using the log-rank test. A two-tailed p-value of less than or equal to 0.05 was considered significant.

Results and Discussion

Expression of SCCRO in Primary Tumors

We have previously established an association between SCCRO and both aggressive tumor and clinical outcome.[1] SCCRO expression in this lung tumor subset is similarly significantly associated with poor outcome (data not shown). Based on expression in tumor greater than 2-fold normal, the lung tumors in this study could be grouped into two subsets: those with high (n=18) and those with low level (n=16) SCCRO expression (Figure 1). This is consistent with our findings in head and neck squamous cell carcinoma and suggests that SCCRO dysregulation plays a significant role in the pathogenesis of a subset of these tumors.

Expression of VEGF-A Correlates with SCCRO in Human Tumors

Our work suggests that the aggressive behavior in SCCRO overexpressing tumors may involve increased angiogenesis, at least in part.[9] Our in vitro data suggests that SCCRO-induced angiogenesis is involved in the up-regulation of VEGF-A.[Talbot et al, unpublished data] A wide range of VEGF-A expression was seen in the primary lung cancers included in this study. The level of VEGF-A expression was correlated with SCCRO expression, with a mean of 7.4-fold induction (relative to 18S rRNA) in the cases with high-level SCCRO, compared with 0.78 in cases with low-level SCCRO expression (p<0.032, Figure 2). The normalized mRNA expression of both SCCRO and VEGF-A also correlates significantly on linear correlation (R2=0.63; Figure 3). This
observed correlation supports the regulation of VEGF-A transcription by SCCRO, but requires further experimental confirmation.

SCCRO and VEGF-A Expression do not Correlate with Microvessel Counts

Microvessel counts are commonly used to assess tumor vascularity as a surrogate marker for angiogenesis. Many papers report microvessel density in both lung and head and neck cancers as an important prognostic indicator, and it is frequently correlated with numerous angiogenic factors;[23;24] however, this is not a universal finding.[25] Our patient samples averaged 29 microvessels per 400x high-power field with a range from 15 to 148. Microvessel counts did not significantly correlate with either SCCRO or VEGF-A expression. Counts were not predictive of metastasis and did not significantly predict disease-specific, recurrence-free, or overall survival. There was, however, a trend towards poorer outcomes in patients with higher vessel counts, which may become significant with larger patient numbers. In this patient cohort it appears that neither SCCRO nor VEGF-A expression correlates with microvessel density or, alternatively, that other factors are involved in the pathway to influence the angiogenic process.

VEGF-A is a Predictor of Poorer Overall Survival

Having established a correlation between SCCRO and VEGF-A, we investigated if VEGF-A expression was a clinical outcome predictor. VEGF-A levels above the mean for the entire cohort were associated with poorer overall survival in those with higher VEGF-A expression ($p<0.032$; Figure 4). This finding is consistent with the role of VEGF-A in angiogenesis and the maintenance of tumor vascularity. We have previously shown the association between SCCRO and survival.[7] Our current findings support VEGF-A as a one possible target mediating SCCRO associated aggressive behavior.

In conclusion, we have confirmed previous in vitro data showing that SCCRO is correlated with VEGF-A in primary human lung squamous cell carcinomas. The expression of both genes is predictive of outcome. This may be an important mechanism for squamous cell carcinoma angiogenesis and the consequential maintenance of tumor viability and represents a potential therapeutic target.

References
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