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Survival in Small Cell Lung Cancer is Independent of Tumor Expression of VEGF and COX-2

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Abstract. *Background: Preclinical data suggests that VEGF and COX-2 are potentially important mediators in the pathogenesis of small cell lung cancer (SCLC). Little is known about the frequency of tumor expression of VEGF and COX-2 in SCLC or the prognostic significance of this expression. Materials and Methods: Clinical records from 54 cases of SCLC were reviewed. Immunohistochemical stains for VEGF and COX-2 were performed on all tumor specimens. Results: Tumor VEGF expression was detected in 43 cases (81%) and COX-2 expression in 11 (20%). No significant association between VEGF or COX-2 expression and survival was observed. Conclusion: This is the first study to assess the frequency and clinical significance of tumor VEGF and COX-2 expression in a large group of patients with SCLC. In this cohort, neither VEGF nor COX-2 expression impacted survival. The frequency of VEGF expression suggests that it merits further investigation as a therapeutic target in SCLC.*

Approximately 30,000 patients are diagnosed each year in the United States with small cell lung cancer (SCLC). The outcome for patients with SCLC is dismal with fewer than 5% of patients surviving 5 years. One clinical feature that typifies SCLC is the early development of widespread metastatic disease. The pathogenetic mechanisms that result in this tumor biology are not defined. One critical step in the development of metastatic disease is angiogenesis. Pioneering work by Folkmann and others has established that the ability of a tumor to develop its own blood supply is necessary for growth, invasion, and distant spread (1). Vascular endothelial growth factor (VEGF) is a cytokine that plays several pivotal roles in angiogenesis. It is known to induce hyperpermeability in pre-existing blood vessels, a step that generally precedes angiogenesis (2). VEGF is also a mitogen for endothelial cells and has been shown to induce endothelial cell migration as well as the formation of vessel precursors in *in vitro* tumor models (3,4). The VEGF expression in tumor specimens can be detected by immunohistochemistry and appears to correlate with increased vascularity or microvessel density of the tumor (5-7). In non-small cell lung cancer (NSCLC), VEGF expression in resected tumors is associated with lymph node spread and advanced tumor stage (5,8). Several studies demonstrate that VEGF expression predicts for poor prognosis in patients with surgically resected NSCLC (6,8-11). Similar observations have been made in breast, colon, ovary, prostate, stomach and head and neck cancers (12-15).

The role that VEGF plays in the development and spread of SCLC has not been established. Lund and colleagues (16) reported that VEGF protein was expressed by all 21 SCLC cell lines tested, although the level of expression was quite variable. More uniform expression was seen *in vivo* in tumor xenografts created from a group of 9 of those cell lines. Salven *et al.* (17) measured the concentration of VEGF in the serum of 68 SCLC patients prior to the initiation of treatment. They found that a lower level of VEGF in the serum was significantly associated with improved tumor response and survival. Ohta and colleagues (18) detected the expression of VEGF mRNA by RT-PCR in 5 out of 7 SCLC tumor specimens. In addition, Lucchi *et al.* (19) reported a series of 87 patients with resected SCLC whose tumors were evaluated for VEGF expression by immunohistochemistry. They found that a high level of VEGF protein expression predicted for inferior disease-free and overall survival. This data suggests that the VEGF expression may be an important prognostic factor for patients with resectable SCLC. However, its utility in patients with more advanced disease has not been established.

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Cyclooxygenase-2 (COX-2) has also been implicated as a mediator of tumor growth and metastasis. COX-2 is an inducible enzyme that generates prostaglandins from arachidonic acid and appears to play a role in inflammation and carcinogenesis. This has been best described in colorectal tumors where COX-2 expression and prostaglandin production have been shown to be crucial for the development and growth of these cancers. One possible mechanism whereby COX-2 induces this effect is through angiogenesis. DuBois and colleagues (20) reported that COX-2 overexpressing colon cancer cells were able to induce migration and tube formation in co-cultured endothelial cells. This was associated with the increased production of mRNA for several angiogenic factors including VEGF. The observed effects on endothelial cells were completely blocked with either selective COX-2 inhibitors or specific antibodies to the induced angiogenic factors. In addition, the selective COX-2 inhibitor celecoxib has been shown to block basic fibroblast growth factor induced angiogenesis in a rat corneal model (21). These data suggest a link between COX-2 expression in tumor cells and angiogenesis.

Several lines of preclinical evidence indicate that prostaglandins are involved in the development, growth and spread of cancer and that COX-2 may represent a useful therapeutic target. Chiabrando *et al.* (22) reported that the Lewis lung carcinoma (LLC) cell line secreted increased levels of prostaglandins as they grew. Young and colleagues (23) observed that non-metastatic LLC cells could induce migration in metastatic LLC cells *in vitro* and that this finding was replicated with the addition of exogenous prostaglandin E_2. The effect was abolished when either indomethacin (a non-selective COX-2 inhibitor) or anti-prostaglandin E_2 antibodies were added to the system. They were also able to demonstrate *in vivo* that, when non-metastatic LLC cells were added to the tumor inoculum in mice or when exogenous prostaglandin E_2 was administered, increased numbers of lung metastases were observed. These effects were similarly blocked by either indomethacin or anti-prostaglandin E_2 antibodies. In addition, the growth of SCLC and NSCLC cell lines was inhibited by the addition of COX-2 inhibitors, and synergy between these inhibitors and conventional chemotherapy agents was also seen in these cell lines (24,25). Furthermore, both selective and non-selective COX-2 inhibitors have been shown to block the development of carcinogen-induced tumors in mice and to prevent the growth of NSCLC xenografts in murine models (21,26,27).

COX-2 gene and protein expression have also been evaluated in human lung cancers. Autopsy and surgical series have shown that COX-2 mRNA expression is increased in NSCLC when compared with normal lung tissue (28-30). In addition, COX-2 protein expression has been detected by immunohistochemistry in 70-90% of human NSCLC surgical specimens (29-32), and COX-2 protein expression was significantly associated with poor survival in a subgroup of patients with resected stage I adenocarcinoma (31). Similarly, Khuri and colleagues (33) have shown that COX-2 mRNA expression was associated with worse disease-free and overall survival in 160 patients with resected stage 1 NSCLC. COX-2 protein expression has been evaluated to only a limited extent in SCLC. One study reported that 4 out of 4 tumor specimens from patients with SCLC had evidence of COX-2 expression by immunohistochemistry (30), and a second observed positive staining for the enzyme in 1 out of 9 tumors (32).

Taken collectively, these data suggest that both VEGF and COX-2 are potentially important mediators in the pathogenesis of SCLC. In the present study, we sought to determine the incidence of VEGF and COX-2 expression in a cohort of patients staged and treated in a similar manner at a single institution, and to identify whether expression of these factors provided significant prognostic information in these patients.

Materials and Methods

Patients. Fifty-four patients treated from 1991-1998 at the Dallas Veterans Affairs Medical Center, Texas, USA were included in this study. Adequate pathology material, clinical data and follow-up information were available for all patients. Biopsy slides were reviewed to confirm the presence of small cell carcinoma. All patients were staged and treated in similar fashion. All patients had computed tomography of the chest, abdomen and pelvis and routine blood work including a complete blood count, creatinine, liver function tests and a lactate dehydrogenase level. Bone scan and brain imaging were performed as clinically indicated. Limited stage patients typically received 6 cycles of cisplatin and etoposide combined with thoracic irradiation. The majority of extensive stage patients received 6 cycles of cisplatin and etoposide. A small number received this regimen alternating with cyclophosphamide, vincristine and adriamycin. The immunohistochemical staining was scored by a single pathologist (R.H.A.) who was blinded to the clinical data. The study was approved by the Institutional Review Board at the Dallas Veterans Affairs Medical Center.

Immunostaining. Study material included 37 transbronchial lung biopsies, 15 lung or mediastinal tissue specimens obtained either *via* thoracotomy or mediastinoscopy, and 2 bone marrow specimens. All tissue was fixed in 10% buffered formalin (bone marrow was additionally exposed to B-5 fixative for 20-45 minutes) and paraffin embedded. Routine histological sections were cut 3 μm thick. All available archived slides were reviewed for each case. Representative paraffin blocks were chosen for immunoperoxidase staining. All immunostaining was performed using VEGF Ab-1 (Neomarkers Inc./Labvision Corparation, Fremont, CA, USA) at a 1:50 dilution with a lung adenocarcinoma positive control and COX-2 (H-62) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a 1:40 dilution with a bladder transitional cell carcinoma positive control. Stains were performed at room temperature on a

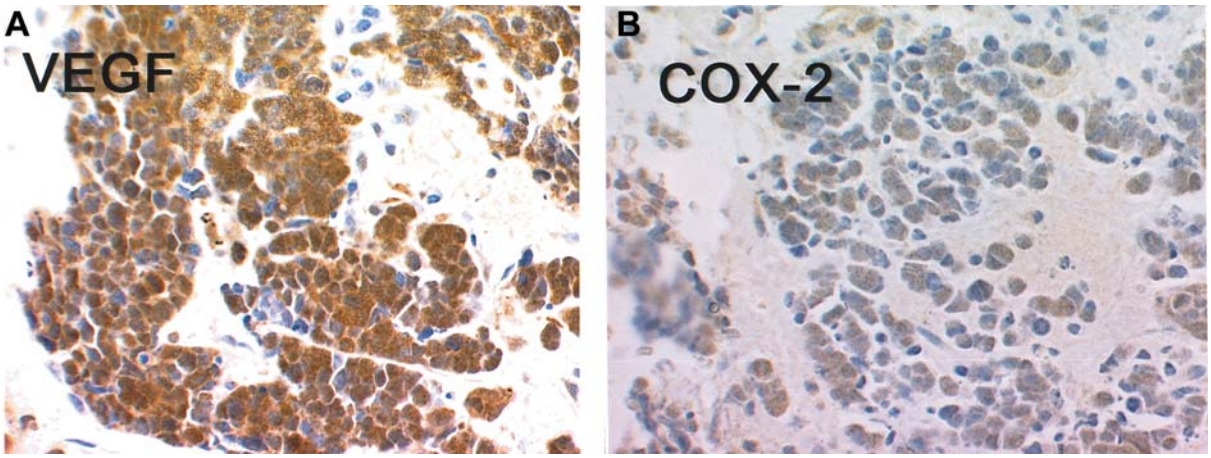

Figure 1. *Examples of immunohistochemical detection of VEGF and COX-2 in SCLC with greater than 95% of the cells staining with the antibodies (Hematoxylin counterstain, original magnification x 40) (1A - VEGF; 1B - COX-2).*

Table I. *Patient characteristics (N=54).*

	Frequency (%)
Sex	
Male	53 (98)
Female	1(2)
Race	
White	43 (80)
Black	11 (20)
Stage	
Limited	20 (37)
Extensive	34 (63)
Liver involvement	15 (28)
Bone involvement	16 (30)
ECOG performance status	
1	23 (43)
2	19 (35)
3	1 (2)
4	1 (2)
Not available	10 (19)
Treatment	
Chemotherapy and radiation	20 (37)
Chemotherapy alone	33 (61)
Radiation alone	1 (2)

Table II. *Frequency of tumor VEGF and COX-2 expression in SCLC (N=54).*

	Frequency (%)
VEGF expression[a]	
None	10 (19%)
1-10% of cells	4 (8%)
11-50% of cells	7 (13%)
> 50% of cells	32 (60%)
COX-2 expression	
None	43 (80%)
1-10% of cells	3 (6%)
11-50% of cells	3 (6%)
> 50% of cells	5 (9%)

[a]Adequate biopsy material was not available to perform VEGF staining for one patient.

Bio Tek Solutions TechMate 1000™ automated immunostainer (Ventana Medical Systems, Tucson, AZ, USA) using the Ultra-streptavidin biotin system with horseradish perioxidase and diaminobenzidine (DAB) chromogen (Signet Laboratories, Dedham, MA, USA). Biobuffer™ (BioPath, Oklahoma City, OK, USA) was used for heat-induced epitope retrieval (HIER). Immunoreactivity was assessed by counting a minimum of 5 high-powered fields (40X objective lens) in non-crushed tumor tissue, and the mean number of positive tumor cells was scored. Stains were graded as negative (0-10% of cells staining), 1+ (11-25% of cells), 2+ (26-50% of cells), 3+ (51-100% of cells). Examples of positive staining for VEGF and COX-2 are shown in Figure 1.

Statistical analysis. The endpoint variable was measured from the time of diagnosis until death (survival time). Statistical comparison of dichotomous levels of clinical factors was accomplished with a one-sided log-rank test, where the alternative hypothesis is that the death rate of patients with VEGF or COX-2 expression is higher. Observed significances not exceeding 0.05 were taken to be statistically significant. Estimates of median survival time and accompanying 95% confidence bounds were obtained using order statistics. Statistical analyses were conducted using StatXact-4 (CYTEL Software Corporation Cambridge, Massachusetts,USA) and S-PLUS 6 (Insightful Corporation Seattle, Washington, USA).

Results

The clinical features of the patient population are shown in Table I. The median age of the group was 65 years (range 50-82). The median survival time from diagnosis of the

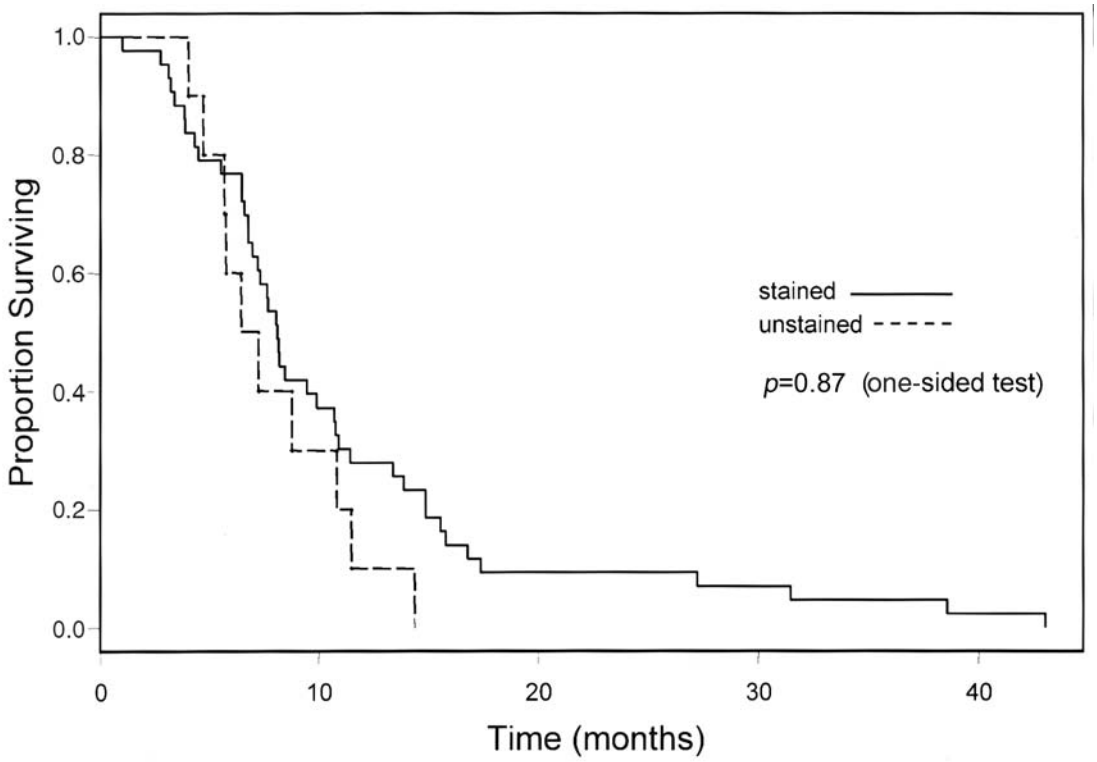

Figure 2. *Overall survival curves for SCLC patients with and without tumor expression of VEGF.*

entire cohort was 7.9 months (95% CI, 6.8-9.9). A univariate analysis of the clinical features showed that only extensive stage ($p<0.001$), liver involvement ($p=0.02$) and bone involvement ($p<0.001$) were significant negative prognostic factors.

The frequency and intensity of staining for VEGF and COX-2 are given in Table II. Forty-three (81%, 95% CI 68-91) of the biopsy specimens showed positive staining for VEGF, and 11 (20%, 95% CI 10-33) were positive for COX-2. The median survival of patients with staining for VEGF was 8.1 months (95% CI, 6.8-10.8), and the median survival of patients without detectable staining for VEGF was 6.8 months (95% CI, 4.7-11.5). This difference was not statistically significant ($p=0.87$) (Figure 2). Thirty-two patients (60%) demonstrated a high level of staining for VEGF (> 50% of cells) (Table II). The survival of this group was compared with that of patients with lower levels of VEGF expression (< 50% of cells), and no significant difference was seen (8.1 months *vs.* 6.9 months, $p=0.15$). The median survivals of patients with and without staining for COX-2 were 8.2 months (95% CI, 3.9-17.4) and 7.3 months (95% CI, 6.5-10.8), respectively. This difference was also not significant ($p=0.71$) (Figure 3). In addition, when limited and extensive stage patients were analyzed separately, neither VEGF nor COX-2 expression was found to be associated with a significant decrease in overall survival.

The prognostic impact of dual staining for VEGF and COX-2 was also assessed. Eleven tumors (20%) expressed both COX-2 and VEGF, and 10 (19%) did not express either factor. Median survivals for these groups were 8.2 months (96% CI, 3.9-17.4) and 6.8 months (95% CI, 4.7-11.5), respectively ($p=0.62$).

Discussion

The majority (81%) of SCLC tumors in the present series expressed VEGF. Lucchi *et al.* (19) published the only prior large series that has evaluated VEGF expression in SCLC. They reported staining only as above or below the median percentage of positive cells for that patient group, and therefore the true prevalence of VEGF expression was not stated. In our patients, VEGF expression had no apparent impact on survival. In contrast, the Italian group found that patients with "high" levels of VEGF expression (above the median for that group) had a significantly

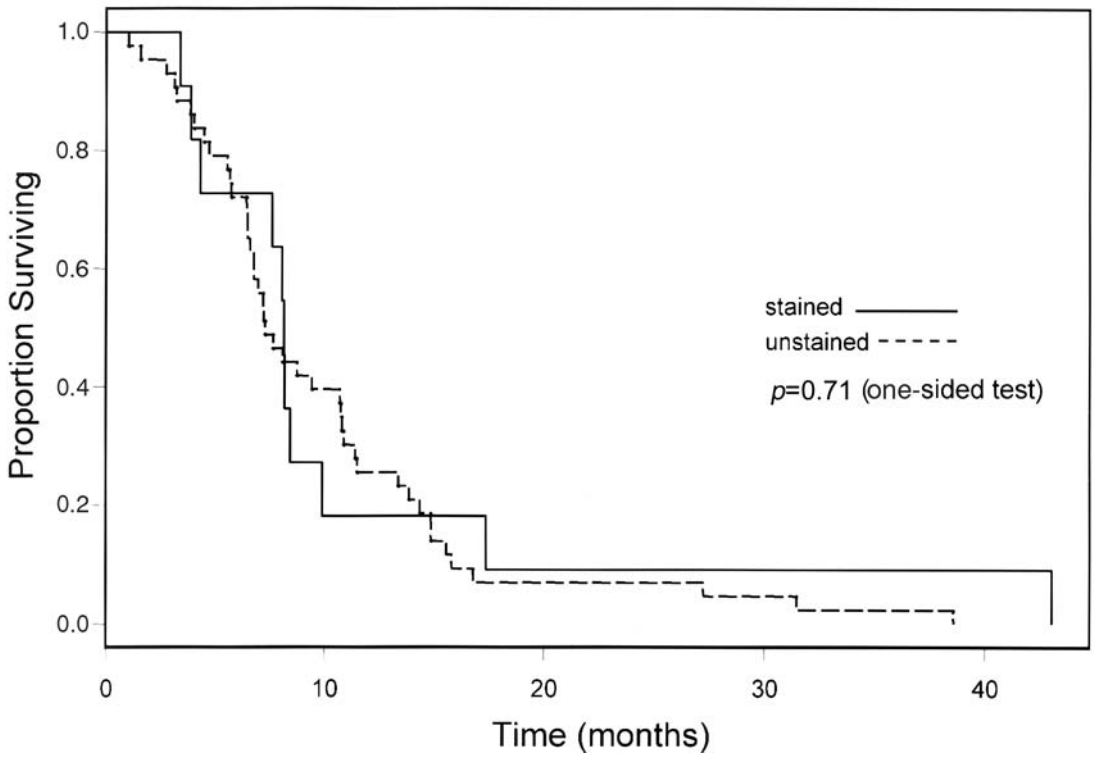

Figure 3. *Overall survival curves for SCLC patients with and without tumor expression of COX-2.*

inferior survival when compared with those patients with a low level of expression. A possible explanation for this discrepancy is that the two series included different patient populations. The Italian series looked only at resected SCLC specimens (a distinctly unusual presentation) and therefore probably included predominantly patients with low tumor burden. Conversely, approximately two-thirds of our patients had extensive stage disease, and the remainder had bulky limited stage presentations that would not have been eligible for a surgical approach. One interpretation of the data is that VEGF expression occurs early in the development and progression of SCLC, and therefore its impact on survival is only seen in those patients with very limited disease. This fits with the belief that VEGF is one factor that facilitates tumor growth and development of metastases, and with the understanding that SCLC acquires the capacity for metastatic spread early in the course of the disease.

Another explanation for the failure to identify any association between VEGF expression and survival in the present cohort may be the relatively small number of patients included. Although the sample size for this series was large in comparison to similar published series in SCLC, the statistical power to detect small, but perhaps clinically meaningful, differences in survival between patient groups was limited. Larger prospective series may better define the true impact of VEGF expression in SCLC.

The prevalence of VEGF expression observed in this cohort suggests that it merits further exploration as a therapeutic target in SCLC. The frequency of expression noted here equals or exceeds that reported for other solid tumors, such as non-small cell lung, breast and colon cancer, and a number of therapeutic strategies directed against VEGF are actively being explored in those malignancies.

This is the first study to estimate the prevalence of COX-2 protein expression in a large cohort of patients with SCLC. In prior publications, a total of only 13 cases of SCLC were analyzed for COX-2 expression by immunohistochemistry, and positive stanining was noted in 5 (38%) (30,32). In the present study, a minority of cases (20%) expressed COX-2 and no significant association between COX-2 expression and patient outcome was evident. Although this suggests that COX-2 expression has a negligible impact on the natural history of SCLC, it may define a subset of SCLC patients in whom therapeutic strategies against COX-2, in

conjunction with chemotherapy, should be explored. Similar approaches are currently being evaluated in several malignancies, including non-small cell lung, breast and colon cancers.

Recently, we discovered that COX-2 was frequently associated with promoter methylation in SCLC but not NSCLC (Miyajima K, Minna J. Personal communication). This could explain the relative lack of expression seen in the present study. This promoter methylation may be present in the precursor cells giving rise to SCLC or acquired in the tumor. The presence in the former might indicate that hypomethylation of COX-2 is an event leading to the development of SCLC.

Understanding of the biology and pathogenesis of SCLC has lagged behind that of many tumors. Part of the difficulty in gaining insight into this malignancy stems from the fact that the usual non-surgical approach to this tumor limits the amount of tissue available for study. Therefore, much of what is known has been derived from studies of cell lines and animal models, which does not always reflect what is observed in actual human tumors. This study is the first in SCLC to utilize biopsy specimens to define the frequency of expression of VEGF and COX-2, two potentially important mediators in the progression of SCLC. In the future, similar approaches, including higher throughput systems such as tissue microarrays, may allow for the identification of additional molecular targets that will ultimately advance the treatment of this deadly disease.

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