GENERAL THORACIC SURGERY

ANGIOGENESIS AS A PREDICTOR OF SURVIVAL AFTER SURGICAL RESECTION FOR STAGE I NON-SMALL-CELL LUNG CANCER

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Objectives: Some patients with surgically resected stage I non-small-cell lung cancer eventually have metastatic disease. A histologic marker of metastatic potential and diminished survival for stage I non-small-cell lung cancer may distinguish this patient population. This study evaluates the degree of angiogenesis as a predictor of cancer-related death after operation for stage I non-small-cell lung cancer. **Methods:** Demographic, surgical, and histopathologic data, including presence of vascular invasion, were reviewed for 106 patients with stage I non-small-cell lung cancer from 1985 through 1990. Visual quantitation of microvessels immunostained with factor VIII-related antigen and CD31 in 5 μ m sections from the paraffin blocks of tissue defined tumor angiogenesis. **Results:** Follow-up was 95.1% complete, mean 5.2 ± 3.0 years. Lung cancer-related mortality rate was 24.4% at 5 years. Mean microvessel counts were 20.7 ± 11.2 for FVIII and 29.6 ± 18.1 for CD31. Univariate analysis revealed an FVIII count of at least 20 ($p = 0.025$) and blood vessel invasion ($p = 0.017$) to be significant predictors of disease-related death. After adjustment for other patient and tumor characteristics, multivariate Cox regression analysis found an FVIII count of at least 20 (hazard ratio 2.9) and blood vessel invasion (hazard ratio 3.7) to be significant independent correlates of lung cancer death ($p = 0.018$ and $p = 0.011$, respectively). CD31 quantitation did not predict survival on univariate or multivariate analyses and did not correlate strongly with FVIII quantitation (Spearman's rank correlation $r = 0.19$). **Conclusions:** This analysis reveals a significant association between tumor neovascularization and cancer-related mortality rate among patients with stage I non-small-cell lung cancer. Microvessel quantitation of FVIII, as an indicator of tumor angiogenesis and metastatic potential, may define a subset of patients with stage I non-small-cell lung cancer who could benefit from adjuvant therapy after surgical resection. (J Thorac Cardiovasc Surg 1998;115:652-9)

Solid tumor growth and development have been associated with an initial prevascular stage (slow, local growth), followed in many cases by a vascular

phase characterized by neovascularization of the tumor mass (rapid, distant spread).¹⁻⁵ Although this angiogenesis alone is not sufficient for metastasis to occur, it has been correlated with increased incidence of metastasis. Immunostaining of endothelial cells from invasive portions of resected oncologic specimens permits visualization of microvessels whose formation is induced by tumor-host cell interactions.^{6,7} Quantitation of immunostained microvessels has since become an indicator of the degree of neovascularization and thus tumor metastatic potential.⁷⁻¹²

The value of angiogenesis as a significant prognostic marker of outcome after resection of solid tumors becomes potentially crucial in assigning therapy to patients with early-stage cancer for whom

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Read at the Seventy-seventh Annual Meeting of The American Association for Thoracic Surgery, Washington, D.C., May 4-7, 1997.

Received for publication May 7, 1997; revisions requested July 15, 1997; revisions received Nov. 11, 1997; accepted for publication Nov. 11, 1997.

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0022-5223/98 \$5.00 + 0 12/6/87520

surgery alone is expected to be curative. Despite a 20% to 40% recurrence rate within 5 years of operation, patients with stage I non-small-cell lung cancer (NSCLC) in particular are typically treated with surgical resection alone.¹³⁻¹⁷ Various biologic markers have been examined as prognostic indicators of recurrent disease and diminished survival in patients with primary lung cancer, including deoxyribonucleic acid ploidy, oncogene derangements, blood group antigens, and tumor cell antigens, among others.¹⁸⁻²¹ Few, however, have examined the significance of neovascularization in a specific subset of patients with early evidence of NSCLC.²²⁻²⁶

This investigation evaluated the prognostic significance of tumor angiogenesis in patients with stage I (T1 N0 M0 and T2 N0 M0) NSCLC who underwent surgical resection alone between 1985 and 1990. Furthermore, we analyzed immunostaining for factor VIII-related antigen (FVIII) and for CD31 platelet-endothelial cell adhesion antigen (CD31) with respect to clinical outcome.^{12, 27}

Materials and methods

Study population. The Emory University Hospital Oncology Registry provided 112 consecutive patients who underwent surgical resection for stage I NSCLC between 1985 and 1990, without postoperative or neoadjuvant therapy. The clinical records of these patients were reviewed to obtain routine demographic information and confirm accurate clinical and pathologic tumor staging.

Operative procedure. The location of the carcinoma was recorded from the preoperative evaluation and adjusted according to intraoperative findings as necessary. In agreement with institutional policy, lobectomies or pneumonectomies were typically performed as dictated by tumor size and location. Wedge resections or segmentectomies were performed in cases of small, peripheral, superficial lesions, or in conditions of diminished pulmonary reserve. Intraoperative staging included hilar and mediastinal lymph node sampling.

Follow-up. Follow-up of the study population was conducted by direct patient contact. Patients were categorized as alive with evidence of disease, alive without disease, dead as a result of lung carcinoma, or dead of unrelated causes. Time in days from the date of the operation to the date of follow-up or death was recorded.

Histopathologic evaluation. After approval was obtained from the Institutional Review Board Human Tissue Use Committee, formalin-fixed, paraffin-embedded blocks of tumor tissue taken at the time of operation were used for this analysis. Maximum size of the tumor in the resected specimens was documented from the original pathologic report. Representative hematoxylin-eosin-stained sections were reviewed to confirm histologic cell type and visceral pleural invasion.

Table 1. Patient and operative demographics (n = 106)

Variable	No.	%
Sex		
Male	66	62.3
Female	40	37.7
Age (yr)		
≤60	36	34
>60	70	66
Site of primary tumor		
Right	61	57.5
Left	45	42.5
Extent of operation		
Wedge/segmentectomy	11	10.3
Lobectomy	89	84
Pneumonectomy	6	5.7
Maximum size (cm)		
≤3	71	69.6
>3	31	30.4
T status		
T ₁	63	59.4
T ₂	43	40.6

Immunohistochemistry. Areas representative of the tumor's invasive component were identified.⁸ Five micrometer sections of formalin-fixed, paraffin-embedded tissue were tested for the presence of immunohistochemically detectable antigens with monoclonal anti-FVIII (prediluted; Ventana Medical Systems, Inc., Tucson, Ariz.) with pepsin digestion and with monoclonal anti-CD31 (1/80; Dako Corporation, Carpinteria, Calif.). An avidin-biotinylated enzyme complex kit (Signet Laboratories, Inc., Dedham, Mass.) was used, with previous heat antigen retrieval with the automated TechMate 1000 immunostaining systems (Ventana Medical Systems).

Sections were removed from paraffin and rehydrated, then steamed in citrate buffer for 20 minutes and cooled for 5 minutes before immunostaining. All tissues were then exposed to blocking antibody for 5 minutes, primary antibody for 25 minutes, biotinylated secondary linking antibody for 25 minutes, hydrogen peroxide solution for 7.5 minutes, avidin-biotinylated enzyme complex for 25 minutes, diaminobenzidine as chromogen for 15 minutes, and hematoxylin as counterstain for 1 minute. These incubations were performed at room temperature; between incubations, sections were washed with buffer. Sections of myometrium with blood vessels were used as positive control preparations; antibody was replaced by buffer in negative control preparations.

Only tissues with high-quality staining of the microvessel component were viewed. Light microscopy at low power (×40) was used to locate areas of tumor containing the most capillaries and small venules, areas of highest neovascularization, to be used for microvessel quantitation. A *microvessel* was defined as any brown-staining endothelial cell cluster separate from adjacent microvessels, tumor cells, and other connective tissue stromal elements. The presence of a vessel lumen was not necessary for a structure to be defined as a microvessel. A single ×200 field, 0.74 mm² area, was simultaneously counted by

Fig. 1. Kaplan-Meier actuarial survival curves for survivors past hospitalization. **A**, Overall survival; **B**, lung cancer-related mortality rate.

two investigators with a double-headed light microscope. Each individual count was recorded, and results were displayed as the mean of the two measurements. The investigators were unaware of the patient's clinical outcome.

Tumor vessel invasion. From the representative hematoxylin-eosin-stained sections, the presence of tumor cell emboli in endothelial-lined channels was determined by identification of intratumoral lymphatic vessel invasion and blood vessel invasion. Lymphatic vessel invasion was differentiated from blood vessel invasion according to previously reported morphologic characteristics.^{28, 29}

Statistical analysis. Univariate correlates of cancer-related mortality rate were found with the log-rank test, and univariate (unadjusted) Kaplan-Meier curves were derived. The multivariate (adjusted) correlates of cancer-

related mortality rate were found with the Cox proportional hazards regression, where the hazard of cancer-related death was modeled. The results were displayed in terms of hazard ratios, ratios of the hazard of cancer-related death for patients with and without a characteristic present, and the 95% confidence interval. All tests were two-tailed. All missing data were assumed to be missing at random. Spearman's rank correlation was used for evaluation of correlation between FVIII and CD31 manual counts. The S-Plus statistical software (MathSoft, Inc., Seattle, Wash.) was used for all analyses.

Results

Six patients were excluded from the original cohort found in the tumor registry because review of

Table II. Histopathologic features (n = 106)

Characteristic	No.	%
Histology of carcinoma		
Adenocarcinoma	63	59.4
Squamous cell	30	28.3
Bronchioalveolar	5	4.7
Large cell	5	4.7
Adenosquamous	3	2.8
Grade of differentiation		
Poor	35	33
Poor to moderate	9	8.5
Moderate	38	35.8
Moderate-to well	3	2.8
Well	11	10.4
Bronchioalveolar		
Serous	4	3.8
Mucinoid	1	0.9
Visceral pleural invasion		
Presence	20	19.2
Absence	84	80.8
Blood vessel invasion		
Presence	11	10.5
Absence	94	89.5
Lymphatic vessel invasion		
Presence	21	20.2
Absence	83	79.8

their medical records and histopathologic reports revealed evidence of disease beyond stage I. A total of 106 patients with stage I NSCLC were available for evaluation. This constitutes 26.6% (106/398) of all patients who underwent operation for primary lung carcinoma at our institution between 1985 and 1990. Operative mortality rate for the study population was 2.8% (3 of 106). Patient and operative demographic data are displayed in Table I.

Follow-up was complete for 95.1% (98/103) of the survivors past hospital stay for a median 5.5 years and as long as 11.1 years. Fig. 1 (A) displays the Kaplan-Meier survival curve for the 98 survivors past hospital stay. Overall survivals were 55.1% at 5 years and 28.7% at 10 years. Lung cancer-related mortality rate was 24.4% at 5 years after the operation, with no cancer-related deaths found after 6 years (Fig. 1, B). An additional three patients were found to be alive with evidence of recurrent or metastatic disease at follow-up.

Histopathologic data are presented in Table II. Degree of angiogenesis, according to microvessel quantitation, is shown in Table III. FVIII quantitation could not be performed for five of the 106 specimens because of the poor quality of immunostaining in those specimens (mean count 20.74 ± 11.2). Similarly, only 103 of the 106 samples could

Table III. Angiogenic activity

Manual counts	No.	%
FVIII		
<20	54	56.3
≥20	42	43.7
CD31		
<30	67	68.4
≥30	31	31.6

be evaluated for CD31 manual quantitation (mean count 29.6 ± 18.1). The range of manual counts of microvessels immunostained for CD31 was broader than for FVIII (8 to 125.5 and 3.5 to 57.5, respectively). Test results for replication reproducibility were strongly positive between the two observers for both FVIII and CD31 manual counts (interclass correlation coefficient 0.97 for each). However, FVIII and CD31 manual counts revealed only a slight positive correlation ($r = 0.19$; Fig. 2).

Univariate analysis revealed a mean FVIII count greater than 20 and the presence of blood vessel invasion to be significant predictors of disease-related death ($p = 0.025$ and $p = 0.017$, respectively; Fig. 3). CD31 manual quantitation, however, failed to predict tumor-related death ($p = 0.34$; Fig. 4). Tumor histology ($p = 0.41$), size greater than 3 cm ($p = 0.72$), grade ($p = 0.68$), presence of visceral pleural invasion ($p = 0.56$), and lymphatic vascular invasion ($p = 0.10$) were similarly not associated with diminished disease-related survival in this cohort. Multivariate Cox regression analysis considered all variables listed in Tables I through III as possibly predicting patient outcome. Tumor angiogenesis, as determined by FVIII count greater than 20 and the presence of blood vessel invasion, were the only significant independent prognostic markers of disease-specific death (Table IV).

Discussion

Despite complete surgical resection of localized stage I NSCLC, as many as 40% of patients have recurrent, typically distant disease and eventually die of lung cancer. A readily obtainable index for the risk of subsequent disease could conceivably define a patient population that could benefit from adjuvant therapy. Oncogenic abnormalities, deoxyribonucleic acid ploidy, cell replication indexes, and other molecular and pathologic tumor characteristics have all been identified as markers of aggressive disease and diminished survival among patients with primary lung carcinoma.¹⁸⁻²⁰ In addition, neovascu-

Fig. 2. Relationship of FVIII and CD31 manual counts.

Fig. 3. Kaplan-Meier survival curves describing freedom from lung cancer-related death. **A**, By FVIII manual quantitation; **B**, by blood vessel invasion (*BVI*).

Fig. 4. Kaplan-Meier curve describing freedom from lung cancer-related death by CD31 manual quantitation.

larization has been described as a critical component of tumor proliferation and metastasis, implicated in the outcome after treatment of a variety of solid tumors.⁷⁻¹²

In our analysis of 106 consecutive patients with stage I (T1 N0 M0 and T2 N0 M0) NSCLC, FVIII manual quantitation as a marker of tumor angiogenesis was revealed to be a significant independent predictor of lung cancer-related death. Blood vessel invasion was also an independent marker for disease-related death. These findings attest to the significance of the development of new capillary blood vessels and vascular invasion in solid tumor dissemination and metastasis. Other histologic variables reviewed, including tumor histology, grade, size, and visceral pleural invasion, were not associated with diminished late survival in these patients with stage I disease. Similarly, patient age, location of primary tumor, and operative procedure performed were not related to survival after resection.

The technique of immunostaining for CD31, rather than FVIII, as the microvessel marker for manual quantitation has recently been promoted on the perceived basis of enhanced sensitivity and specificity of endothelial cell staining.^{12, 27} FVIII is known to cross-react with megakaryocytes, platelets, and lymphatic endothelial cells. In an analysis of 103 patients with primary breast cancer, Horak and colleagues²⁷ describe the CD 31 monoclonal antibody as more specific than FVIII immunostaining

Table IV. Significant independent predictors of lung cancer-related death

Characteristic	Hazard ratio	95% Confidence Interval	p Value
FVIII count ≥ 20	2.9	1.2, 6.8	0.018
Blood vessel invasion	3.7	1.4, 10.1	0.011

for the determination of angiogenesis. They report, however, that CD31 is also expressed during myelomonocytic cellular differentiation, and consequently may cross-react with plasma cells, platelets, neutrophils, peripheral T cells, and mantle zone B cells. Use of CD31 monoclonal antibody immunostaining for assessment of lung cancer neovascularization has not been previously described. In our review, manual quantitation of CD31-stained microvessels proved cumbersome, with higher counts than for FVIII. This was most likely because of both the increased sensitivity to endothelial cells and the inability to discriminate possible cross-reaction with plasma cells, tumor cells, and fibroblasts. As such, there was a wide range of FVIII values for any given CD31 manual count, with only a weak correlation between the two techniques. Most significantly, angiogenesis as assessed by CD31 microvessel quantitation was not predictive of late death from lung cancer in this cohort.

The degree of tumor neovascularization and the

presence of blood vessel invasion were predictive of late disease-related death for patients with stage I NSCLC. Manual quantitation of microvessels after FVIII immunostaining but not CD31 immunostaining was a reliable marker of angiogenesis as it related to late outcome. Tumor angiogenesis may serve to mark patients at risk of recurrent disease after complete surgical resection and should be considered in future prospective studies selecting patients for adjuvant therapy.

REFERENCES

- Hart IR, Saini A. Biology of tumor metastasis. *Lancet* 1992;339:1453-7.
- Weidner N. Tumor angiogenesis: review of current applications in tumor prognostication. *Semin Diagn Pathol* 1993;10:302-13.
- Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990;82:4-6.
- Teicher BA. Angiogenesis and cancer metastases: therapeutic approaches. *Crit Rev Oncol Hematol* 1995;20:9-39.
- Dorudi S, Hart IR. Mechanisms underlying invasion and metastasis. *Curr Opin Oncol* 1993;5:130-5.
- Liotta LA. Cancer cell invasion and metastasis. *Sci Am* 1992;Feb;54-63.
- Srivastava A, Laidler P, Davies RP, Horgan K, Hughes LE. The prognostic significance of tumor vascularity in intermediate-thickness (0.76-4.0 mm thick) skin melanoma: a quantitative histologic study. *Am J Pathol* 1988;133:419-23.
- Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Engl J Med* 1991;324:1-8.
- Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993;143:401-9.
- Frank RE, Saclarides TJ, Leurgans S, Speziale NJ, Drab EA, Rubin DB. Tumor angiogenesis as a predictor of recurrence and survival in patients with node-negative colon cancer. *Ann Surg* 1995;222:695-9.
- Williams JK, Carlson GW, Cohen C, DeRose PB, Hunter S, Jurkiewicz. Tumor angiogenesis as a prognostic factor in oral cavity tumors. *Am J Surg* 1994;168:373-80.
- Gaspmini G, Weidner N, Maluta S, Pozza S, Boracchi P, Mezzetti, et al. Intratumoral microvessel density and p53 protein: correlation with metastasis in head-and-neck squamous cell carcinoma. *Int J Cancer* 1993;55:739-44.
- Mountain CF. A new international staging system for lung cancer. *Chest* 1986;89(suppl):225S-33S.
- Harpole DH Jr, Herndon JE 2nd, Young WG, Wolfe WG, Sabiston DC. Stage I nonsmall cell lung cancer: a multivariate analysis of treatment methods and patterns of recurrence. *Cancer* 1995;76:787-96.
- Nesbitt JC, Putnam JB, Walsh GL, Roth JA, Mountain CF. Survival in early-stage non-small cell lung cancer. *Ann Thorac Surg* 1995;60:466-72.
- Ginsberg RJ, Rubinstein LV. Randomized trial of lobectomy versus limited resection for T1 N0 non-small cell lung cancer. Lung Cancer Study Group. *Ann Thorac Surg* 1995;60:615-23.
- Martini N, Bains MS, Butt ME, Zakowski MF, McCormack P, Rusch VW, et al. Incidence of local recurrence and second primary tumors in resected stage I lung cancer. *J Thorac Cardiovasc Surg* 1995;109:120-9.
- Smit EF, Groen JM, Splinter TAW, Ebels T, Postmus PE. New prognostic factors in resectable non-small cell lung cancer. *Thorax* 1996;51:638-46.
- Mountain CF. New prognostic factors in lung cancer: biologic prophets of cancer cell aggression. *Chest* 1995;108:246-54.
- Van Zandwijk N, Mooi WJ, Rodenhuis S. Prognostic factors in NSCLC: recent experiences. *Lung Cancer* 1995;12:527-33.
- Macchiarini P, Fontanini G, Hardin MJ, Chuanchieh H, Bigini D, Vignati S, et al. Blood vessel invasion by tumor cells predicts recurrence in completely resected T1 N0 M0 non-small-cell lung cancer. *J Thorac Cardiovasc Surg* 1993;106:80-9.
- Macchiarini P, Fontanini G, Hardin MJ, Squartini F, Angeletti CA. Relation of neovascularisation to metastasis of non-small-cell lung cancer. *Lancet* 1992;340:145-6.
- Fontanini G, Bigini D, Vignati S, Basolo F, Mussi A, Lucchi M, et al. Microvessel count predicts metastatic disease and survival in non-small cell lung cancer. *J Pathol* 1995;177:57-63.
- Macchiarini P, Fontanini G, Dulmet E, de Montpreville V, Chapelier AR, Cerrina J, et al. Angiogenesis: an indicator of metastasis in non-small cell lung cancer invading the thoracic inlet. *Ann Thorac Surg* 1994;57:1534-9.
- Yarnazaki K, Abe S, Takekawa H, Sukoh N, Watanabe N, Ogura S, et al. Tumor angiogenesis in human lung adenocarcinoma. *Cancer* 1994;74:2245-50.
- Harpole DH, Richards WG, Herndon JE 2nd, Sugarbaker DJ. Angiogenesis and molecular biologic substaging in patients with stage I non-small cell lung cancer. *Ann Thorac Surg* 1996;61:1470-6.
- Horak ER, Leek R, Klenk N, LeJeune S, Smith K, Stuart N, et al. Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. *Lancet* 1992;340:1120-4.
- Rosen PP. Tumor emboli in intramammary lymphatics in breast cancer: pathologic criteria for diagnostic and clinical significance. *Pathol Annu* 1983;18:215-32.
- Weigand RA, Isenberg WM, Russo J, Brennan MJ, Rich MA. Blood vessel invasion and axillary lymph node involvement as prognostic indicators for human breast cancer. *Cancer* 1982;50:962-9.

Discussion

Dr. E. Carmack Holmes (*Los Angeles, Calif.*). The Emory group has looked at the prognostic significance of tumor angiogenesis in 106 patients with resected stage I lung cancer, and they have used two monoclonal antibodies directed against FVIII and CD31, both of which have specificity for endothelial cells. These two antibodies can be used to quantitate angiogenesis, and this article concludes that angiogenesis is the strongest independent indicator of cancer-free survival in patients with resected stage I lung cancer.

Patients with stage I lung cancer that stained positively with FVIII antibody had a cancer-free survival of 66%, and those with negative stain results had a survival of 83%. That is almost a 20% higher survival in marker-negative patients.

Now, evidence is rapidly accumulating that molecular markers may replace standard histopathology in predict-

ing survival. This article is the sixth in recent years to show a strong survival correlation with immunostaining for angiogenesis. If you add other prognostic molecular markers, such as the proto-oncogene *HER-2/neu* and the tumor suppressor gene *p53*, then the prognostic power is greatly enhanced.

In a recent article by Harpole and coworkers, they showed that when none of the markers that I mentioned before in addition to angiogenesis were positive, survival in stage I was 80%; when all four molecular markers were positive, survival dropped to 50%. This is a full 30% difference in the marker-negative patients, and the marker-positive patients probably should receive adjuvant therapy. Indeed, it is becoming quite clear that measurements of tissue molecular markers will more directly assess the aggressiveness of a tumor than will standard histologic examinations.

Dr. Duarte, have you looked at other molecular markers? This certainly will enhance the power of the predictability of the molecular markers.

Also, I am perplexed as to the reason that the CD31 antibody did not correlate and the staining was unreliable. You referred to this in your presentation and proposed certain reasons why this was the case, but I might suggest another, and perhaps you would comment on it. As you know, Horak and colleagues showed a high correlation with metastatic disease with the CD31 antibody, at least in breast cancer. I suggest that some techniques different between your study and their study might explain the differences in your findings. First, the antibody concentrations in your study and their study were different; also, the retrieval method, that is, pepsin in your study and protease type 24 in theirs, may well have contributed to the differences in the two studies.

Finally, I would like to make a comment. I think this article would be greatly enhanced, Dr. Duarte, if there were a more vigorous discussion comparing your study and its results with previous publications on the same subject.

Dr. Ali Nur Rahman (*Manchester, England*). We are naturally interested in this important piece of work, because England harbors one of the highest incidences of cancer in the world.

I am just wondering about the mechanism of reduced survival in these cases. If it is by angiogenesis, has it already metastasized? If so, has there been any evidence of magnetic resonance imaging scanning, even though the renal glands, I assume, were negative? Is there any way of indicating whether it has already metastasized? How do these patients die in the end, from a second disease after the tumor has been removed, I assume, completely?

Dr. Mark K. Ferguson (*Chicago, Ill.*). Have you analyzed whether there is a correlation between FVIII staining and blood vessel invasion?

Dr. Duarte. I will try to answer Dr. Holmes' questions in order. With respect to whether we have looked at other markers, at this time we have not. Indeed, he referred to Harpole and the group from Boston, where *p53* abnormalities and *c-erbB2* proto-oncogene overexpression, as well as angiogenesis, were found to be significant inde-

pendent predictors. We have not done that at this time. It is something we are considering doing in subsequent studies, perhaps including it in a model of selecting patients who may benefit from adjuvant therapy.

With respect to the CD31 antibody, we found differences in our technique of immunostaining and of tissue digestion from those of Horak and coworkers, who first described using CD31 monoclonal antibody in patients with breast cancer. As I have shown, however, we were able to have effective, adequate immunostaining in 103 of the 106 lung cancer tissue specimens with monoclonal CD31. Perhaps some degree of the differences in results can be attributed to protein digestion differences and dilutional differences in the use of this antibody, but I suspect not.

In addition, Horak and coworkers reported in that article that CD31 is considered more sensitive for endothelial cells. Noel Weidner and the group from Boston, however, have reported (as does the antibody manufacturing sheet that comes with it) that it also stains megakaryocytes, platelets, tumor cells, fibroblasts, and particularly plasma cells. In doing the actual manual quantitation, it was sometimes difficult to discriminate which were plasma cells, fibroblasts, and so on, versus tumor cells stained. As a measure of reproducibility between the two observers, the interclass correlation coefficient was 0.97. So, despite this cross-reaction, the two observers were counting the same immunostains as a vessel or as some other cross-reactivity cell. I suspect, though, that the failure of CD31 to show significance here may have been caused primarily by that cross-reaction.

Last, with respect to CD31, personal communications from other individuals, particularly those in the department of pathology that have used CD31 specifically in lung cancers, have also noted failure to show a significant positive relationship between CD31 and survival. In fact, our pathologists at Emory recently reported an inverse relationship, actually, with image cytometry rather than manual quantitation.

With respect to the mechanism of angiogenesis raised by the other discussant, angiogenesis is considered one of the earlier phases of tumor development and growth. It precedes the development of metastasis. It is the process by which the tumor induces blood vessels to form to it, typically from the parenchymal capillaries and postcapillary venules. Different modalities, rather than simple histologic analysis of nodes from the intraoperative dissection, have been used to try to identify positive nodal metastases in patients by means of particular immunostains and antibodies. The association between microvessel quantitation, angiogenesis, and nodal metastasis or systemic micrometastasis with these sensitive techniques has not yet been evaluated. In all of these cases, the nodes that were all reexamined were all negative for cancer.

With respect to Dr. Ferguson's question, there was no significant association between FVIII counts and the presence of blood vessel invasion, nor with the presence of lymphatic vessel invasion, tumor size, or T status. It was truly an independent predictor of outcome.