Evaluation of Epidermal Growth Factor-related Growth Factors and Receptors and of Neoangiogenesis in Completely Resected Stage I-IIIA Non-Small-Cell Lung Cancer: Amphiregulin and Microvessel Count Are Independent Prognostic Indicators of Survival[1]

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ABSTRACT

We have determined the expression of transforming growth factor α (TGFα), amphiregulin (AR), CRIPTO, the epidermal growth factor receptor (EGFR), *erb*B-2, *erb*B-3, and tumor angiogenesis in a series of 195 patients with stage I–IIIA non-small cell lung cancer (NSCLC) treated with radical surgery to define their usefulness as prognostic indicators of survival. A variable degree of specific staining in cancer cells was observed for the three growth factors and for the three growth factor receptors in the majority of NSCLC patients. A statistically significant association between overexpression of TGFα, AR, and CRIPTO was observed. Enhanced expression of AR was significantly correlated with enhanced expression of *erb*B-2 and advanced T-stage. A direct association was also detected for overexpression of TGFα and of *erb*B-2 or *erb*B-3, respectively. Sex, tumor size, nodal status, stage, microvessel count, as a measure of neovascularization, and AR overexpression significantly correlated with overall survival at univariate analysis. In a Cox multivariate analysis, the only characteristics with an independent prognostic effect on OAS were microvessel count [relative hazard (RH), 6.61; $P < 0.00001$), nodal status (RH, 1.59; $P = 0.0013$), and AR overexpression (RH, 1.72; $P = 0.02$). These results suggest that evaluation of neoangiogenesis and of certain growth factors, such as AR, can be useful in addition to conventional pathological staging to select high-risk NSCLC patients who may benefit from postsurgical systemic therapies.

INTRODUCTION

Complete surgical resection is the only potentially curative treatment for NSCLC[3] patients. Pathological staging of NSCLC represents the most accurate evaluation currently available of clinical outcome in patients who have undergone surgical resection. However, the survival of NSCLC patients with early stage operable disease is not satisfactory. Approximately 30–40% of pathological stage I patients have disease recurrence and eventually die following apparently curative surgery. To improve the results of surgery, systemic adjuvant chemotherapy following radical surgery has been proposed in NSCLC patients. However, the impact of adjuvant systemic therapies on survival is not completely defined (1). In this respect, the identification of novel pathological and/or biological prognostic variables in addition to disease stage could allow the selection of high-risk NSCLC patients who may benefit of adjuvant systemic postsurgical treatment (2).

The potential prognostic role of several biological parameters, including oncogenes, such as Ki-*ras* (3–5), tumor suppressor genes, such as p53 (6–9), and cancer cell proliferative activity (10–13), has been investigated in NSCLC. However, conflicting results on the independent prognostic role of these variables have been reported (2). Peptide growth factors are involved in regulating normal epithelial cell proliferation and differentiation (14). EGF and structurally related growth factors, such as TGFα and AR, are potent mitogens for several human

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; MVC, microvessel count; EGF, epidermal growth factor; EGFR, EGF receptor; TGFα, transforming growth factor α; AR, amphiregulin; PCNA, proliferating cell nuclear antigen; RH, relative hazard; CI, confidence interval; OAS, overall survival.

epithelial cell types, including breast, colon, ovary, and lung, and have been implicated in human cancer development and progression through autocrine and paracrine pathways (15). EGF, TGFα, and AR bind to EGFR (15). It has been recently shown that EGFR dimerization or heterodimerization with either *erb*B-2, *erb*B-3, or *erb*B-4, which are EGFR-related growth factor receptors, is needed for intracellular signal transduction induced by these ligands (16). Although structurally related to EGF-like peptides, CRIPTO is a growth factor that acts through a not yet identified cell membrane receptor (15). CRIPTO is overexpressed in human colorectal and breast cancers as compared to normal epithelium and is an autocrine growth factor for colon and breast cancer cells (17–22). EGFR or *erb*B-2 overexpression has been correlated with a worse prognosis in NSCLC patients, although their independent prognostic role is still controversial (23–27). However, no extensive study on the frequency of expression and on the prognostic value of the various EGF-like growth factors and of their specific receptors has been performed in NSCLC.

Several peptide growth factors that are locally secreted by tumor cells, including the EGF-like growth factors, also regulate cancer development and progression by inducing tumor neovascularization through the paracrine stimulation of normal endothelial cell proliferation (28). In this respect, the formation of novel blood vessels is an important step in cancer development and progression because it is essential for providing adequate oxygen and nutrient supply to the growing tumor mass and for initiating metastatic spreading (29). Furthermore, the count of microvessels in the most intense areas of neovascularization has been suggested as a prognostic marker in patients with different human cancers, including malignant melanoma, breast, head and neck, prostatic, ovarian, gastric, and lung carcinomas (29–37).

In the present study, we evaluated the expression of TGFα, AR, CRIPTO, EGFR, *erb*B-2, and *erb*B-3 and the intensity of tumor angiogenesis in a series of 195 stage I–IIIA NSCLC patients treated with radical surgery without any pre- or postsurgical local or systemic therapy in an attempt to define their usefulness as prognosticators of survival in operable NSCLC.

PATIENTS AND METHODS

Patients and Tumor Tissues. One hundred ninety-five consecutive NSCLC patients who had undergone curative surgical resection at the Department of Surgery, University of Pisa, from March 1991 to December 1994 were studied. There were 175 males and 20 females with a median age of 64 years (range, 41–80 years). As of August 31, 1996, the median follow-up period for living patients was 36 months (range, 19–64 months). The patients presented no detectable metastases in distant organs at the moment of surgery. They had not received either chemotherapy or radiation therapy before surgery. No adjuvant treatment was given following radical surgery. Survival was calculated as the period from surgery to death. Tumors specimens obtained at surgery were formalin-fixed and paraffin-embedded for histological and immunohistochemical processing. Tumors were classified according to the WHO histological classification (38) and following the guidelines of the American Joint Committee on Cancer Staging.

Immunohistochemistry and Evaluation of Immunoperoxidase Staining. Formalin-fixed, paraffin-embedded tissue sections (5 μm) were deparaffinized in xylene and rehydrated in a graded series of ethanol. The slides were then treated for 30 min a 20°C with methanol containing 0.3% hydrogen peroxide to block any endogenous peroxidase activity. After several washes with PBS, the sections were blocked for 45 min with 10% goat serum or with 10% horse serum, washed with PBS, and incubated overnight with the appropriate primary antibody at 4°C. Sections were then washed three times with PBS and treated with an appropriate secondary biotinylated goat antibody (1:200 dilution, Vectastain ABC kit, Vector Laboratories, Burlingame, CA) for 30 min, as previously reported (23). Following several washes with PBS, the slides were reacted for 30 min with avidin-biotinylated horseradish peroxidase H complex, rinsed twice in PBS, and incubated for 2 min in 0.05% diaminobenzidine and in 0.01% hydrogen peroxide as described previously (23). The slides were then rinsed in distilled water, counterstained with hematoxilin, and mounted. The following antibodies were used in this study. An anti-CRIPTO rabbit antiserum, CR-67, was raised against a 17-mer peptide corresponding to amino acid residues 97–113 of the human CRIPTO protein, which represent the COOH terminus of the 37 amino acid EGF-like region, and was used at a 1:400 dilution, as described previously (22). An anti-AR rabbit antiserum, AR56, was generated against a 17-mer peptide corresponding to amino acid residues 159–175 of the rat AR protein and was used at a 1:200 dilution (22). An anti-TGFα mouse monoclonal antibody that was generated using human recombinant TGFα as an immunogen (Ab-2, Oncogene Science, Manhasset, NY) was used at a 1:100 dilution. Each antibody was specific for TGFα, AR, or CRIPTO and did not cross-react with the other two EGF-related peptides (22). An anti-*erb*B-2 rabbit antiserum that was raised against a synthetic peptide (21N) representing residues 1243–1255 of the predicted protein sequence was used at a 1:200 dilution (22). An anti-EGFR mouse monoclonal antibody that was generated using human EGFR as an immunogen (Triton, Alameda, CA) was used at a 1:100 dilution (23). An anti-*erb*B-3 mouse monoclonal antibody, RTJ2, was raised against a synthetic peptide corresponding to a portion of the cytoplasmic domain of the human c-*erb*B-3 protein and was used at a 1:200 dilution, as described previously (39). Each antibody was specific for EGFR, *erb*B-2, or *erb*B-3 and did not cross-react with the other two related receptors (22, 23, 39). An anti-CD34 monoclonal antibody (clone QB-END10, Novocastra, Newcastle, United Kingdom) was used for microvessel staining at a 1:100 dilution (40). For evaluation of tumor cell proliferative activity, the anti-PCNA PC10 mouse monoclonal antibody (Novocastra) was used at a 1:200 dilution. Nonspecific staining was evaluated for each specimen using either a similar concentration of preimmune rabbit serum or IgG, or by adsorbing the primary antibody with the appropriate immunogenic peptide. To determine the percentage of positive cells, at least 1000 cancer cells per slide were counted and scored. For MVC determination, a single microvessel was defined as any brown immunostained endothelial cell separated from adjacent microvessels, tumor cells, and mesenchymal cells. Each sample was examined under low power (×10 objective lens and ×10 ocular lens) to identify the three regions of the section with the

highest number of microvessels. A ×250 field (×25 objective lens and ×10 ocular lens; 0.74 mm^2 per field) in each of these three areas was counted, and the average counts of the three fields were recorded. Large vessels with thick muscular walls were excluded from the counts.

Statistical Analysis. The association between the different pathological and biological characteristics was studied by the use of contingency tables. Statistical significance was evaluated by the Pearson χ^2 test. Univariate survival analysis for the role of each prognostic variable on survival was estimated according to the Kaplan-Meier product-limit method (41). The statistical significance of the differences in survival distribution among prognostic groups was evaluated by the log-rank test (42). For multicategorical ordinal variables, a test for trend was used. For TGFα, AR, CRIPTO, EGFR, *erb*B-2, *erb*B-3, and PCNA, the cutoff values in the series of 195 NSCLC patients were used to distinguish between low- and high-expressing tumors. MVC was considered either as a dichotomous variable, using the median value of 20 microvessels in the series of 195 NSCLC patients as a cutoff or using five groups of increasing microvessel number/sample (1–10, 11–20, 21–30, 31–40, and 41–100), Multivariate analysis was carried out by the Cox proportional hazards regression model (43). The Cox model was used to determine among the clinicopathological and biological characteristics that had significantly affected survival at the univariate analysis, such as stage, T status, nodal status, MVC, and AR, those with an independent prognostic role. Covariate selection was performed by a stepwise procedure using a maximum likelihood ratio test for backward elimination. MVC was introduced into this model as a continuous variable after a log-transformation due to its skewed distribution. For covariates retained into the final model, RHs with 95% CIs were estimated. All *P*s represent two-sided tests of statistical significance. *P*s ≤ 0.05 were considered statistically significant. All analyses were performed with the BMDP New System statistical package, version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA).

RESULTS

Clinicopathological Characteristics. One hundred ninety-five NSCLC patients (175 males and 20 females) with a median age of 64 years (range, 41–80) were studied. There were 116 patients with squamous cell carcinoma (59.5%), 66 patients with adenocarcinoma (33.8%), and 13 patients with large cell anaplastic carcinoma (6.7%). According to the degree of differentiation, 44 (22.6%) were well differentiated (G1) cancers, 77 (39.5%) were moderately differentiated (G2) cancers, and 74 (37.9%) were poorly differentiated (G3) carcinomas. Forty-two (21.5%) tumors were classified as T1, 132 (67.7%) as T2, and 21 (10.8%) as T3. Metastatic involvement of hilar lymph nodes (N1) was present in 30 (15.4%) patients, whereas mediastinal lymph nodes (N2) were involved in 40 (20.5%) patients. Absence of lymph node metastatic involvement (N0) was observed in 125 (64.1%) patients. The majority of the cases was classified as stage I (115, 59%), whereas 25 (12.8%) cases were stage II, and 55 (28.2%) cases were stage IIIA. At the time of analysis, 122 (62.6%) patients were alive and 73 (37.4%) patients were dead.

Table 1 Expression of TGFα, AR, CRIPTO, EGFR, *erb*B-2, and *erb*B-3 in 195 NSCLC patients

Immunohistochemical evaluation was performed as described in "Patients and Methods." Samples were considered positive when specific immunostaining was detected in ≥5% cancer cells.

	Positive/total cases (%)	% positive tumor cells, median (range)
TGFα	193/195 (99)	65 (0–80)
AR	152/195 (77.9)	35 (0–75)
CRIPTO	178/195 (91.3)	50 (0–90)
EGFR	158/195 (81)	45 (0–90)
*erb*B-2	119/195 (61)	25 (0–80)
*erb*B-3	166/195 (85.1)	45 (0–80)

Expression of Growth Factors, Growth Factor Receptors, and Measurement of Neovascularization. TGFα, AR, CRIPTO, EGFR, *erb*B-2, and *erb*B-3 expression was evaluated by immunohistochemistry in the series of 195 NSCLC patients. As shown in Table 1, the majority of lung cancer samples expressed specific immunostaining for the three EGF-like growth factors and for the three *erb*B-related growth factor receptors. The intensity of staining within a single cancer specimen was heterogeneous for all proteins, ranging from 0 to 75–90% of the cancer cells. Immunoreactivity was limited to cancer cells because there was very little or no specific staining in the surrounding stroma or in capillary endothelial cells (data not shown). We next evaluated whether any correlation could exist between growth factor or growth factor receptor expression in cancer specimens and the clinicopathological characteristics of the 195 NSCLC patients. As illustrated in Table 2, a statistically significant direct association between overexpression of TGFα, AR, and CRIPTO was observed. Furthermore, enhanced expression of AR was significantly correlated with enhanced expression of *erb*B-2 and advanced T stage. A direct association was also detected for overexpression of TGFα and *erb*B-2 or *erb*B-3, respectively. A higher frequency of CRIPTO overexpression was found in adenocarcinomas, whereas EGFR-overexpressing tumors were significantly associated with the squamous cell carcinoma histological subtype.

MVC, as an indicator of neovascularization within the primary tumor, was determined by immunohistochemical staining of endothelial vessels using an anti-CD34 monoclonal antibody (40). Heterogeneous MVC distribution ranging between 3 and 100 microvessels/tumor sample with a median of 20 was observed in the NSCLC patients. Higher MVC was significantly associated with lymph nodal involvement ($P = 0.0004$) but not with any other clinicopathological or biological variable analyzed (data not shown).

Univariate Analysis of Association of Clinicopathological and Biological Characteristics with Survival. The prognostic impact of clinicopathological and biological parameters on patient survival was evaluated by univariate analysis. As shown in Table 3 and Fig. 1, female sex ($P = 0.032$), greater tumor size ($P = 0.008$), nodal status ($P < 0.00001$), and advanced stage ($P < 0.00001$) were significantly associated with a worse OAS. MVC, analyzed either as a dichotomous variable using the median value of 20 microvessels as a cutoff or as five groups of increasing microvessel number/

Table 2 Association of TGFα, AR, CRIPTO, EGFR, *erb*B-2, and *erb*B-3 expression with clinicopathological and biological characteristics

Associations between pairs of parameters were evaluated using contingency tables and were analyzed for statistical significance with the Pearson's χ^2 test. The median values of MVC, TGFα, AR, CRIPTO, EGFR, *erb*B-2, and *erb*B-3 were used as cutoffs to discriminate between high- and low-expressing tumors. Data are presented as percentage of high-expressing tumors for TGFα, AR, CRIPTO, EGFR, *erb*B-2, and *erb*B-3 within each clinicopathological or biological parameter. *P*s ≤ 0.05 were considered as statistically significant.

	n[a]	TGFα	AR	CRIPTO	EGFR	*erb*B-2	*erb*B-3	MVC
Tumor status								
T1	42	57.1%	26.2%	42.8%	40.5%	50%	50%	40.5%
T2	132	43.2%	52.3%	41.7%	50.8%	50%	47.7%	48.5%
T3	21	42.8%	47.6%	47.6%	38.1%	52.4%	57.1%	61.9%
		P = 0.27	*P* = 0.013	*P* = 0.88	*P* = 0.34	*P* = 0.98	*P* = 0.72	*P* = 0.27
Node status								
N0	125	48%	42.4	40.8%	41.6%	50.4%	51.2%	37.6%
N1	30	40%	53.3	50%	60%	56.7%	53.3%	70%
N2	40	45%	52.5	42.5%	55%	45%	40%	65%
		P = 0.72	*P* = 0.37	*P* = 0.66	*P* = 0.10	*P* = 0.63	*P* = 0.41	*P* = 0.0004
Histology								
Squamous	116	42.2%	39.6%	36.2%	56.9%	49.1%	52.6%	43.1%
Adenocarcinoma	66	53%	57.6%	57.6%	34.8%	54.5%	40.9%	54.5%
Anaplastic	13	46.1%	46.1%	23.1%	23.1%	38.5%	61.5%	61.5%
		P = 0.37	*P* = 0.07	*P* = 0.007	*P* = 0.003	*P* = 0.53	*P* = 0.21	*P* = 0.20
Grading								
G1	44	47.7%	45.5%	45.5%	50%	50%	50%	45.5%
G2	77	51.9%	48%	44.1%	49.4%	55.8%	50.6%	46.7%
G3	74	39.2%	44.6%	39.2%	43.2%	44.6%	47.3%	51.4%
		P = 0.28	*P* = 0.91	*P* = 0.75	*P* = 0.69	*P* = 0.38	*P* = 0.91	*P* = 0.78
MVC								
≤20	101	47.5%	46.5%	42.6%	47.5%	51.5%	48.5%	
>20	94	44.7%	45.7%	42.5%	46.8%	48.9%	50%	
		P = 0.68	*P* = 0.91	*P* = 0.99	*P* = 0.92	*P* = 0.72	*P* = 0.83	
TGFα								
≤65	105		36.2%	34.3%	47.6%	41.9%	41.9%	49.5%
>65	90		57.8%	52.2%	46.7%	60%	57.8%	46.7%
			P = 0.003	*P* = 0.012	*P* = 0.89	*P* = 0.012	*P* = 0.027	*P* = 0.69
AR								
≤35	105	36.2%		29.5%	46.7%	40.9%	45.7%	48.6%
>35	90	57.8%		57.8%	47.8%	61.1%	53.3%	47.8%
		P = 0.003		*P* = 0.0001	*P* = 0.88	*P* = 0.005	*P* = 0.29	*P* = 0.91
CRIPTO								
≤50	112	38.4%	33.9%		49.1%	51.8%	51.8%	48.2%
>50	83	56.6%	62.6%		44.6%	48.2%	45.8%	48.2%
		P = 0.012	*P* = 0.0001		*P* = 0.53	*P* = 0.62	*P* = 0.41	*P* = 0.99
EGFR								
≤45	103	46.6%	45.6%	44.7%		54.4%	46.6%	48.5%
>45	92	45.6%	46.7%	40.2%		45.6%	52.2%	47.8%
		P = 0.89	*P* = 0.88	*P* = 0.53		*P* = 0.22	*P* = 0.44	*P* = 0.92
*erb*B-2								
≤25	97	37.1%	36.1%	44.3%	51.5%		43.3%	49.5%
>25	98	55.1%	56.1%	40.8%	40.8%		55.15	46.9%
		P = 0.012	*P* = 0.005	*P* = 0.62	*P* = 0.22		*P* = 0.09	*P* = 0.72
*erb*B-3								
≤45	99	38.4%	42.4%	45.4%	44.4%	44.4%		47.5%
>45	96	54.2%	50%	39.6%	50%	56.2%		48.5%
		P = 0.027	*P* = 0.29	*P* = 0.41	*P* = 0.44	*P* = 0.09		*P* = 0.83

[a] No. of patients.

sample (1–10, 11–20, 21–30, 31–40, and 41–100), was a highly significant predictor of OAS ($P < 0.00001$; Table 3 and Fig. 1, *C* and *D*). Age, histological sybtype, histological grading, and tumor proliferative activity, assessed by PCNA nuclear staining, were not able to predict disease outcome (Table 3). Among the EGF-like growth factors and the *erb*B-related receptors, AR overexpression was the only parameter significantly associated with a worse OAS ($P = 0.01$; Table 3 and Fig. 1*E*).

Multivariate Analysis of Association of Clinicopathological and Biological Characteristics with Survival. A multivariate analysis was performed to define the variables with independent prognostic value with respect to survival. The clinicopathological parameters that were associated with OAS at univariate analysis, such as stage, T status, nodal status, MVC, and AR, were introduced in a Cox proportional hazards regression analysis. Using a backward stepwise procedure, covariates without independent prognostic significance were eliminated

Table 3 Univariate analysis for OAS

The five categories for MVC are as follows: 1, 3–10; 2, 11–20; 3, 21–30; 4, 31–40; 5, 41–100 microvessels tumor sample. For TGFα, AR, CRIPTO, EGFR, *erb*B-2, *erb*B-3, and PCNA, the cutoff values in the 195 NSCLC patients series were used to distinguish between low- and high-expressing tumors. The statistical significance of the differences in survival distribution among prognostic groups was evaluated by the log-rank test. For multicategorical ordinal variables a test for trend was used. *P*s ≤ 0.05 were considered statistically significant.

	Cases	Observed/expected deaths	*P*
Sex			
Male	175	0.98	0.032
Female	20	1.88	
Age			
≤64 years	109	0.91	0.39
>64 years	86	1.11	
Tumor status			
T1	42	0.48	0.008[a]
T2	132	1.15	
T3	21	1.43	
Node status			
N0	125	0.67	<0.00001[a]
N1	30	1.50	
N2	40	2.16	
Stage			
I	115	0.66	<0.00001[a]
II	25	1.36	
IIIA	55	1.81	
Histology			
Squamous	116	0.85	0.19
Adenocarcinoma	66	1.28	
Anaplastic	13	1.02	
Grading			
G1	44	0.99	0.78
G2	77	0.92	
G3	74	1.10	
MVC			
≤20	101	0.46	<0.00001
>20	94	1.79	
MVC			
1	38	0.55	<0.00001[a]
2	63	0.41	
3	38	1.35	
4	40	2.20	
5	16	2.03	
TGFα			
≤65	105	0.97	0.8
>65	90	1.03	
AR			
≤35	105	0.74	0.01
>35	90	1.33	
CRIPTO			
≤50	112	0.84	0.14
>50	83	1.19	
EGFR			
≤45	103	0.97	0.80
>45	92	1.03	
*erb*B-2			
≤25	97	0.94	0.62
>25	98	1.06	
*erb*B-3			
≤45	99	0.91	0.43
>45	96	1.09	
PCNA			
≤35	106	1.13	0.22
>35	89	0.84	

[a] Test for trend.

from the model. Nodal status, MVC, and AR were the only characteristics to retain an independent prognostic influence on OAS (Table 4), with MVC having the strongest impact (RH, 6.61; $P < 0.0001$) as compared to nodal status (RH, 1.59; $P = 0.0013$) or to AR (RH, 1.72; $P = 0.02$).

DISCUSSION

Growth factors can regulate cancer development and progression through several mechanisms. These include autonomous uncontrolled growth as a result of the autocrine production of endogenous growth factors that activate specific receptors localized on cancer cell membrane and induction of tumor vascularization due to paracrine stimulation of normal host endothelial cells by angiogenic growth factors secreted by cancer cells. The EGF family of growth factors, including EGF, TGFα, AR, and CRIPTO, plays an important role in human breast and colorectal cancer (15). Experimental evidence has been provided that TGFα and AR, through the activation of EGFR, function as autocrine growth factors also for both normal and malignant human bronchial epithelial cells (44). Moreover, EGFR expression is generally low in normal bronchial epithelium, whereas it is enhanced in preneoplastic and neoplastic bronchial lesions, and therefore, it has been proposed as an early marker of neoplastic transformation (45). The potential prognostic role of TGFα, AR, and EGFR and of the closely related *erb*B-2 growth factor receptor has been investigated in human NSCLC in preliminary pilot studies (15). However, no conclusive information on the prognostic impact of these biological parameters has been obtained thus far. Furthermore, no data are yet available on the expression of the EGF-like growth factor CRIPTO and of the EGFR-related *erb*B-3 growth factor receptor in human NSCLC.

To our knowledge, this is the first extensive immunohistochemical analysis of the expression of TGFα, AR, CRIPTO, EGFR, *erb*B-2, and *erb*B-3 in a large group of stage I–IIIA completely resected NSCLC patients for which survival data and several clinicopathological and biological parameters, including evaluation of tumor proliferative activity and tumor angiogenesis, were evaluated. In this study, we found that the majority of NSCLC samples expressed relatively high levels of the three EGF-like growth factors and of the three EGFR-related cell membrane receptors. Furthermore, a statistically significant direct correlation between overexpression of TGFα, AR, and CRIPTO and between overexpression of TGFα and *erb*B-2 or *erb*B-3 was observed in this series of patients. These results are similar to those previously reported for human breast and colorectal cancer (18–19, 22). Among these biological parameters, enhanced AR expression was the only variable to be significantly correlated with a reduced OAS in the series of 195 stage I–IIIA NSCLC patients at a univariate analysis. The other clinicopathological and biological characteristics that correlated with survival at univariate analysis were sex, postsurgical staging, tumor size, metastatic lymph node involvement, and MVC, as a measure of tumor angiogenesis. More importantly, only nodal status, MVC, and AR overexpression were shown to be as independent prognostic indicators of OAS.

These results are in support of the clinical relevance of measuring tumor-induced neovascularization in NSCLC. We

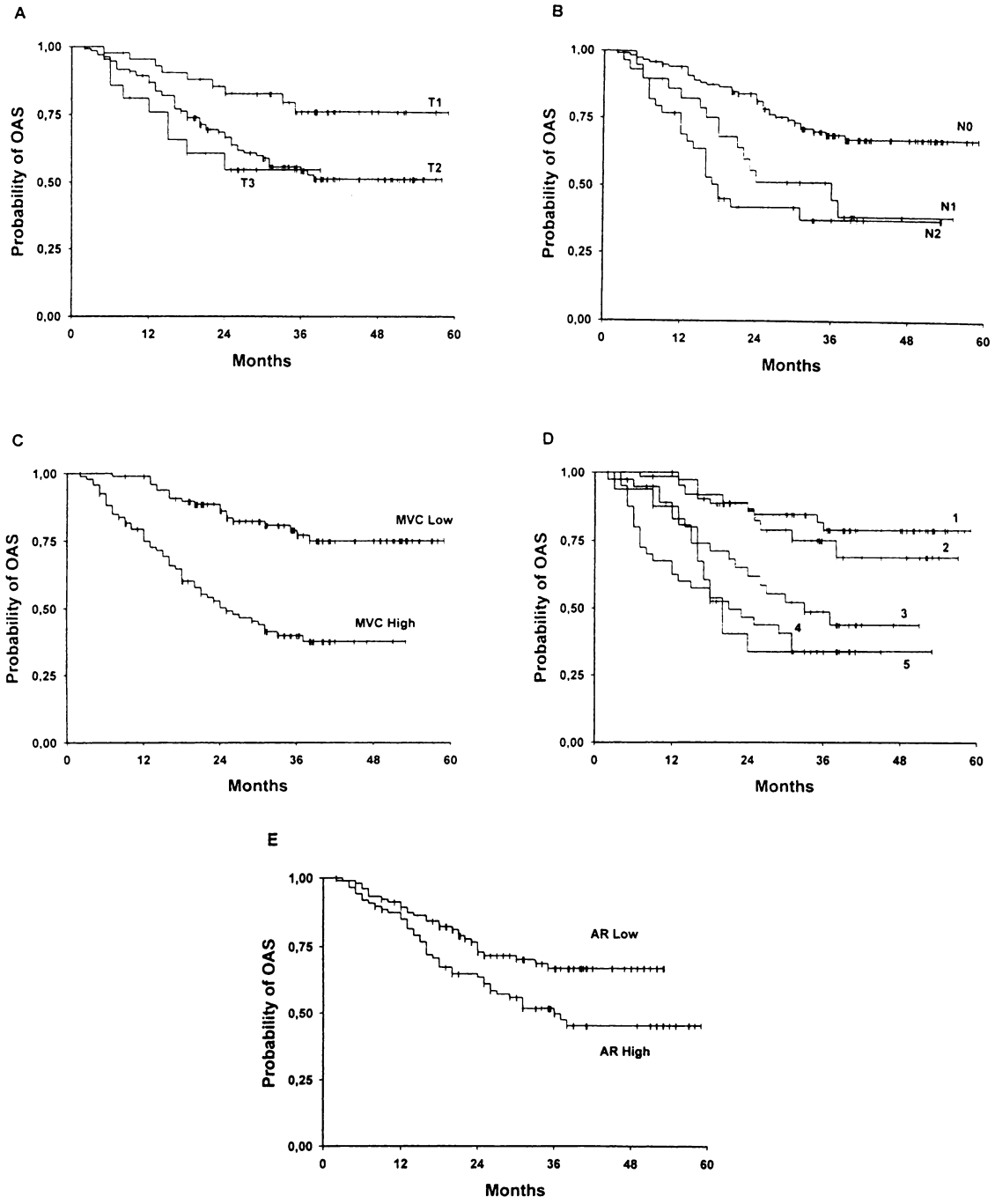

Fig. 1 Kaplan-Meier OAS estimates in relationship to tumor status (*A*), nodal status (*B*), MVC considered as a dichotomous variable (*C*) or as a five-category variable (*D*), and AR expression (*E*). A MVC of 20 was the median cutoff value in the series of 195 NSCLC patients for considering high or low tumor angiogenesis. *D*, the five categories for MVC were as follows: *1*, 3–10; *2*, 11–20; *3*, 21–30; *4*, 31–40; and *5*, 41–100 microvessels/tumor sample. Thirty-five % positive tumor cells was the median cutoff in the series of 195 NSCLC patients for considering low or high AR expressing tumors. The *P*s for each estimate were as follows: *A*, $P = 0.008$; *B*, $P < 0.00001$; *C*, $P < 0.00001$; *D*, $P < 0.00001$; and *E*, $P = 0.01$. *P*s ≤ 0.05 were considered statistically significant.

have previously reported that high MVC, assessed by tumor staining with a monoclonal antibody against factor VIII-related antigen, was significantly correlated with metastatic spreading and with a worse prognosis in selected or relatively small series of NSCLC patients (32–33, 37). For example, in a group of 70 stage I–III NSCLC patients with a median follow-up of 46 months in which some biological parameters, such as p53, bcl-2 expression, and tumor proliferative activity, were evaluated,

Table 4 Multivariate analysis for OAS

The three variables that retained an independent prognostic effect on survival using the Cox proportional hazards model are represented in the final model. Stage was considered as I, II, and IIIA. Node status was evaluated as N0, N1, and N2. MVC was considered as a continuous variable after a log transformation. AR was considered as a dichotomous variable using the median of 35% positive tumor cells/sample as cutoff for low- and high-expressing tumors. β, regression coefficient. *P*s ≤ 0.05 were considered statistically significant.

Covariate	β	SE	χ^2	*P*	RH (95% CI)
Starting model					
Stage			1.15	0.28	
T status			2.09	0.15	
Nodal status			6.24	0.01	
MVC			19.42	<0.00001	
AR			3.72	0.05	
Final model					
Nodal status	0.4658	0.1403	10.36	0.0013	1.59 (1.20–2.11)
MVC	1.8879	0.4336	19.65	<0.00001	6.61 (2.78–15.72)
AR	0.5437	0.2398	5.24	0.02	1.72 (1.07–2.78)

MVC was the only biological variable with an independent prognostic impact on survival (35). Harpole *et al.* (36) have recently demonstrated that tumor angiogenesis was the most significant independent prognostic factor in a retrospective analysis of 275 stage I completely resected NSCLC patients in which *erb*B-2 and p53 expression were also found as independent prognosticators. However, our study failed to demonstrate a prognostic effect of *erb*B-2 expression, as well as of p53 expression[4] on patient survival. Giatromanolaki *et al.* (34) have shown that in 107 stage I NSCLC patients, highly vascularized tumors as evaluated by anti-CD31 staining significantly correlated with a worse survival. We have recently obtained similar results in a series of 227 stage I NSCLC patients (46).

The results of the present study demonstrate that in stages I–IIIA NSCLC patients who were treated with potentially curative surgery, the evaluation of some biological characteristics of the primary tumor may be helpful in defining groups of patients with a high risk of relapse who could benefit of adjuvant systemic treatments. MVC is the more important biological variable, and it deserves to be evaluated in larger prospective studies and in clinical trials of postsurgical adjuvant therapy. In this respect, we have recently demonstrated in a prospective study in which 407 NSCLC patients were evaluated that MVC is an independent prognostic indicator of survival (47). Furthermore, the present report is the first to demonstrate the independent prognostic role of AR overexpression in NSCLC. It is interesting to note that although AR overexpression was significantly correlated with a more advanced tumor size, AR overexpression, but not tumor size, maintained an independent prognostic value in a Cox model of survival. Our results need to be validated prospectively in other independent series of patients. However, a clinically relevant aspect of our findings is the observation that autocrine and paracrine regulatory pathways involving EGF-like growth factors and EGFR-related receptors are frequently operative in human NSCLC. With the clinical development of specific drugs, such as anti-growth factor receptor humanized monoclonal antibodies and modified antisense oligodeoxynucleotides, that interfere with these growth stimulatory pathways (48–52) and of specific antiangiogenic drugs that can block endothelial cell proliferation (53), evaluation of the expression of growth factors and their receptors and assessment of tumor-induced neovascularization could allow researchers to select NSCLC patients in whom to test the antitumor activity of these agents used alone or in combination with conventional cytotoxic drugs.

[4] Unpublished results.

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