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Tumor angiogenesis and biologic markers in resected stage I NSCLC

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Abstract

Objective: Microvessel count (MC), as a measure of tumor angiogenesis, has been shown to be significantly correlated with metastatic disease in cutaneous, mammary, prostatic, head and neck cancer. We have previously assessed the role of intensity of angiogenesis as predictor of metastasis in surgically resected T1N0M0 NSCLC. We needed to confirm its value, in a prospective larger study on Stage I NSCLC, before its utilization as a prognostic tool for further clinical investigations. **Methods:** In the present report we prospectively investigated 227 patients (206 males, 21 females; median age 65 years) with Stage I NSCLC treated only by radical surgery between March 1991 and December 1994 with utmost care for some biological characteristics (proliferative activity, the blood vessel invasion, angiogenesis and the p53 protein expression). **Results:** The operative procedures consisted of 62 pneumonectomies, 148 lobectomies and 17 segmentectomies or wedge resections. With a median follow-up of 36 months (range 15–60), eighty patients have already experienced a local ($n = 22$) or systemic ($n = 58$) relapse. Univariate analysis revealed that T factor (T1 versus T2)($P = 0.008$) and angiogenesis count ($\leq$ versus $>$ median, 17) ($P = 0.0006$) were significant predictors of survival. The same variables were also significant predictors of long Disease Free Survival ($P = 0.006$ and $P = 0.004$, respectively). On multivariate analysis, however, only the microvessel count retained its level of prognostic significance as regards both overall ($P < 0.01$) and disease-free survival ($P < 0.01$). **Conclusions:** The present study corroborates the role of angiogenesis in the metastatic spread of NSCLC and emphasizes its value in the identification of patients in whom surgery should be supplemented by systemic treatment. © 1997 Elsevier Science B.V.

Keywords: Non-small cell lung cancer; Surgery; Prognostic factors; Angiogenesis; Survival

1. Introduction

Lung cancer is a neoplasm with different clinical behaviours and diverse growth rates and metastatic potential. Actually the most effective predictor of recurrence and survival is the stage of the disease at diagnosis and treatment [23,24]. However, the different clinical behaviour, as regards local recurrence and distant metastases, also in early-stage lung cancer [3,19], requires the identification of additional prognostic factors. This is so because the patients, within a staging group, identified to be at high risk of recurrence, may obtain a greater benefit from the available treatments and future biologically based therapeutic approaches [10].

Research on tumor angiogenesis began in Folkman's animal models 30 years ago [5], and from then on, it has exponentially increased, being now a main field in cancer research [4,6]. In 1991 we have retrospectively assessed the role of intensity of angiogenesis as predictor of metastasis in surgically resected T1N0M0 NSCLC [22].

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At that moment we planned to perform a prospective confirmatory study on Stage I (T1-2N0M0) NSCLC to validate properly the real prognostic value of intratumoral microvessel count, before reaching the stage of clinical application as a routine clinical practice in selecting high-risk early-stage NSCLC-patients who might benefit from adjuvant therapies.

2. Materials and methods

2.1. Patients

A total of 227 patients, resected for Non-Small Cell Lung Cancer Stage I (T1-2N0) at our Department, from March 1991 to December 1994, were prospectively investigated. Patients (206 males and 21 females with a median age of 65 years) had a clinical follow-up ranging from 15 to 60 months (median 36 months). Tumor staging was performed according to the TNM staging system and to the World Health Organization Histological Classification [1,23,33], after a radical lymphadenectomy was performed and the bronchial suture was postoperatively confirmed negative.

2.2. Immunohistochemical procedures

The p53 protein expression was assessed in frozen tissue samples using immunohistochemistry. PAB 1801 is a monoclonal antibody which recognizes an epitope in p53 protein between amino acids 32–79. The avidin-biotin peroxidase method was used developing immunoreaction with diaminobenzidine. Simultaneous staining of the known p53 positive case was employed as positive control for p53. Incubation of parallel slides omitting the first antibody was performed as negative control. The count of p53 immunoreactive cells was made by scoring a minimum of five high-power fields ($40\times$ objective lens) and counting in each field the number of immunoreactive cells on the overall number of neoplastic cells. We considered p53 immunostaining both as continuous- and dichotomous-variable and assumed 8% of positive cells as the cut-off to distinguish between the negative and positive p53-tumors.

Table 1
Operative procedures

Features		Numbers
Side of operation	Right vs. Left	123/104
Extent of operation	Pneumonectomy	62
	Lobectomy	148
	Wedge resection/Segmentectomy	17

Table 2
Tumor characteristics

Features		Numbers
Tumor size (cm)	Mean $\pm$ S.D.	4.28 ± 2.37
	Median	4
Tumor histology	Squamous vs. Non Squamous	137/90
Tumor status	T1 vs. T2	75/152
Grading	G1 vs. G2–3	57/170
P53	Mean $\pm$ S.D.	22.82 ± 27.24
	Median	8
Tumor proliferation (PCNA)	Mean $\pm$ S.D.	33.56 ± 21.04
	Median	30
BVI	Absent vs. Present	191/36
Microvessel Count ($\times 200$ field)	Mean $\pm$ S.D.	21.63 ± 14.03
	Median	17

Microvessel Count (MC) was determined on methacarn-fixed and paraffin embedded tumour samples at the time of resection, using the anti-CD34 monoclonal antibody diluted 1:100 overnight, displayed by the ABC method. Anti-CD34 MAb labels the vascular endothelium and provides an easy identification of the most intense areas of neovascularisation in the tumours. A single microvessel was defined as any brown immunostained endothelial cell separated from adjacent microvessels, tumour cells and other connective tissue elements. By means of the same tecnique, the identification of neoplastic emboli within tumor vessels was easily performed and the blood vessel invasion (BVI) was determined.

Areas of highest neovascularisation were found by scanning sections at low power ($\times 10$ objective lens and $\times 10$ ocular lens) and microvessels were carefully counted on a $\times 200$ field ($\times 25$ objective lens and $\times 8$ ocular lens, 0.74mm^2 per field). The MC was expressed as the highest number of microvessels identified within the $\times 200$ field. In all cases, MC was determined independently by two pathologists. Each pathologist evaluated the slides without any knowledge of the counts made by the other pathologist. The counts from the two pathologists correlated very well ($R = 0.98$; two-sided $P < 0.0001$). When conflicting data were obtained, we used mean values. We considered MC both as continuos and dichotomous variable assuming the median value of 17 vessels as cut-off value to distinguish low from high MC.

Proliferative activity of the tumors was determined by estimating the expression of the proliferating cell nuclear antigen with monoclonal antibody PC10 (Novocastra Laboratoires, Newcastle UK). PCNA immunolocalization, including its spatial distribution within cells, was assessed and its count was made by scoring a minimum of five high-power fields.

Table 3
Tumor characteristics and operative procedures in relation to development or not of metastasis

Features		No metastasis ($n = 147$)	Metastasis ($n = 80$)	P
Tumor size (cm)		4.15 ± 2.58	4.51 ± 1.90	NS
		3.5	4	
Tumor histology	Squamous vs. Non Squamous	84/63	53/27	NS
Extent of operation	WR + Lobectomy vs. Pneumonectomy	108/39	59/21	NS
T status	T1 vs. T2	58/89	17/63	0.0053
Grading	G1 vs. G2–3	39/108	18/62	NS
P53		23.12 ± 27.04	22.35 ± 27.758	NS
		8		
Tumor proliferation (PCNA)		33.13 ± 31.34	34.38 ± 20.57	NS
		30	32.5	
BVI	Absent vs. Present	123/24	68/12	NS
Microvessel Count ($\times 200$ field)		19.38 ± 11.78	25.76 ± 16.73	0.001
		16	24.5	

Data are expressed as mean $\pm$ S.D. and below, as median.

2.3. Statistical analysis.

All statistical analysis were carried out by the Statistica (Stat-Soft) software system. Univariate analysis by the Mann-Whitney U test for continuous variables and the χ^2 test for discrete variables were used to assess differences of tumor-characteristics between patients who had developed metastasis and those who had not.

Overall survival was calculated from the date of operation until death or the date of last follow-up (censored). Four patients who died due to cause other than primary NSCLC without evidence of disease were censored at death.

The median value ($n = 17$) of the microvessel count was chosen as the cut-off point.

Survival was estimated by the product-limit method [16] and the differences in their distributions were evaluated by the log-rank test, for univariate analysis. The influence of significant variables generated by the univariate analysis was then assessed by the Cox proportional hazards stepwise model. The level of significance was a priori set at $P < 0.05$; all tests were two-sided.

Numbers were expressed either as mean $\pm$ S.D. of n number of observations or as median.

3. Results

The operative procedures and tumor characteristics of the 227 patients included in this study are illustrated in Table 1 and Table 2. The follow-up lasted until March 1996. With a 36-month median follow-up (range 15–60), 80 patients have already experienced a local ($n = 22$) or systemic ($n = 58$) relapse, while 4, out of the 54 dead patients, died due to a cause other than primary NSCLC without evidence of disease. The 50 patients, who died of their disease, experienced a local relapse in 8 cases or a recurrence in extratoracic sites in 42 cases. Among the 30 patients actually living with metastasis, 14 had local and 16 systemic relapse. Table 3 shows the different tumor characteristics in relation to development or not of metastasis. As regards survival, at univariate analysis, patients with T1 tumors ($P = 0.008$) and low angiogenesis count ($= 17$) ($P =$

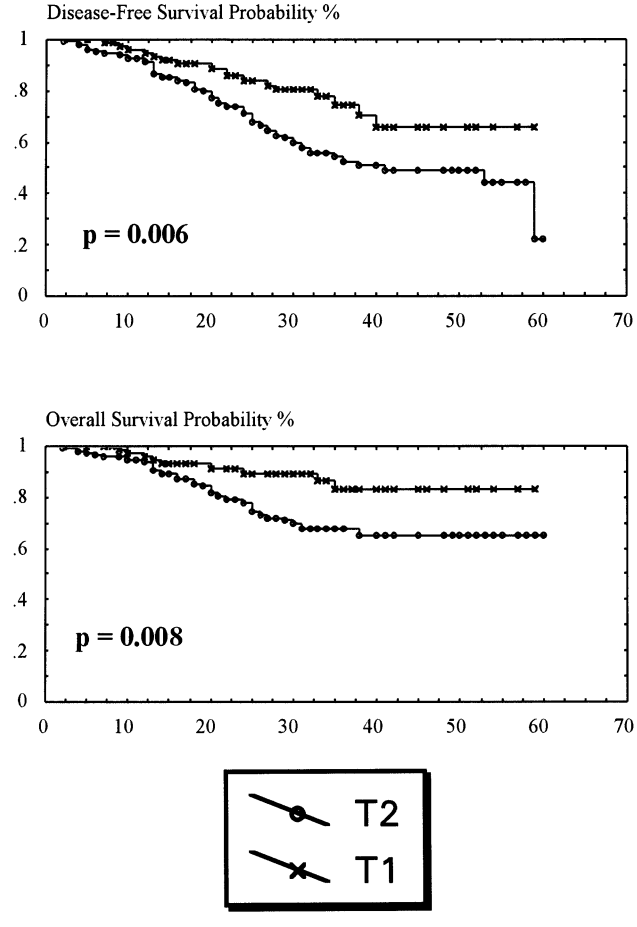

Fig. 1. Survival curves according to the tumor status.

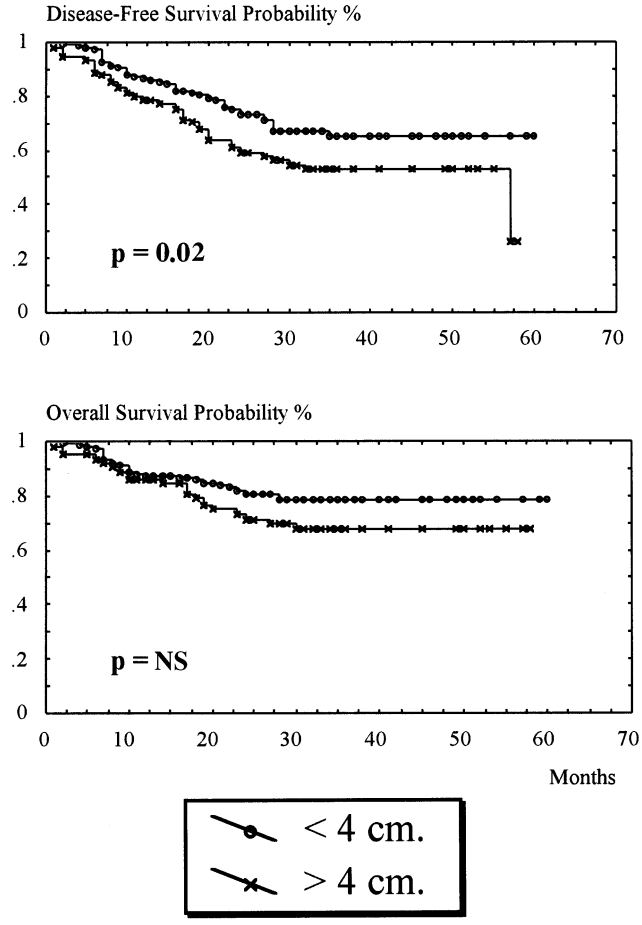

Fig. 2. Survival curves according to the maximum diameter of the tumor ($\leq$ vs. $>$ median).

0.0006) had better prognosis. The same variables ($P = 0.006$ and $P = 0.004$, respectively) and the size of the tumor ($P = 0.02$) (Figs. 1–3) were predictors of longer Disease Free Survival (Table 4).

The joint effect of the different variables was compared with overall survival and disease-free interval using a Cox proportional hazards stepwise model.

On multivariate analysis the T status ($P < 0.05$), the histology ($P < 0.05$), and the microvessel count ($P < 0.01$) were independent markers of disease-free survival while the microvessel count ($P < 0.01$) was the sole predictor of overall survival when compared to the other parameters affecting survival in the univariate analysis (Table 5).

4. Discussion

Having been established that TNM is actually the most important factor for planning therapeutic strategies for NSCLC, and for predicting survival in resected patients [22], the factors accounting for diverse outcomes among patients with the same stage of disease are, however, generally unknown [3]. Indeed, in early-stage NSCLC (Stage I), despite a complete surgical resection, the 5-year survival rate is 60–80% [3,10,19,24,28]. In the last 20 years no therapeutic improvement has been verified except for the results of the Lung Cancer Study Group in the field of the optimal surgical resection to be performed for NSCLC [20].

Biological characterization has been advocated to overcome this obstacle, offering a useful selection tool for both surgeons and oncologists [7,8].

Tumor angiogenesis is one of the most important biological factors involved in the development and progression of NSCLC [2,8,34] as well as other solid tumors [11–13,29–32]. At the beginning, we verified the above mentioned hypotesis retrospectively analysing a homogenous group of patients (87 patients T1N0M0) [22]. On this basis, at that time (March 1991), we programmed a prospective confirmatory study extending the investigation to a larger number of patients resected for Stage I (T1-2N0M0) NSCLC.

As soon as we achieved the minimum follow-up for a right analysis we correlated data, coming from our pathologists, concerning conventional and new biologi-

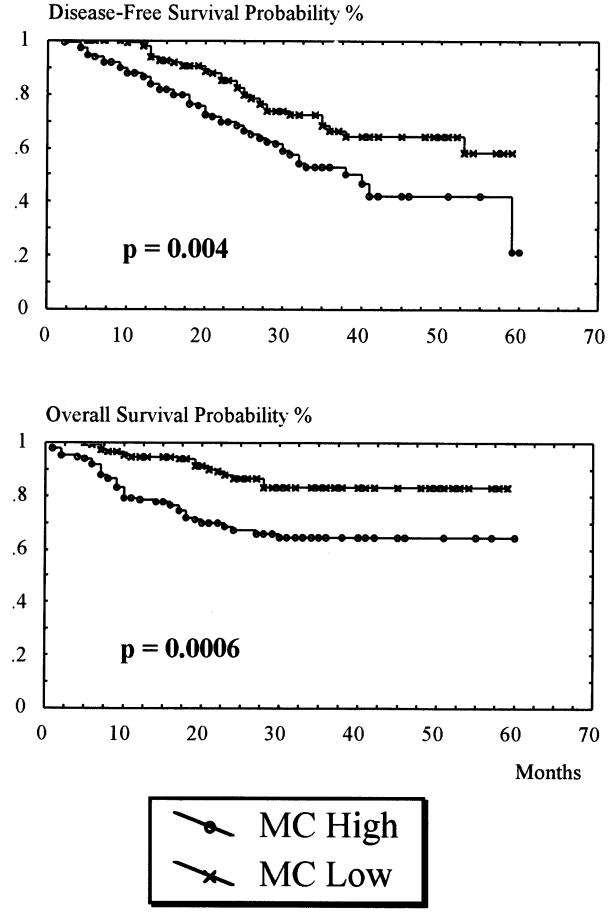

Fig. 3. Survival curves according to Microvessel Count ($\leq$ vs. $>$ median).

Table 4
Univariate survival analysis

Features		Disease free survival (P value)	Overall survival (P value)
Tumor size	≤vs.> median (4cm)	0.02	NS
T status	T1 vs. T2	0.006	0.008
Tumor histology	Squamous vs. Non Squamous	NS	NS
Extent of operation	WR + Lobectomy vs. Pneumonectomy	NS	NS
Grading	G1 vs. G2–3	NS	NS
Tumor proliferation (PCNA)	≤vs.>median (30)	NS	NS
P53	≤vs.>median (8)	NS	NS
BVI	Absent vs. Present	NS	NS
Microvessel Count(×200 field)	≤vs.>median (17)	0.004	0.0006

NS, not statistically significant.

cal factors as angiogenesis, PCNA, BVI and p53 expression, with the clinical results in terms of disease-free and overall survival.

The overall survival reported in this study is in line with most of the largest studies reported in the surgical literature [19,28].

Tumor-status and the microvessel count were found to predict both overall and disease-free survival.

It is generally accepted that, within stage I NSCLC, T1 tumors have a better outcome than T2 tumors [28]. However, in the current study, as in the literature, the T status loses its significance in the multivariate analysis confirming that the T1 and T2 tumors must be considered together in stage I NSCLC.

Similarly, the tumor size within the tumor stage does not influence the outcome significantly even if larger tumors (> 4cm) had a longer disease-free survival.

Unexpectedly, data concerning BVI from the current study, are apparently in contrast to our previous retrospective experience [21]. We did not verify any significant correlation between BVI and overall or disease-free survival.

However, considering only T1N0M0, BVI influence both overall and disease-free survival ($P = 0.02$ and $P = 0.04$ respectively). This finding may confirm the value of BVI in the initial tumoral process, but, on the other side, it emphasizes its poor value as discriminant factor of high- and low-risk patients in overall stage I NSCLC. Similarly, high levels of p53 protein, previously related to metastatic nodal involvement and to shorter overall survival [9,27], failed to be significant predictors of survival in stage I NSCLC.

Indeed, what arises from our study is that the most important difference in clinical outcome is associated with the degree of vascularization of the primary tumor. A biological explanation may lie in the interaction between tumor cells and stroma (connective tissue, stromal cells and vessels). Neovascularisation is necessary for tumor growth and progression by providing tissue perfusion to the tumor cells. Moreover, as O'Reilly and Folkman have demonstrated in a experimental model [26], angiogenesis plays a critical role in the development of metastasis. They isolated a 38-KDa fragment of plasminogen, named angiostatin, which is a potent angiogenesis inhibitor in the murine Lewis lung carcinoma.

In this animal model the resection of the primary tumor was followed by the depletion of the circulating angiostatin and, as a consequence, by the development of metastasis. This experiment provides a reasonable explanation of what happens in the common practice of all the thoracic surgeons, when the surgical removal of

Table 5
Multivariate disease-free and overall survival analysis

Features	Disease-free survival				Overall survival			
	β	S.E.	t-value	P-value	β	S.E.	t-value	P-value
Age	0.016517	0.0182483	0.90515	NS	−0.000620	0.0217491	−0.02851	NS
T status	−0.867794	0.3581970	−2.42267	<0.05	−0.826824	0.4390382	−1.88326	NS
Size	−0.032017	0.0674162	−0.47492	NS	−0.016289	0.0785239	−0.20744	NS
Histology	−0.746093	0.2956080	−2.52393	<0.05	−0.565006	0.3473008	−1.62685	NS
BVI	−0.190738	0.2072356	−0.92039	NS	−0.232037	0.2475114	−0.93748	NS
PCNA	−0.009304	0.0066024	−1.40921	NS	−0.005505	0.0072601	−0.75825	NS
P53	−0.001471	0.0015446	−0.95209	NS	−0.001810	0.0020990	−0.86234	NS
Microvessel Count(×200 field)	0.029122	0.0082538	3.52829	<0.01	0.034951	0.0093751	3.72804	<0.01

lung tumors, sometimes also in early-stage, is followed by the rapid growth of distant metastases.

The present study confirms the results of the Lung Cancer Research Laboratory at the Dana Farber Cancer Institute [12] on a retrospective series of stage I NSCLC although routine histopathologic variables and molecular biologic markers were separately considered in the multivariate survival analysis and some controversies exist regarding the prognostic value of blood vessel invasion and P53 expression.

Our confirmatory results may have important future therapeutic implications when ongoing phase I/II clinical studies on antiangiogenic drugs [15,17,35] will be completed; it will be of great interest to test them in an adjuvant setting in stage I NSCLC.

Indeed, as recently reported by Lafitte and colleagues [18] in a prospective, randomized study on Stage I T2N0M0 non-small cell lung carcinomas, there was no difference in disease-free and overall survival with or without radiotherapy in a adjuvant setting. The higher incidence of distant recurrence in resected T1-2N0M0 NSCLCs, as previously showed by Feld and the Lung Cancer Study Group [3], is inducing many authors to suggest systemic adjuvant treatments in prospective stratified studies including pathological and biological factors accounting for the early or late appearance of distant metastases.

There is a general consensus that improper patient selection, may be responsable of the absence of impact on survival and recurrence rate of the adjuvant chemotherapy in stage I NSCLC [14].

Our next step will be to perform a clinically controlled and randomized trial of adjuvant therapy for patients at high risk (high MC) with inhibitors of angiogenesis alone or in combination with conventional cytotoxic chemotherapy.

Even if the magnitude of improvement expected with the introduction of adjuvant therapies and new biology-based therapeutic approaches might turn out to be small; in view of the increasing incidence of lung cancer in most parts of the world (excluding the US) [25], such modest improvement might traslate into the prolongation of survival of several patients. Therefore, in the fight against lung cancer, we should leave no stone unturned to execute it.

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Appendix A. Conference discussion

Dr Grodzki (*Pisa*, *Italy*): Did you try to include the cytological examination of pleural lavage as a prognostic factor?. Many papers indicate that it is important because the positive lavage means a worse survival rate than the negative.

Dr Lucchi: That's right, but we didn't. We may consider it in a next investigation where cytological, hystological and clinical features will be tested.

Dr Lerut (*Leuven*, *Belgium*): You said that the cut-off of the microvessel count was 17. How was that decided. Was that retrospectively or prospectively decided?

Dr Lucchi: The cut-off of the microvessel count was prospectively decided, choosing the median value = 17 to obtain two comparable groups for disease-free and overall survival actuarial analysis. We could also choose 20 microvessels or more as the cut-off and the results do not change. On the contrary, in this way we may select a smaller group of patients in which the risk of recurrence is even higher.

Dr Lerut: So that contains a bias because in fact, it is a matter of defining something in a subjective way. Are you really measuring neo-angiogenesis or are you measuring growth of vessels, which then is leading to the question, is it primary or simply a secondary effect?

Dr Lucchi: Angiogenesis is the process leading to the formation of new blood vessels. Physiologic angiogenesis is present in embryonic development, ovulation and repair of wound healing when it is activated for brief periods and then completely inhibited. Angiogenesis plays a role in the physiopathology of some non-neoplastic disease, but the most dramatic angiogenesis-dependant disease is cancer. Microvessel count, by immunohistochemical techniques, as a measure of tumor angiogenesis, is a well-standardized method. It is easy to perform. It is reproducible and quite economical. It should be considered in non-small cell lung cancer, as well as in other solid tumors, as an effective marker of metastatic propensity.