Osteopontin Expression and Prognostic Significance in Non–Small Cell Lung Cancer

Valentina Donati,[1] Laura Boldrini,[1] Matteo Dell'Omodarme,[4] Maria C. Prati,[4] Pinuccia Faviana,[1] Tiziano Camacci,[1] Marco Lucchi,[2] Alfredo Mussi,[2] Massimo Santoro,[5] Fulvio Basolo,[3] and Gabriella Fontanini[3]

Abstract

Purpose: The survival rate of non–small cell lung cancer patients is very low, and knowledge of predictors of outcome is inadequate. To improve the curability of lung cancer, we need to identify new specific molecules involved in tumorigenesis and progression. The purpose of this study was to better define the role of osteopontin in non–small cell lung cancer biology by determining its prognostic significance.

Experimental Design: Osteopontin expression was evaluated by immunohistochemistry, as percentage of neoplastic cells with cytoplasmic immunoreactivity, in a wide series of patients with stage I-IIIA non–small cell lung cancer (207 cases). The median value of this series (20% of positive cells) was used as the cutoff value to distinguish tumors with low (<20%) from tumors with high (≥20%) osteopontin expression.

Results: Taking the series of patients as a whole (207 cases), osteopontin expression was associated with neither overall survival ($P = 0.14$) nor disease-free survival ($P = 0.074$). However, among patients with at least 6 years of follow-up (163 cases), 6-year overall survival and disease-free survival were significantly reduced if osteopontin expression was high ($P = 0.0085$ for overall survival, $P = 0.0023$ for disease-free survival). Moreover, a statistically significant correlation between high levels of osteopontin and shorter overall survival ($P = 0.034$) and disease-free survival ($P = 0.011$) in patients with stage I tumors (136 cases) was shown.

Conclusions: Our results support the hypothesis of an association between high osteopontin expression and poor survival of patients with stage I non–small cell lung cancer, suggesting that osteopontin could be a candidate target for cancer therapy.

Lung cancer is currently the most frequently diagnosed solid tumor in the world as well as the most common cause of cancer mortality worldwide: an estimated 1.2 million people are diagnosed annually with lung cancer and 1.1 million of them die from the disease. Non–small cell lung cancer (NSCLC) comprises >80% of lung cancers, and complete surgical resection of primary tumors in early-stage disease is the only potentially curative treatment. Despite improvements in detection and in surgical and medical treatments during the past two decades, the 5-year survival rate for patients with NSCLC ranges from 9% to 61% following resection depending on clinical stage; survival rates after surgery (pathologic stage) range from 25% to 67%. One area of intense research in early-stage NSCLC regards the identification of molecular markers to complement tumor-node-metastasis staging to fully assess the prognosis of patients and to define innovative strategies (1, 2). Numerous prognostic factors have been identified in patients with early-stage NSCLC that might enable classification of such patients into different subsets corresponding to different risks of recurrence following complete resection.

One of the factors that have recently been shown to be linked to cancer development, progression, and metastasis in different malignancies is a multifunctional protein named osteopontin. Osteopontin, which is a phosphorylated acidic glycoprotein (3), is both a multifunctional cytokine and an adhesion protein. In fact, although structurally resembling a matrix protein because of its arginine-glycine-aspartic acid domain, it has been recognized to be a key cytokine involved in the regulation of cellular migration, cell-mediated immunity (4), tissue repair, and remodeling. Indeed, osteopontin, which regulates maintenance or reconfiguration of tissue integrity during inflammatory processes, takes part in a broad range of physiologic events (5–8) and pathologic processes, including cancer metastasis (9). In these apparently very different processes, osteopontin stimulates motility (chemotaxis) and controls cell-survival pathways, like inhibition of apoptosis, via

Authors' Affiliations: Division of Surgical Pathology, Departments of [1]Surgery, [2]Cardio-Thoracic Surgery, and [3]Oncology, Transplants and New Technologies in Medicine, University of Pisa; [4]Scuola Normale Superiore, Pisa, Italy and [5]Dipartimento di Biologia e Patologia Cellulare e Molecolare, University "Federico II," Naples, Italy

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Requests for reprints: Gabriella Fontanini, Anatomia Patologica III, University of Pisa, Via Roma 57, 56126 Pisa, Italy. Phone: 39-50-992983; E-mail: g.fontanini@med.unipi.it.

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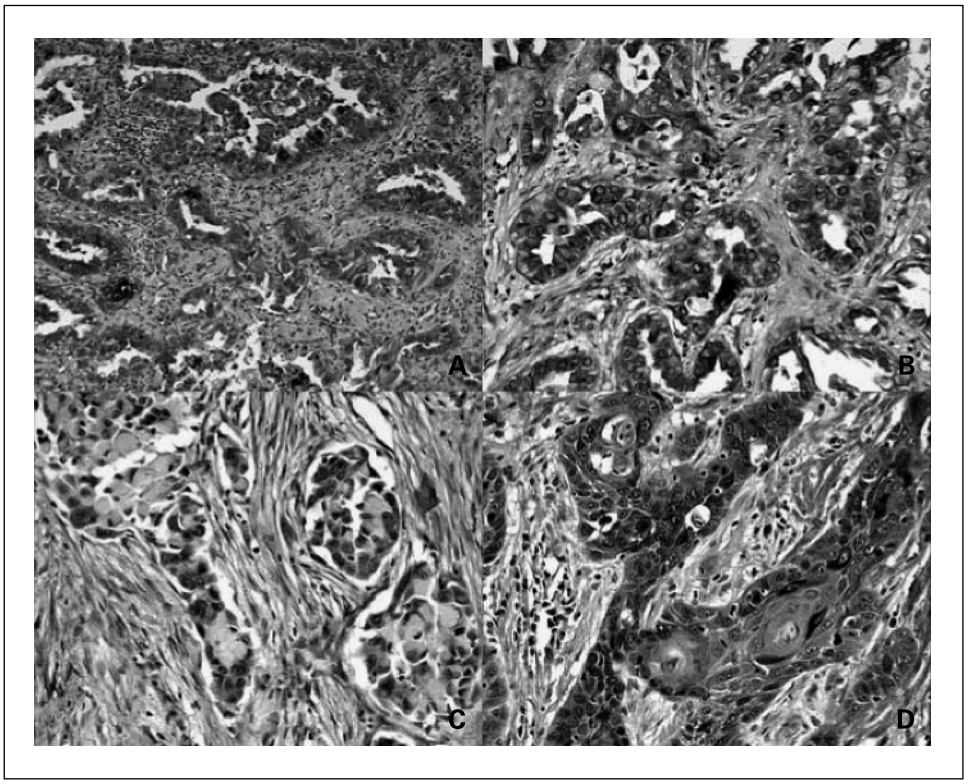

Fig. 1. Osteopontin expression detected by immunohistochemical staining in three adenocarcinomas (*A-C*) and one squamous cell carcinoma (*D*) of the lung. Magnification, ×100 (*A*) and ×200 (*B-D*).

cell-cell or cell-matrix interactions with adhesive receptors. Receptors for osteopontin are the integrins $\alpha_v(\beta_1, \beta_3, \text{or } \beta_5)$ and $(\alpha_4, \alpha_5, \alpha_8, \text{or } \alpha_9)\beta_1$, and the hyaluronic acid CD44 (10) and/or its variant forms (specifically v6 and/or v7) in conjunction with a β_1 integrin (11).

Osteopontin protein or gene expression levels are increased in many human tumors, including breast (12, 13), lung (14), prostate (15), colon (16), ovarian (17), and gastric (18) cancers, confirming the hypothesis that it plays an important role in tumorigenesis, tumor progression, and metastasis (19, 20) and suggesting that osteopontin could be a specific target for anticancer therapy. In fact, both osteopontin and its main receptors in tumor cells can be suppressed in different ways: osteopontin gene expression can be inhibited on the level of the RNA message by ribozyme cleavage (21) and hybridization with antisense oligonucleotides (22); osteopontin protein can be blocked with monoclonal antibodies or synthetic peptides (23); regarding CD44, therapeutic strategies include inhibiting its expression, blocking receptor-ligand binding, and suppressing associated signal transduction (23); small-molecule inhibitors of the receptor integrin $\alpha_v\beta_3$, which is involved in tumor cell dissemination, angiogenesis, and osteolysis in bone metastasis, are under study (23).

Therefore, in spite of the numerous studies concerning osteopontin expression (14, 24, 25), the effect of osteopontin on NSCLC outcome remains unclear. We decided to investigate osteopontin expression in a large series (207 cases) of stage I-IIIA NSCLC, focusing in particular on stage I patients (136 cases), to clarify whether osteopontin could be a prognostic marker in identifying subsets of NSCLC patients with high risk of recurrence and therefore a candidate target for cancer therapy.

Materials and Methods

Patients and clinical data. Two hundred seven patients with NSCLC, who consecutively underwent radical surgical resection at the Department of Cardio-Thoracic Surgery of the University of Pisa from December 1991 to December 1994, were retrospectively studied. No detectable metastases in distal organs were present at the time of surgery. No patient had received chemotherapy or radiotherapy before surgery. The cohort of patients included 188 (90.8%) males and 19 (9.2%) females, with a combined median age of 65 years (mean age, 64.1 years; range, 41-88 years). Follow-up lasted through June 30, 2003, with a median follow-up period of 49 months for living patients (range, 2-137 months). Disease-free survival and overall survival rates were calculated as the period from surgery until the date of disease relapse or of death, respectively.

Specimens. Neoplastic specimens were removed from the periphery of the tumor masses, because the central region of a cancer is more often subject to regressive alterations, and were formalin-fixed and paraffin-embedded for histologic and immunohistochemical analysis. The pathologic features of the samples were classified according to WHO histologic criteria (26), and tumor staging was done according to the International Union Against Cancer tumor-node-metastasis classification (1).

Immunohistochemistry. Osteopontin expression was detected by immunohistochemistry using a polyclonal antibody anti-osteopontin (AF808; R&D Systems, Inc., Minneapolis, MN; dilution at 1:40). The antibody was applied to 5 μm sections from the most representative formalin-fixed, paraffin-embedded tumor tissue specimen obtained from each of the 207 patients with NSCLC using the avidin-biotin-peroxidase complex method (Vectastatin Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA) following the manufacturer's instructions. The immunostaining was done manually at room temperature. The sections, mounted on glass slides, were deparaffinized through serial baths in xylene and rehydrated in a graded series of alcohol and water. To remove any endogenous peroxidase activity

and nonspecific background staining, the sections were soaked in absolute methanol containing 0.3% hydrogen peroxide for 30 minutes at room temperature. After being washed with TBS for 5 minutes, slides were blocked with nonimmune rabbit serum for 30 minutes to inhibit nonspecific binding followed by incubation with the anti-osteopontin primary antibody for 60 minutes at room temperature. After rinsing with TBS for 5 minutes, sections were subsequently incubated with biotin-conjugated goat anti-mouse IgG antibody for 30 minutes. Then, after being washed again with TBS for 5 minutes, slides were incubated with avidin-biotin-peroxidase complex for 30 minutes and washed again with TBS. Finally, the sections were incubated with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) and then rinsed in distilled water. All slides were lightly counterstained with Mayer's hematoxylin for 30 seconds, washed in running water, dehydrated, and mounted with Canadian balsam. No antigen retrieval was done. A section of thyroid papillary carcinoma, classic variant, proven previously to be osteopontin positive by Western blot, was used as positive control.

The immunohistochemical expression of osteopontin was evaluated as the percentage of tumor cells with cytoplasmic immunoreactivity, counting at least 1,000 cancer cells (100 cells in 10 high-power fields) for each section (Fig. 1). The median value of this series (20% of positive cells) was used as the cutoff value to distinguish tumors with low (<20%) from tumors with high (≥20%) osteopontin expression. Moreover, we analyzed the staining intensity by distinguishing four categories: one negative (0), one with a weak staining (+), one with an intermediate staining (++), and one with a strong staining (+++).

Statistical analysis. Statistical analysis was carried out using R 1.8.1 (27). Univariate analysis was done by modeling Kaplan-Meier survival curves. Log-rank test was used to evaluate the statistical significance of differences in survival distributions. Multivariate analysis was carried out using Cox proportional hazards model. The proportional hazards assumption was tested as proposed in Grambsch and Therneau (28). Mann-Whitney and Kruskal-Wallis tests were used to evaluate the associations between the continuous test variable-gene expression and patients' clinicopathologic variables (Mann-Whitney test for dichotomous variables: gender; age, <65 versus ≥65 years; stage, I versus II-IIIA; squamous cell carcinomas versus nonsquamous cell carcinomas; relapse versus no relapse; Kruskal-Wallis test for nondichotomous variables: T, N, and grade). All tests used are described in Armitage et al. (29). Results were considered statistically significant if $P < 0.05$.

Table 1. Univariate analysis of the associations between prognostic variables and overall survival or disease-free survival in 207 cases of NSCLC

Patient and tumor characteristics	No. cases	Overall survival *P* value	Disease-free survival *P* value
Gender			
Male	188	0.59	0.62
Female	19		
Age (y)			
<65	105	0.86	0.82
≥65	102		
Tumor grade			
1	54	0.0075	0.0018
2	83		
3	70		
Histology			
Squamous	122	0.23	0.613
Nonsquamous	85		
Tumor size			
T_1	66	0.019	0.00017
T_2	121		
T_3	20		
Nodal status			
N_0	145	<0.00001	0.000057
N_1	32		
N_2	30		
Stage			
I	136	<0.000001	<0.000001
II-IIIA	71		
Osteopontin expression (OPN%)			
Low (<0.20)	101	0.14	0.074
High (≥0.20)	106		
Osteopontin (OPN+)			
0	61	0.68	0.44
+	62		
++	52		
+++	32		

Table 2. Multivariate analysis of overall survival according to Cox's model

	β	exp(β)	SE exp(β)	*z*	*P*
Stage I	−0.97	0.379	0.201	−4.83	1.4×10^{-6}

NOTE: Final model obtained with a backward stepwise regression. Likelihood ratio test = 22.2 on 1 *df*, $P = 0.0000025$, $n = 207$. Proportional hazards test $P = 0.15$. β, Cox regression's coefficient; exp(β), risk ratio; *z*, Wald statistics; *P*, Wald's test *P*.

Results

Clinicopathologic characteristics. The mean age of the 188 male and 19 female patients was 64.1 years (range, 41-88 years; median age, 65 years). The most common histologic type of tumor was squamous cell carcinoma (58.9%; 122 cases) followed by adenocarcinoma (31.4%; 65 cases), large-cell anaplastic carcinoma (6.3%; 13 cases), and bronchioloalveolar carcinoma (3.4%; 7 cases). According to the degree of differentiation, the primary tumors were histopathologically graded as well-differentiated (grade 1, 26.1%), moderately differentiated (grade 2, 40.1%), and poorly differentiated

Table 3. Multivariate analysis of disease-free survival according to Cox's model

	β	exp(β)	SE exp(β)	*z*	*P*
Stage I	−0.989	0.372	0.201	−4.92	8.7×10^{-7}
OPN% high	0.400	1.491	0.203	1.97	0.049

NOTE: Final model obtained with a backward stepwise regression. Likelihood ratio test = 26.1 on 2 *df*, $P = 0.0000021$, $n = 207$. Proportional hazards test $P = 0.13$.

Table 4. *P* values of Mann-Whitney *t* test and Kruskal-Wallis tests comparing osteopontin expression and different clinicopathologic characteristics in 207 cases of NSCLCs

	OPN%
Gender	0.24
Age	0.48
Histology	0.74
T	0.55
N	0.80
Grade	0.0010
Stage	0.93
Relapse	0.13

(grade 3, 33.8%). With respect to tumor size, 66 (31.9%) cancers were classified as T_1, 121 (58.4%) were classified as T_2, and 20 (9.7%) were classified as T_3. One hundred forty-five (70%) patients showed no metastasis to the regional lymph node (N_0), whereas metastatic involvement of hilar lymph nodes (N_1) was present in 32 (15.5%) patients, and metastases to the mediastinal lymph nodes (N_2) were observed in 30 (14.5%) cases. Most tumors were classified as stage I (136; 65.7%), whereas 25 (12.1%) were stage II and 46 (22.2%) were stage IIIA. One hundred one (48.8%) patients presented relapse during follow-up: 24 of them developed local recurrence, whereas 77 developed distant metastases. At the end of the follow-up, 114 (55.1%) were alive, whereas 93 (44.9%) had died.

Stage I-IIIA patients (207 cases): association between clinicopathologic characteristics and survival. Among the clinicopathologic variables, higher tumor grade ($P = 0.0075$ for overall survival, $P = 0.0018$ for disease-free survival), greater tumor size ($P = 0.019$ for overall survival, $P = 0.00017$ for disease-free survival), metastatic nodal involvement at the time of diagnosis ($P < 0.00001$ for overall survival, $P = 0.000057$ for disease-free survival), and advanced stage ($P < 0.000001$ both for overall survival and disease-free survival) were significantly associated with worse overall survival and shorter disease-free survival. The prognostic effect of the variables on overall survival and disease-free survival, evaluated by univariate analysis on all 207 patients, is summarized in Table 1.

Multivariate analysis was done using a backward stepwise regression technique (Cox proportional hazards model). For overall survival, it showed that only the stage (stage I versus II-IIIA) has a significant prognostic influence ($P < 0.00001$). The proportional hazards assumption cannot be rejected ($P = 0.15$; Table 2).

For disease-free survival, the best statistical model had two covariates: stage ($P < 0.00001$) and osteopontin expression ($P = 0.049$; Table 3).

Stage I-IIIA patients (207 cases): association of osteopontin expression with clinicopathologic characteristics and survival. One hundred forty-six (70.5%) tumors showed cytoplasmic immunoreactivity for osteopontin. Osteopontin expression was analyzed as a dichotomous variable using the median value of 20% as the cutoff point and distinguishing two categories: one with a high osteopontin expression (≥20%) and one with a low or null osteopontin expression (<20%). One hundred six (51.2%) cases showed high osteopontin expression, whereas in 101 (48.8%) of them osteopontin expression was low. Regarding the staining intensity, 62 tumors showed a weak immunoreactivity, 52 an intermediate staining, and 32 a strong staining (Table 1).

There was a highly significant association between osteopontin expression and tumor grade ($P = 0.001$): patients with poorly differentiated (grade 3) tumors showed a significantly lower osteopontin expression. On the other hand, there were no significant associations between osteopontin expression and any of the following variables: gender ($P = 0.24$), age ($P = 0.48$), histology ($P = 0.74$), T ($P = 0.55$), N ($P = 0.80$), stage ($P = 0.93$), and relapse ($P = 0.13$; Table 4).

In univariate analysis, neither osteopontin expression (OPN%; $P = 0.14$ for overall survival, $P = 0.074$ for disease-free survival) nor osteopontin staining intensity (OPN+; $P = 0.68$ for overall survival, $P = 0.44$ for disease-free survival) showed an association with patients' outcome (Table 1). However, as stated previously, in multivariate analysis for disease-free survival, one of the two covariates of the best statistical model was osteopontin expression ($P = 0.049$; Table 3).

Interestingly, in a sample of 163 patients with at least 6 years of follow-up (Table 5), we observed that for both overall survival and disease-free survival a high value of osteopontin expression was a significantly unfavorable prognostic factor ($P = 0.0085$ for overall survival, $P = 0.0023$ for disease-free survival).

Stage I patients (136 non–small cell lung cancer): association between clinicopathologic characteristics and survival. In the sample of stage I patients (136 cases), among the clinicopathologic characteristics, only tumor grade was significantly associated with disease-free survival ($P = 0.0059$). Indeed, none of the other variables showed a statistically significant correlation with outcome (Table 6).

Stage I patients (136 non–small cell lung cancer): association of osteopontin expression with clinicopathologic characteristics and survival. Osteopontin expression had a highly significant correlation with gender ($P = 0.006$) and tumor grade ($P = 0.00004$) and was significantly correlated with relapse ($P = 0.02$). In fact, a significantly lower osteopontin expression was observed in female (10 of 136) patients, in poorly

Table 5. Patients with at least 6 years of follow-up (163 cases): osteopontin expression as prognostic factor for overall survival and disease-free survival

Overall survival	Osteopontin low	Osteopontin high	*P*	Disease-free survival	Osteopontin low	Osteopontin high	*P*
Dead	37	49	0.0085	Relapse	40	54	0.0023
Alive	49	28		Free	46	23	

Table 6. Univariate analysis of the associations between prognostic variables and overall survival or disease-free survival in 136 cases of stage I NSCLC

Patient and tumor characteristics	No. cases	Overall survival *P* value	Disease-free survival *P* value
Gender			
Male	126	0.37	0.56
Female	10		
Age (y)			
≤65	64	0.48	0.47
>65	72		
Tumor grade			
1	39	0.070	0.0059
2	57		
3	40		
Histology			
Squamous	84	0.79	0.88
Nonsquamous	52		
Tumor size			
T_1	54	0.39	0.37
T_2	82		
Osteopontin expression (OPN%)			
Low (<0.2)	65	0.034	0.011
High (≥0.2)	71		
Osteopontin (OPN+)			
0	38	0.11	0.090
+	40		
++	36		
+++	22		

differentiated (grade 3) tumors, and in patients who did not relapse. On the other hand, there were no significant associations of osteopontin expression with age ($P = 0.25$) and histology ($P = 0.75$; Table 7).

Univariate analysis showed that osteopontin expression was significantly correlated with overall survival ($P = 0.034$) and disease-free survival ($P = 0.011$) (Table 6). In multivariate analysis, both Cox models contain only osteopontin expression. A high value of osteopontin expression is unfavorable to both survival (risk ratio, 1.88:1; $P = 0.037$; Table 8) and relapse (risk ratio, 2.08:1; $P = 0.013$; Table 9).

Table 7. *P* values of Mann-Whitney *t* test and Kruskal-Wallis tests comparing osteopontin expression and different clinicopathologic characteristics in 136 cases of stage I NSCLCs

	OPN%
Gender	0.006
Age	0.25
Histology	0.75
Grade	0.00004
Relapse	0.02

Figures 2 and 3 represent Kaplan-Meier curves for overall survival and disease-free survival, respectively. Patients were divided according to stage I or II-IIIA classification and according to high or low osteopontin expression. One can see that stage I patients were significantly split by osteopontin expression for both overall survival ($P = 0.034$) and disease-free survival ($P = 0.011$), whereas stage II-IIIA patients were not. In fact, stage I patients with high osteopontin expression had shorter overall survival and disease-free survival than those with low osteopontin expression.

Discussion

Osteopontin has long been implicated in the process of carcinogenesis, progression, and metastatic dissemination of several human tumors, such as breast (12, 13), prostate (15), colon (16), ovarian (17), gastric (18), and lung (14) cancers and more recently in many other tumors, such as pancreatic, renal, endometrial, esophageal, and head and neck carcinomas (30).

In the present study, we decided to focus on osteopontin protein expression detected by immunohistochemistry in a large sample (207 cases) of NSCLCs, because these tumors still have a poor prognosis in spite of the notable advances in diagnosis, staging, treatment, and biological characterization. We analyzed the correlations between osteopontin expression and many clinicopathologic variables to clarify its possible prognostic role. As far as we know, our study is the largest retrospective analysis of the prognostic role of osteopontin expression in patients with NSCLC treated with curative surgery. Moreover, we decided to give particular attention to NSCLC with stage I ($T_1/2N_0$) tumor, because a new prognostic factor might enable classification of such patients into different subsets corresponding to different risks of recurrence following complete resection.

In our work, osteopontin expression was analyzed as a dichotomous variable using the median value of 20% as the cutoff point to distinguish the samples with a high osteopontin expression (≥20%) from those with a low osteopontin expression (<20%). According to the criteria we adopted, we found that 106 (51.2%) NSCLC had a high osteopontin expression, whereas in 101 (48.8%) of them osteopontin expression was low.

We also evaluated the staining intensity, but only as a descriptive variable, in contrast to recent studies (24, 25, 30), because we considered a score that combines the percentage of immunoreactive tumor cells with their staining intensity as a subjective index. Regarding the relationship between osteopontin and clinical outcome in the whole series of patients (207 cases), we did not find a statistically significant correlation

Table 8. Multivariate analysis of overall survival according to Cox's model for stage I patients

	β	exp(β)	SE exp(β)	*z*	*P*
OPN% high	0.631	1.88	0.303	2.08	0.037

NOTE: Final model obtained with a backward stepwise regression. Likelihood ratio test = 4.53 on 1 *df*, $P = 0.033$, $n = 136$. Proportional hazards test $P = 0.09$.

Table 9. Multivariate analysis of disease-free survival according to Cox's model for stage I patients

	β	exp(β)	SE exp(β)	*z*	*P*
OPN% high	0.733	2.08	0.295	2.48	0.013

NOTE: Final model obtained with a backward stepwise regression. Likelihood ratio test = 6.6 on 1 *df*, $P = 0.010$, $n = 136$. Proportional hazards test $P = 0.11$.

between osteopontin expression either disease-free survival ($P = 0.074$) or overall survival ($P = 0.14$). This result contrasts in part with the conclusions of Schneider et al. (31) and Chambers et al. (14) concerning a statistically significant association between high osteopontin expression and shorter survival in NSCLC patients. These discrepancies could be explained by the fact that, although Schneider et al. assessed osteopontin expression levels by quantitative real-time reverse transcription-PCR analysis, which is a more sensitive and reproducible methodology compared with semiquantitative techniques, such as the immunohistochemical staining we used, the number of patients they analyzed (82 and 25 cases, respectively) was clearly lower than ours (207 patients). In contrast to us, Chambers et al. considered as osteopontin positive not only the tumors with immunoreactivity within tumor cells but also those with osteopontin immunostaining detected in tumor-infiltrating macrophages and necrotic areas; moreover, they evaluated as osteopontin immunopositive the tumors with ≥1% of the section showing osteopontin staining and as osteopontin negative those with <1% of the section showing osteopontin staining without using the median value of osteopontin expression as the cutoff value to distinguish tumors with high from tumors with low osteopontin expression, as we did.

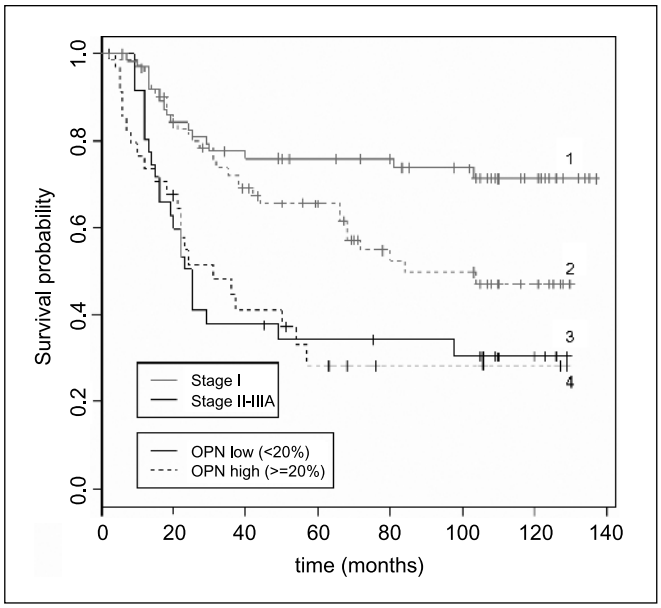

Fig. 2. Kaplan-Meier curves for overall survival. *Curve 1,* tumor stage I, low osteopontin (*OPN*) expression, mean survival (SE), 107 (6) months; *curve 2,* stage I, high osteopontin, mean survival (SE), 84 (6) months; *curve 3,* stage II-IIIA, low osteopontin, mean survival (SE), 56 (9) months; *curve 4,* stage II-IIIA, high osteopontin, mean survival (SE), 54 (9) months.

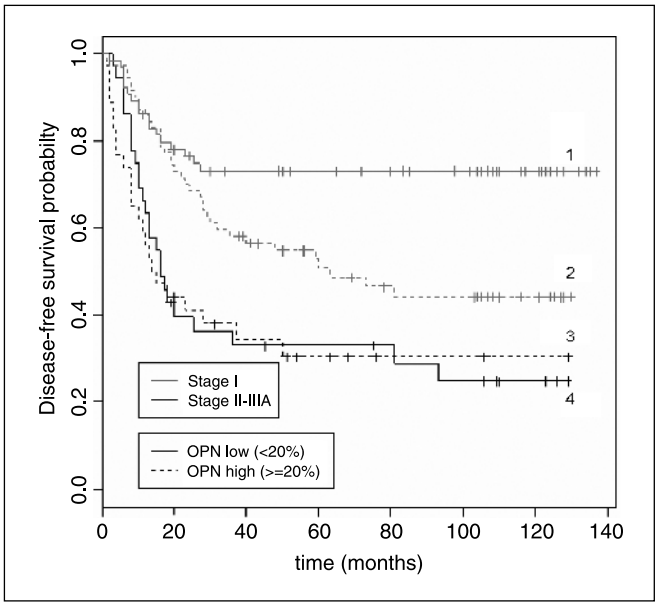

Fig. 3. Kaplan-Meier curves for disease-free survival. *Curve 1,* tumor stage I, low osteopontin expression, mean survival (SE), 104 (7) months; *curve 2,* stage I, high osteopontin, mean survival (SE), 73 (7) months; *curve 3,* stage II-IIIA, high osteopontin, mean survival (SE), 48 (9) months; *curve 4,* stage II-IIIA, low osteopontin, mean survival (SE), 48 (9) months.

Interestingly, in a sample of 163 patients with at least 6 years of follow-up, we observed that for both overall survival and disease-free survival a high value of osteopontin expression was a significantly unfavorable prognostic factor ($P = 0.0085$ for overall survival, $P = 0.0023$ for disease-free survival). This observation could be explained by the fact that this subgroup of patients is made mostly of stage I patients, whose osteopontin expression correlates with outcome. In fact, in this subgroup (stage I) of patients, osteopontin expression was significantly correlated with overall survival and disease-free survival in both univariate and multivariate analyses; therefore, a high value of osteopontin expression results unfavorable for both survival (risk ratio, 1.88:1) and relapse (risk ratio, 2.08:1).

As concerns the association between osteopontin expression and clinicopathologic variables, such as age, gender, histologic type, primary tumor size, involvement of regional lymph node, presence of distant metastases at diagnosis, histologic grading, staging, relapse during follow-up, and status (alive versus dead), we observed statistically significant correlations between osteopontin expression and grading both in the whole series ($P = 0.001$) and in the stage I subgroup ($P = 0.00004$), whereas only in the stage I patients did we notice associations of osteopontin expression with gender ($P = 0.006$) and relapse ($P = 0.02$).

The fact that patients with poorly differentiated (grade 3) tumors showed a significantly lower osteopontin expression than those with well-differentiated (grade 1) or moderately differentiated (grade 2) carcinomas could be explained by considering that in highly undifferentiated tumors the loss of cellular differentiation could be responsible for an increasing reduction of osteopontin protein expression.

The observation that osteopontin was expressed at lower levels in stage I female compared with male patients needs to be considered with caution, because in our study there was an

imbalance in sample collection between men (126 cases) and women (10 cases); this imbalance did not depend on the way we selected our case history, because we chose to study a cohort of patients who had consecutively undergone surgical resection.

Interestingly, we did not describe significant differences between histologic subgroups in terms of osteopontin expression either in the whole series of patients ($P = 0.74$) or in the stage I patients ($P = 0.75$). Our conclusion differs from those made by Zhang et al. (24), who observed a preferential osteopontin expression in squamous cell carcinomas (osteopontin immunoreactivity in 68.8% of squamous cell carcinomas versus 20.8% of adenocarcinoma), and from those of Shijubo et al. (25), who described a significantly worse prognosis of stage I adenocarcinomas compared with other groups, but matches that of the Schneider et al. study (31). The difference in the percentage of osteopontin-positive cancer cells in NSCLC histotypes in the various reports may be due to the difference in the percentage of various histologic subgroups in the different series; moreover, staining conditions, including the antibody used, may influence the results.

To sum up, in our study, a high osteopontin expression resulted as an unfavorable prognostic factor for relapse and outcome in stage I patients and is a valid variable to split this subpopulation into two groups for both overall survival and disease-free survival. This conclusion has notable importance in terms of both the biological characterization of early-stage tumors and new therapeutic opportunities.

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