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Enhanced Expression of SPARC/Osteonectin in the Tumor-associated Stroma of Non-Small Cell Lung Cancer Is Correlated with Markers of Hypoxia/Acidity and with Poor Prognosis of Patients[1]

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ABSTRACT

Secreted Protein Acidic and Rich in Cystein (SPARC)/osteonectin is a nonstructural matricellular protein involved in cell-matrix interaction during tissue remodeling and embryonic development. Using a novel monoclonal antibody (10-255), we examined immunohistochemically the patterns of SPARC expression in non-small cell lung cancer (NSCLC). High levels of SPARC in normal lung were confined exclusively to the bronchial cartilage. In NSCLC tissues, cancer cells were unreactive in 107 of 113 cases analyzed (95%), whereas substantial production of SPARC by stromal fibroblasts was noted in 42 of 113 cases (37%). Stromal SPARC was linked with tumor necrosis ($P = 0.01$) and, marginally, with node metastasis ($P = 0.07$), as well as with high levels of carbonic anhydrase 9 and LDH in cancer cells ($P = 0.0001$ and $P = 0.01$, respectively). SPARC was also coincident with enhanced levels of cancer cell differentiated embryo-chondrocyte expressed gene 1, hypoxia inducible factor 2α, and thymidine phosphorylase ($P = 0.01$, $P = 0.05$, and $P = 0.03$, respectively). Although endothelial reactivity for SPARC was noted only in small, immature vessels, SPARC production by stroma cells supported a high degree of vascular maturation (indicated by the presence of subendothelial lamina lucida). Survival analysis revealed a significant association of stromal SPARC with poor prognosis ($P = 0.006$), a finding that was also confirmed in multivariate models. In NSCLC, SPARC is selectively synthesized by the cells of the tumoral stroma. The strong association of this feature with markers of intratumoral hypoxia and acidity indicates an interesting link between cancer cell metabolism and the induction of a supportive stroma that favors cancer cell invasion and migration that lead to an ominous clinical outcome.

INTRODUCTION

SPARC, also termed osteonectin and BM-40, is a nonstructural matricellular 32-kDa glycoprotein (reviewed in Ref. 1). Human SPARC, located on chromosome 5q31–33 (2), is involved in cell-matrix interaction during tissue remodeling and embryonic development. SPARC is produced by chondrocytes, osteoblasts, and fibroblasts and can be associated with the extracellular matrix after binding to fibril-forming and basement membrane collagens (3). Various reports suggest inductive or inhibitory effects on epithelial and endothelial proliferation, because the function of SPARC is strongly dependent on the presence or absence of other stromal proteins, such as matrix metalloproteinases (4, 5). After the binding of SPARC to endothelial and smooth muscle cell surface proteins, tyrosine phosphorylation has been demonstrated, but whether specific SPARC receptors exist is unknown (6–8).

In a recent study of infiltrating breast carcinoma, SPARC was identified as the main protein specifically expressed by the juxtatumoral stromal cells, a finding indicating an important role in tumor invasion (9). In the present study, using a novel mAb, we examined the distribution of SPARC in NSCLC, and we investigated its association with several variables including tumor hypoxia, acidity, and angiogenesis.

MATERIALS AND METHODS

We examined 113 tumor samples from patients with early operable NSCLC[3] (stages T_{1-2}, N_{0-1}, M_0; 77 squamous and 36 adenocarcinomas). Paraffin-embedded material was obtained from the archives of the Department of Pathology, University of Oxford (United Kingdom). The same material has been used in previous studies involving the prognostic role of various oncoproteins and angiogenesis-related parameters (10–17). Forty-eight patients had T_1 stage and 65 had T_2 stage disease. Node involvement (N_1 stage) was present in 38 of 113 patients. Histological grade 3 (poorly differentiated neoplasms) was noted in 60 of 113, whereas the remaining cases were grouped in one category of grade 1/2 (well/moderate degree of differentiation). All patients were treated with surgery alone, without postoperative radiotherapy or chemotherapy. Patients dying within 60 days after operation were excluded to avoid bias from perioperative death. Survival analysis (overall survival) was performed in 102 of 113 patients. The follow up of patients at the time of analysis ranged from 60 to 2500 days (median 1237 days). For surviving patients, the median follow up was 1676 days (range 621 to 2500).

Although the cancer tissue samples examined were chosen from the tumor periphery, such that normal lung was included, specific conditions around the tumor (hypoxia, acidity, and high growth factor concentration) could have affected the SPARC-expression status in the adjacent lung. To avoid such a bias, we further assessed the expression of SPARC protein in 10 samples from apparently normal lung (located away from the tumor) obtained from patients who underwent pneumonectomy. These samples were retrieved from the archives of the Department of Pathology, Democritus University of Alexandroupolis (Greece).

Assessment of SPARC Protein Immunohistochemical Expression. The level of SPARC protein was assessed using the mouse anti-SPARC mAb (mAb 10-255).[4] Briefly, SPARC-null mice were immunized with recombinant human (rhu) SPARC (18), and splenocytes from the mice were harvested and fused with mouse myeloma P3 × 63AG8.653 cells (American Type Culture Collection, Rockville, MD) as described (19). Hybridomas were screened for reactivity with rhuSPARC by indirect ELISA using standard techniques. Clones showing strong ELISA reactivity toward rhuSPARC were further characterized for reactivity on paraffin-embedded sections of human testis, a tissue known to be positive for SPARC.

Staining for SPARC was performed on 3-μm paraffin sections, mounted on

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; mAb, monoclonal antibody; TBS, Tris-buffered saline; HIF, hypoxia inducible factor; DEC-1, differentiated embryo-chondrocyte expressed gene 1 protein; LDH, lactate dehydrogenase; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.

[4] R. A. Brekken, E. H. Sage, manuscript in preparation.

Table 1 *Antibodies, dilutions, and antigen-retrieval methods*

Primary antibody	Dilution (incubation time)	Antigen retrieval	Specificity	Source	Ref.
JC70 (CD31)	1:50 (30 min[a])	Protease XXIV	Endothelium	Dako, Denmark	10
ESEE 122	1:20 (90 min[a])	MW[b]	HIF-1α	Oxford University	11
EP 190b	Neat (90 min[a])	MW	HIF-2α	Oxford University	11
M75	1:20	MW	CA9	Oxford University	12
VG1	1:4 (90 min[a])	MW	VEGF	Oxford University	13
11B5	1:3 (60 min[a])	MW	VEGF/KDR	University of Texas Southwestern	13
P-GF.44C	1:4 (60 min[a])	None	TP	Oxford University	14
FGF-2(147)-6	1:20 (90 min[a])	MW	bFGF	Santa Cruz Biotech	15
Bek(C-17)-6	1:20 (90 min[a])	MW	bek-bFGFR	Santa Cruz Biotech	15
CW27	1:100 (overnight)	MW	DEC1	Oxford University	16
Ki67	1:50 (overnight)	MW	Ki67	Oxford University	17
Ab9002	1:200 (overnight)	MW	LDH-5	Abcam, United Kingdom	Unpublished

[a] At room temperature.
[b] MW, microwave heating.

poly-L-lysine-coated slides. Sections were dewaxed, rehydrated, and predigested with protease type XXIV (Dako, Denmark) for 15 min at 37°C. The mAb 10-255 was applied at room temperature overnight, at a concentration of 10 μg/ml. After washes in TBS, rabbit antimouse antibody (Dako, Denmark) at a dilution of 1:50 in TBS was applied for 30 min, followed by mouse alkaline phosphatase anti-alkaline phosphatase complex (DAKO, Denmark) 1:1 for 30 min. After washes in TBS, the last two steps were repeated for 10 min each. The color was developed by 20-min incubation with New Fuchsin solution (DAKO, Denmark).

The percentage of cancer cells with cytoplasmic SPARC reactivity was recorded after inspection of all fields in the tissue sample. The percentage of positive cells was recorded in each ×200 field, and the final score for each case was the median value obtained. The extent of staining for SPARC in the tumor stroma was obtained after the assessment of the percentage of ×200 optical fields exhibiting strong stromal reactivity (number of fields with high stromal reactivity for SPARC per number of fields assessed). Cytoplasmic SPARC reactivity in >50% of fibroblasts identified in an ×200 optical field was defined as "strong" stromal reactivity in the field.

Other Immunohistochemistry. For the tissue samples analyzed for SPARC reactivity, immunohistochemical data regarding a variety of proteins related to hypoxia (HIF-1α, and HIF-1β, and DEC-1) and tumor acidity (carbonic anhydrase-9, LDH-5), oxidative stress (thymidine phosphorylase), and angiogenesis (microvessel density, VEGF, VEGF-kinase domain receptor complex endothelial expression, bFGF, bek-bFGF-receptor) were available from previous studies (10–17). SPARC reactivity was examined in parallel tissue sections cut from the same tissue blocks used for previous immunohistochemistry studies. Table 1 shows the antibodies and details of the immunohistochemical procedures used to detect the expression of various oncoproteins and growth factors/receptors, as well as references in which the immunohistochemical methods have been extensively described. The methods used for staining, assessment, and grouping have been published previously (10–17).

Assessment of Necrosis. Tissue samples used for immunohistochemistry were chosen from the tumor periphery to examine the most viable parts of the tumor area and to avoid inner tumor areas where necrosis occurs more frequently. The percentage of optical fields (×200) with necrosis was recorded by two observers separately. Necrotic areas in >50% of the number of examined fields, which was the mean value of fields with necrosis given by the observers, were scored as extensive; necrotic areas in <50% were scored as limited (11–49%) or absent (0–10%). Even small areas of necrosis within a field characterized the field as necrotic.

Statistical Analysis. Statistical analysis and graphic presentation were performed using the GraphPad Prism 2.01 package (GraphPad, San Diego CA[5]). The Fisher's exact test, the χ^2 t test, or the unpaired two-tailed t test was used for testing relationships among categorical variables as appropriate. Spearman analysis was used to assess correlation among continuous variables. Survival curves were plotted using the method of Kaplan-Meier, and the log-rank test was used to determine statistical differences among life tables. A Cox proportional hazard model was used to assess the effects of patient and tumor variables on overall survival. A value of $P \geq 0.05$ was considered significant.

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RESULTS

Normal Lung SPARC Reactivity. SPARC protein was weakly expressed in the cytoplasm of normal bronchial epithelium (Fig. 1*a*), whereas type I and type II alveolar cells were unreactive. Alveolar vessels and the submucosa stroma (fibroblasts and vessels) were negative. Expression of SPARC was persistently found in chondrocytes of the bronchial cartilage (Fig. 1*b*). These patterns of expression were noted both in the normal lung adjacent to the tumor and in tissue samples from normal lungs obtained from sites distant from the tumor.

Patterns of SPARC Expression in NSCLC. SPARC immunoreactivity in cancer cells was decreased in comparison to that in the normal bronchi (Fig. 1*d*). High levels of cytoplasmic SPARC in cancer cells were rare (noted in 6 of 113 cases examined). However, when present, SPARC expressing cancer cells were located adjacent to necrotic areas.

In contrast to cancer cells, stromal fibroblasts were frequently and strongly reactive for SPARC (Fig. 1, *d* and *e*). The staining included the cytoplasm and less frequently the nuclei of fibroblasts, and the extracellular fibroblast matrix was also stained. Lack of SPARC stromal reactivity was noted in 32 of 113 (28.3%) cases. Extensive SPARC reactivity in 60–100% of the optical fields examined was noted in 42 of 113 (37.2%) cases, whereas an intermediate extent of SPARC stromal reactivity (10–50% of optical fields examined) was noted in 39 of 113 (34.5%) cases. The fibroblast extracellular matrix was also reactive with SPARC, whenever fibroblasts were seen to produce the protein.

A varying extent of strong staining was also noted in tumor infiltrating plasma cells, but not in tumor-infiltrating macrophages. Large intratumoral vessels were negative (Fig. 1*c*), whereas SPARC was frequently noted in endothelial cells of small immature vessels (Fig. 1*f*).

Association of SPARC with Histopathological Variables. High levels of SPARC production in the stroma were linked with extensive tumor necrosis ($P = 0.01$). There was a trend, although not statistically significant, for high levels of SPARC to correlate with node metastasis ($P = 0.07$). No association with tumor stage, histology type, histology grade, or Ki67 proliferation index was noted.

Association of SPARC with Hypoxia and Acidity Markers. Table 2 shows the association of SPARC with hypoxia and acidity. A particularly strong association of stromal SPARC with CA9 and LDH in cancer cells was noted ($P = 0.0001$ and $P = 0.01$, respectively). SPARC was also linked with high differentiated embryo-chondrocyte expressed gene 1 and hypoxia inducible factor 2α in cancer cells ($P = 0.01$ and $P = 0.05$, respectively).

Analysis of Survival and Association of SPARC with Angiogenesis. No correlation of SPARC with microvessel density, VEGF/kinase domain receptor activated microvessel density, or bFGF expression was noted. In contrast, a significant association with high

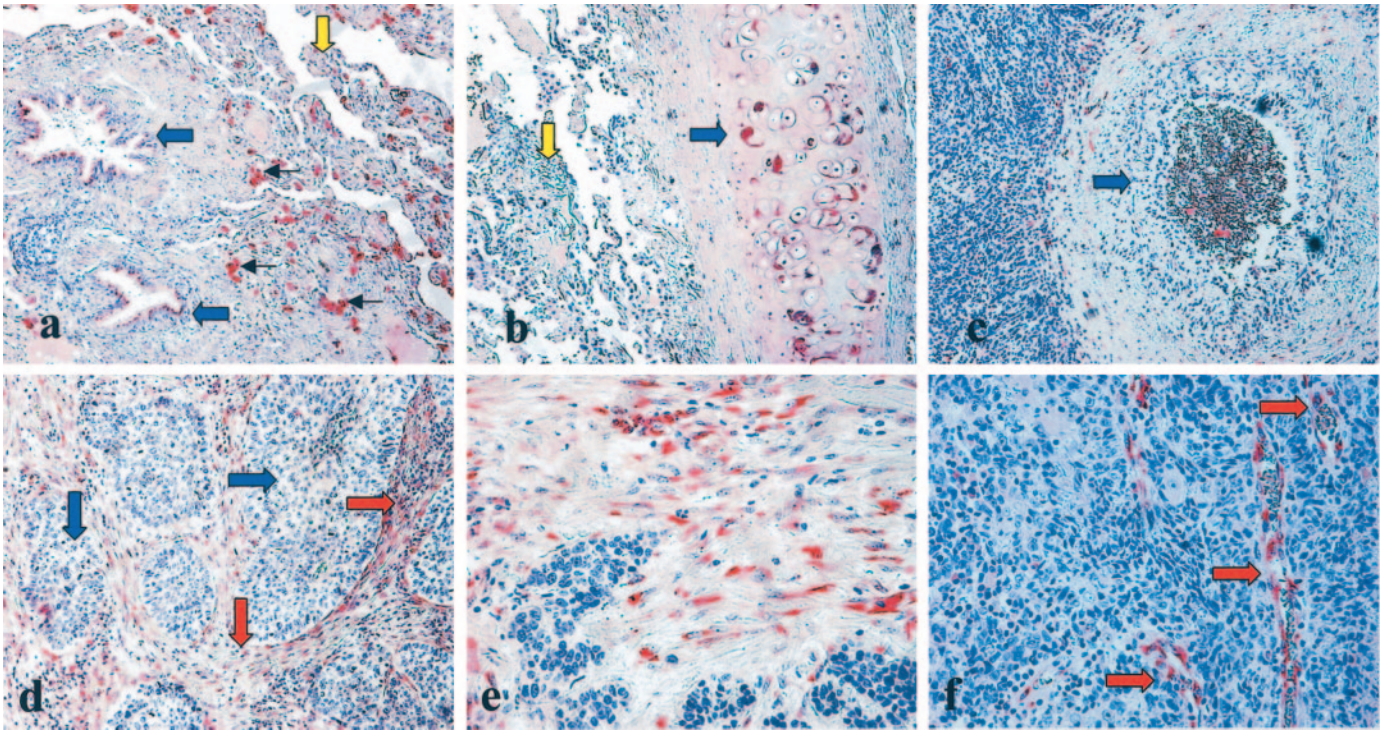

Fig. 1. Immunohistochemical expression of SPARC protein in normal and malignant lung tissues. *a*, normal bronchi and alveolar tissue adjacent to tumor; note the weak expression of SPARC by the bronchial epithelium (*blue arrows*) and the lack of SPARC reactivity in the alveolar cells (*yellow arrow*). Alveolar macrophages show a prominent SPARC reactivity (*black arrows*); ×200 magnification. *b*, strong expression (cytoplasmic and nuclear) of SPARC by chondrocytes (*blue arrow*), whereas adjacent alveolar tissue is negative (*yellow arrow*); ×200 magnification. *c*, mature large vessels did not express SPARC (*blue arrow*); ×200 magnification. *d*, prominent SPARC expression by stroma fibroblasts (*red arrows*) in a background of negative cancer cell reactivity (*blue arrows*); ×200 magnification. *e*, strong reactivity for SPARC in stromal fibroblasts; ×400 magnification. *f*, strong reactivity for SPARC in endothelial cells in immature small intratumoral vessels (*red arrows*) in a cancer cell nest that shows no reactivity with anti-SPARC IgG; ×200 magnification.

Table 2 *Association of SPARC in lung cancer stroma with proteins related to hypoxia and acidity in NSCLC*

	Relative levels of SPARC in tumor stroma			
	Absent	Intermediate	Extensive	*P*
HIF-1α				
Low	11	18	11	0.10[a]/0.15[b]
High	22	19	31	
HIF-2α				
Low	19	20	15	0.11[a]/**0.05**[b]
High	14	17	27	
DEC-1				
Low	24	25	19	**0.03**[a]/**0.01**[b]
High	9	12	23	
LDH-5				
Low	17	17	10	**0.03**[a]/**0.01**[b]
High	16	21	32	
CA-9				
Low	30	27	16	**<0.0001**
High	3	11	26	
Acidity[c]				
Low	15	15	0	**<0.0001**
High	18	23	42	
Thymidine phosphorylase				
Low	25	29	24	0.10[a]/**0.03**[b]
High	8	9	18	

[a] Refers to χ^2 comparing all three SPARC groups.
[b] Refers to Fisher's exact test for absent/intermediate vs. extensive SPARC reactivity.
[c] Low acidity refers to patients with low LDH and low CA-9 levels.
Bold, statistiscally significant.

levels of cancer cell thymidine phosphorylase was noted ($P = 0.03$; Table 2), as well as with a high LH39 vascular maturation index (Fig. 2).

Analysis of survival revealed a significant association of stromal SPARC with poor survival ($P = 0.002$; Fig. 3). In the several multivariate models tested, high levels of SPARC in the tumoral stroma retained a significant independent prognostic value as shown in Table 3.

DISCUSSION

The function of SPARC protein in the pathogenesis and behavior of human cancer is complex. Increased synthesis of SPARC in submucosal fibroblasts has been reported in experimental studies during the early steps of chemical carcinogenesis (20). SPARC protein is suppressed in mature human tissues. Unlike fetal colon, normal adult colon does not express SPARC, whereas a re-expression occurs in the stroma of colonic carcinomas (21). Similarly, SPARC is undetectable in normal hepatic and benign liver diseases, whereas substantial amounts of mRNA and protein are detected in the stroma of hepatocellular carcinoma (22). The stroma of gastric carcinomas express substantial levels of SPARC in contrast to benign stomach tissues, which do not (23). High levels of the SPARC protein consistently

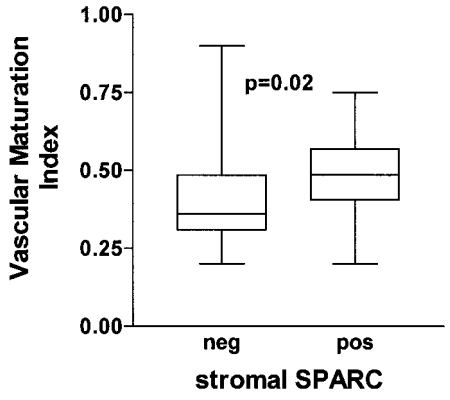

Fig. 2. Schematic representation of the vascular maturation index (LH39 immunostaining) in cases with positive and negative SPARC reactivity in the stroma. *Bars* represent the range, the edge of boxes represent the 25% and 75th percentile and the *line in the boxes* represents the median value.

occurring in invasive meningiomas are not seen in benign noninvasive meningeal tumors, data indicating a potential role of SPARC in tumor invasion (24). The putative function of SPARC as a mediator of prostate cancer cell invasion into bone has been also suggested by Jacob *et al.* (25).

The importance of SPARC protein in the pathogenesis and clinical behavior of NSCLC is unknown. In the present study we used a novel mAb, raised against recombinant human SPARC, to investigate the expression patterns of SPARC in normal lung, squamous lung carcinomas, and adenocarcinomas. In normal lung, SPARC was confined to the bronchial epithelium, showing a rather weak expression, whereas a strong reactivity was noted in the cytoplasm and nuclei of the bronchial cartilage. The expression of SPARC by chondrocytes has been reported (26, 27). Alveolar tissue and bronchial submucosa, including the relevant vasculature, were unreactive. This finding is in accordance with other studies showing a lack of SPARC expression in normal liver, colon, stomach, and kidneys (21–23, 28). Although an extensive study on SPARC in normal tissues is not available, it seems that expression of this protein may persist in some mature human tissues, such as ovarian and prostate epithelium, chondrocytes, and placental trophoblasts (29–31).

The weak cytoplasmic reactivity noted in the normal bronchial mucosa was abolished in the cancerous epithelium, regardless of the histology type and degree of tumor differentiation. Strong expression of SPARC in the cytoplasm of cancer cells was an exception (4% of cases), and when SPARC was present, it was confined in areas adjacent to necrosis. In direct contrast, the tumoral stroma was strongly reactive for SPARC in the majority of cases (72%). Stromal fibroblasts expressed SPARC both in the cytoplasm and nuclei, and the extracellular matrix also stained for SPARC exclusively in areas of these fibroblasts. Nuclear patterns of SPARC expression have also been reported in embryonic chick cells (32). Tumor-infiltrating macrophages were unreactive, whereas plasma cells showed various degrees of SPARC reactivity. These data indicate that the main source of stromal SPARC in NSCLC is the stroma fibroblasts. Indeed, experimental data show that SPARC is accumulated in the stroma and that collagen may serve as storage site for SPARC (32–34).

We examined whether the degree of stromal reactivity for SPARC was related to lung cancer growth and the metastatic potential. Stromal reactivity for SPARC was not related to the tumor burden or the Ki67 cancer cell proliferation index. Node metastasis was more frequent in SPARC-positive cases. A significant association of SPARC in stroma with extensive necrosis was also confirmed. This suggests that increased levels of SPARC in fibroblasts may be a secondary event triggered by unfavorable intratumoral conditions. This hypothesis is supported by the association of SPARC with tissue hypoxia and acidity (*i.e.* carbonic anhydrase 9, LDH, and the hypoxia inducible factors) in cancer cells. Overexpression of cancer cell thymidine phosphorylase, indicative of a strong intratumoral oxidative stress (35), also paralleled stromal SPARC up-regulation.

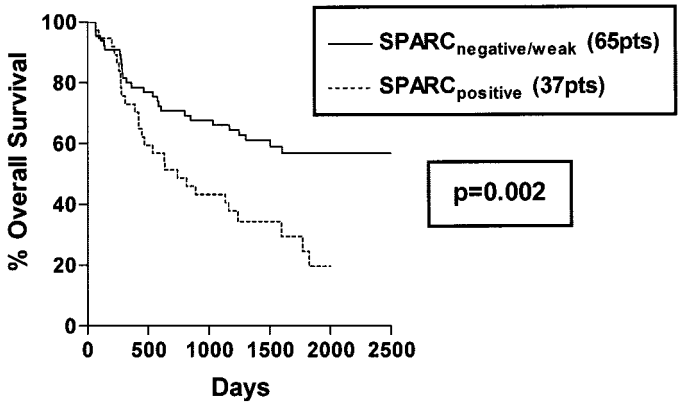

Fig. 3. Kaplan-Meier survival curves stratified for SPARC stroma reactivity.

Table 3 *Multivariate analysis of death events*

	Multivariate 1[a]		Multivariate 2[b]	
	P	*t*	*P*	*t*
Parameter				
SPARC	0.02	2.24	0.01	2.51
Tumor stage	0.21	1.25	0.27	1.01
Nodal stage	0.006	2.78	0.03	2.18
Histology	0.28	1.06	0.13	1.51
Grade	0.77	0.28	0.54	0.60
HIF1-α			0.69	0.39
HIF2-α			0.40	0.83
CA-9			0.23	1.21
LDH			0.60	0.51
MVD			0.34	0.94
aMVD			0.004	2.96
VEGF			0.32	0.99
TP[c]			0.70	0.38

[a] Model 1 comprises SPARC together with the traditional histological variables.
[b] Model 2 comprises all the variables examined in the study.
[c] TP, thymidine phosphorylase.

The intratumoral vascular density and the expression of the angiogenic factors VEGF and bFGF were apparently not related to production of SPARC in the stroma. Surprisingly, high stromal SPARC was linked to a high density of mature vessels, as assessed with the LH39 mAb recognizing the lamina lucida of vessels. Large mature vessels structurally identified in the normal and in malignant lung tissue, however, were unreactive (both endothelium and vascular sheath). On the contrary, small immature intratumoral vessels exhibited strong endothelial reactivity for SPARC, even in the absence of stromal fibroblast reactivity. These findings suggest a complex role for SPARC in the development of tumoral vasculature. It could be suggested that SPARC is important during the first steps of endothelial cell migration and growth into tube-like structures, where an immature status of the endothelium is demanded. SPARC may no longer be necessary when vessels reach a certain degree of maturation. Similar findings have been reported by Porter *et al.,* who found strong reactivity in endothelial cells involved in tissue repair (31). On the other hand, high levels of stromal SPARC seem to favor the maturation process of vessels or may promote vascular survival under the unfavorable hypoxic and acidic intratumoral conditions in which SPARC is increased. Whether the previously reported endothelial cell proliferation inhibition by SPARC is a step toward vascular maturation requires further investigation (36).

With respect to the prognostic relevance of SPARC in the stroma of tumors, patients bearing SPARC-positive tumors had a significantly poorer postoperative overall survival. The prognostic role of SPARC was independent of tumor and nodal stage, histology, and differentiation. Moreover, the prognostic role of SPARC was independent of all of the angiogenesis- and hypoxia-related variables assessed in the study. The important adverse effect of stromal overexpression of SPARC in the prognosis has been also confirmed in bladder cancer (37).

We conclude that the SPARC protein strongly characterizes the tumor-associated stroma of lung carcinomas, a feature that is absent from normal lung. The strong association of SPARC reactivity in the stroma with markers of hypoxia and intratumoral acidity suggest an interesting link between cancer cell metabolic particularities and induction of a supportive stroma that favors cancer invasion and migration. Evidence also favors the importance of SPARC stromal activity as a prognostic marker in lung cancer.

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