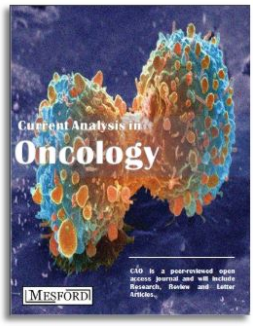
Retrospective Study to Examine the Relationship between Secreted Protein Acid and rich in Cysteine Expression and Prognosis in Lung Cancer Using Surgical Resection Specimens



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Abstract:

An extracellular matrix non-structural secretory glycoprotein called secreted protein, acidic and rich in cysteine (SPARC) plays a significant role in promoting cancer cell activity and altering a tumor's microenvironment. Studies of SPARC expression in lung cancer have been few compared to those of other organ malignancies. Consequently, we performed this study to elucidate the relationship between SPARC expression of a resected tumor and a post-operative prognosis.

Methods: Postoperative patients with pulmonary squamous carcinoma or adenocarcinoma at Iwate Medical University Hospital from 2008 to 2012 were enrolled in this analysis. Operations enrolled patients underwent were restricted to lobectomy and lymph node dissection. SPARC expression in formalin-fixed paraffin-embedded sections obtained from specimens by surgical removal were evaluated by immunohistochemistry using a SPARC antibody. Data on patients' characteristics and survival were collected from individual medical records.

Result: A total of 152 patients (87 adenocarcinoma and 65 squamous) were eligible for this analysis. Compared to cancer cells, the tumor stroma was predominately stained by an anti-SPARC antibody in immunohistochemical staining. The positive SPARC expression rate in squamous cell carcinoma and adenocarcinoma was 97% (63/65) and 62% (54/87), respectively. High SPARC expression of squamous cell carcinoma was related to poorer survival, but not of adenocarcinoma. This result was also observed in stage I squamous cell carcinoma. Conclusion: SPARC was higher expressed in squamous cell carcinoma than in adenocarcinoma. High expression of SPARC in squamous cell carcinoma was related to a poor prognosis. This suggests SPARC may be a prognostic marker of pulmonary squamous cell carcinoma.

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Keywords:
SPARC, NSCLC, adenocarcinoma, squamous cell carcinoma, post-operation, immunohistochemistry, prognosis, stroma

INTRODUCTION

An acidic cysteine rich secreted protein (SPARC), also called BM - 40 or osteonectin, is a glycoprotein with a molecular weight of 43,000 that was first discovered as a bone-specific protein. SPARC is involved in tissue repair [1], cell differentiation [2], cell proliferation [3], cell migration [4], and angiogenesis [5] through interactions with the extracellular matrix and cytokines. SPARC is highly expressed in tissues, including bone and teeth, which need remodeling in response to aging and external stimulation. In adult, SPARC is expressed in bone, kidney, testis, ovary, hematopoietic tissue, the central nervous system and cochlea. In cancer tissues, SPARC is expressed in fibroblasts of tumors [6]. SPARC is considered to promote tumor growth, invasion, and vascular hyperplasia in the tumor through cytokines, and metalloproteases [7, 8].

Studies of SPARC in several malignancies including human breast cancer has been actively performed. Increased SPARC expression is related to aggressiveness and may serve as a prognostic factor for triple-negative breast cancer [9]. An association between SPARC and lung cancer has been shown in in vivo studies in the mouse [10]: SPARC-transfected adenocarcinoma cells subcutaneously inoculated in the mouse developed more rapidly than untransfected cells. In a human study, patients having a lung tumor showing a high expression of SPARC as evaluated by immunohistochemical analysis showed a poorer prognosis [11].

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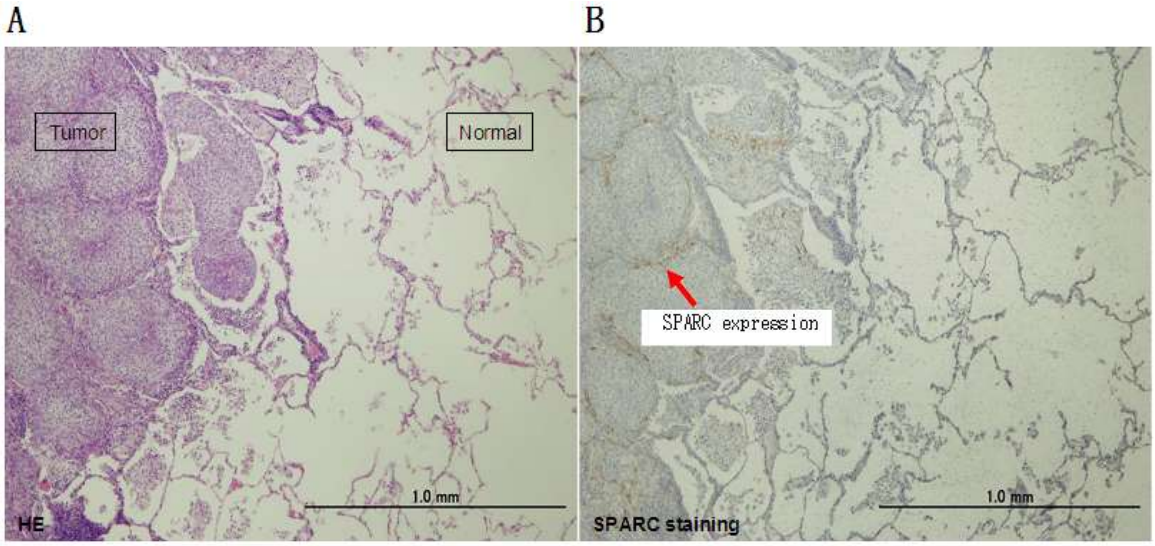


Fig. (1). Hematoxylin & eosin (HE) staining showed tumor tissue adjacent to normal lung tissue in a resected adenocarcinoma section (A). Immunohistochemical staining for SPARC expression revealed expression in the tumor's stromal tissues (arrow) (B). The immunostain was counterstained using resorcin–fuchsin and hematoxylin. Scale bar, 1.0 mm. This specimen was specifically stained in stroma, 0-25 % of tumor, with high intensity (total score = 3).

This current study analyzed differences in SPARC expression among histological types of lung cancer and evaluated the relationship between the expression of SPARC and the prognosis among histological types. This study will add new inspects to our knowledge of relation between SPARC expression in lung tumor and prognosis and may to lead to a new therapeutic strategy targeting SPARC in lung cancer.

MATERIALS AND METHODS

Patients

In this study, both patients with pulmonary squamous carcinoma receiving surgery from 2008 to 2012 and patients with pulmonary adenocarcinoma receiving surgery from 2011 to 2012 at Iwate Medical University Hospital were enrolled. Eligible patients were selected according to the following main criteria: Patients have a stage IA, IB, IIA, IIB or IIIA non-small cell lung cancer. Histologic types other than adenocarcinoma or squamous cell carcinoma were excluded. Multiple mediastinal lymph node involvement (multiple N2) and extensive mediastinal lymph node involvement (bulky N2) were excluded. Surgical procedures were restricted to lobectomy and lymph node dissection.

Immunohistochemical Staining

Paraffin was removed from formalin-fixed paraffin-embedded sections and the sections exposed to a mouse anti-SPARC monoclonal antibody (AON-5031, Santa Cruz, Dallas, TX, USA) as a primary antibody, followed by a secondary antibody (histofine simple stain MAX-PO [Multi] animal species: goat). After sections were washed with phosphate buffered saline (PBS), slides were developed in 3,3-diaminobenzidine solution. For the evaluation of SPARC expression in lung cancer lesions, the expression intensity and ratio were evaluated in accordance with a previous paper. The evaluation of SPARC expression in lung tumors was carried out as follows: The Intensity of positively staining cells was defined as: negative intensity = 0, weak = 1, moderate = 2, high = 3. The percentage of positively stained cells among total cells in the tumor was defined as 0% = 0, 0 – 25% = 1, 25% – 50% = 2, 50% – 75% = 3, 75% – 100% = 4. The total score (0 – 7) was characterized as the sum of both intensity and the percentage of staining. A total score of 0 – 2 was defined as a weakly expressing SPARC group and a total score of 3 – 7 was defined as a highly expressing SPARC group (Fig. **1**).

Comparison of Survival

Survival analysis was performed using the method of Kaplan and Meier. The differences between the survival curves were assessed using a log–rank test. Each ratio among groups was evaluated with Fisher's chi-square test. SPSS (IBM, Armonk, NY, USA) was used for the statistical analysis of our data.

RESULTS

Patients and expression of SPARC

Patients with adenocarcinoma or squamous cell carcinoma and who underwent surgery during defined periods were enrolled in this study. Excluded cases included 25 patients with pulmonary adenocarcinoma due to an inappropriate specimen; 26 adenocarcinoma and 13 squamous carcinoma cases were excluded as they underwent only a partial resection. One hundred and fifty-two patients (87 adenocarcinoma and 65 squamous) were eligible for analysis in this study (Fig. **2**). All enrolled patients were Japanese. The total SPARC expression rate of pulmonary adenocarcinoma was 62% (54/87). Whereas, the total SPARC expression rate of squamous cell carcinoma was 97% (63/65), with most positive cells in the tumor stroma. A large proportion of squamous cell carcinoma cases stained negatively (Table **1**). The distribution of staining scores among histological types are shown in Supplementary Figs. **1A** & **B**. Fig. (**3**) shows a Kaplan–Meier curve of postoperative survival

for all patients, including both histological subtypes. The weak SPARC expression group showed a significantly longer survival than the high expression group (log-rank test p = 0.002).

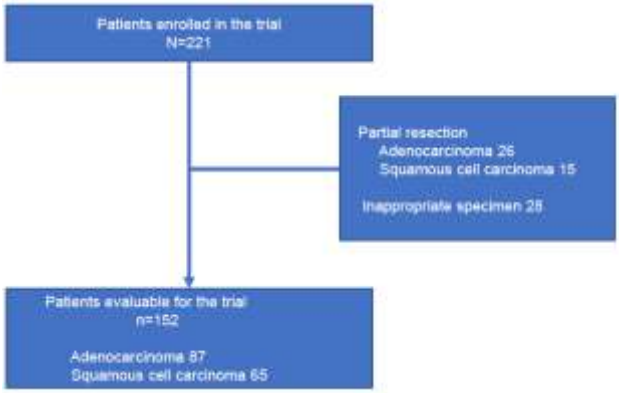


Fig. (2). Two hundred and twenty-one patients had adenocarcinoma or squamous cell carcinoma and received surgery during defined periods. Excluded cases were 25 patients with pulmonary adenocarcinoma who had an inappropriate specimen, 26 cases with a partial resection, and 13 patients with a pulmonary adenocarcinoma. One hundred and fifty-two patients (87 adenocarcinoma and 65 squamous) were eligible for the analysis of survival and background factors.

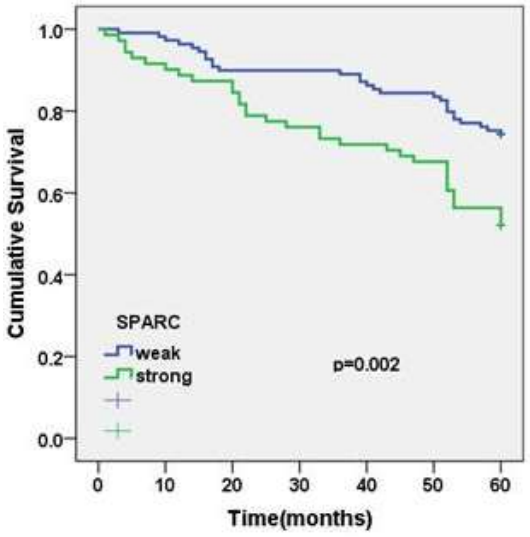


Fig. (3). Kaplan–Meier curve of survival for all patients showed a better prognosis for the weak (blue) SPARC expression rather than the high SPARC expression group (green). The P value was determined using a log-rank test.

Adenocarcinoma

In an analysis between clinical characteristics of cases with adenocarcinoma and SPARC expression, a significant relationship with smoking history, postoperative pathological stage, or invasiveness was not noted (Table **2**). However, younger and male patients more frequently showed high SPARC expression tumors (Table **2**). Though an imbalance of age may act as a better prognosis for the low expression group, fortunately it was considered as less of an influence due to the lack of significant difference found in survival in this study. In terms of survival analysis, the existence of an EGFR mutation is crucial. Twenty-seven EGFR mutation positive cases (exon 19 deletion: 15 cases, exon 21 L 858 R: 11 cases, exon 18 G 719 A: 1 case) existed among adenocarcinoma cases. A significant association between EGFR mutation positive lung cancer and SPARC expression was not noted.

Table1. Sites of Expression Among Histological Types.

SPARC Expression	Adenocarcinoma	Squamous Cell Carcinoma
Stroma	54 (62%)	61 (94%)
Cell + Stroma	7 (8%)	2 (3%)
Positive	61 (70%)	63 (97%)
Negative	26 (30%)	2 (3%)

Table 2. Characteristics of Cases with Adenocarcinoma.

		SPARC		
Clinical Parameters		**Weak**	**Strong**	**P value**
Age.				006
	＜65	18	13	
	≧65	15	41	
Sex				0.002
	Male	8	32	
	Female	25	22	
Smoking history				0.652
	No	22	33	
	Yes	11	21	
Pathological Staging				0.096
	I	19	41	
	II-IV	14	13	
Invasion				0.269
	none	18	22	
	invasive	15	32	
EGFR Mutation				0.630
	positive	5	22	
	negative	10	30	
	unknown	18	2	

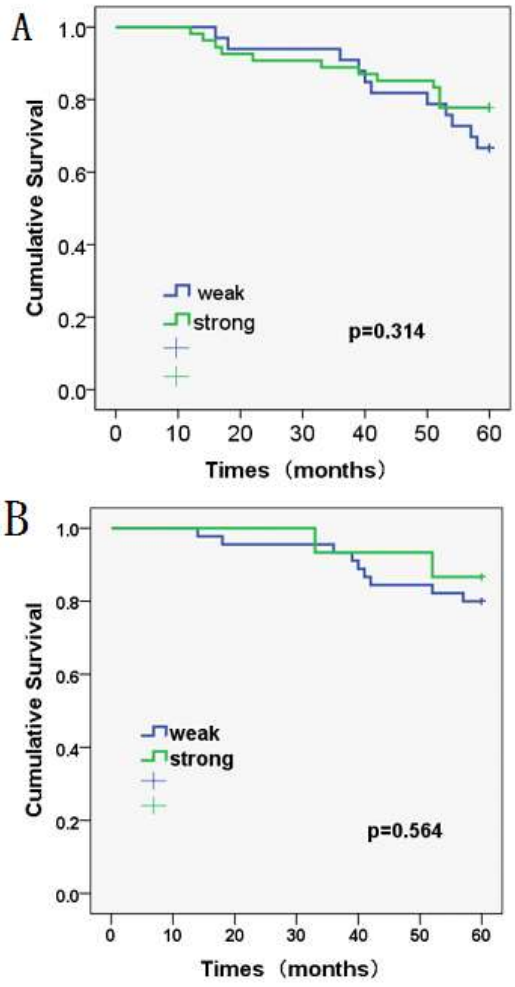


Fig. (4). Kaplan–Meier curves for all patients (A) and stage I patients (B) with adenocarcinoma showed a difference between high (green) or weak SPARC expression (blue), with weak SPARC expression associated with longer survival. The P value was determined using a log-rank test.

In the adenocarcinoma group, 33 cases showed weak expression of SPARC and 54 cases had high expression (Table **2**). SPARC was expressed mainly in the stromal tissue in the tumor (Table **1**). SPARC expression only in stromal tissue was observed in 47 (54%) cases while positive expression in tumor cells was seen in 7 (8%) cases, were also all positive for the stroma. Negative staining in both tumor cells and the stroma was observed in 33 (38%) cases. The postoperative survival curve for adenocarcinoma showed a lack of significant difference between the weak and high expression groups (50.2 months, 50.5 months, respectively, p = 0.314; Fig. **4A**). A significant difference was similarly not seen in stage I patients (p = 0.564; Fig. **4B**). Thus, in adenocarcinoma, it was postulated that SPARC expression in tumor tissue was not related to the prognosis after surgery.

Squamous Cell Carcinoma

In squamous cell carcinoma, 65 eligible cases were evaluated for survival and patients' characteristics. A significant association was not observed between SPARC expression and smoking history, postoperative pathological stage, age, sex, and lymph node metastasis, except pathological stage ($p < 0.01$; Table **3**). This suggests that the increased expression of SPARC was likely associated with stage progression. In patients with squamous cell carcinoma, weak expression of SPARC was found in 26 cases and high expression in 39 cases. The total SPARC expression rate for squamous cell carcinoma was 97% (63/65). SPARC was expressed mainly in the stroma in squamous cell carcinoma similar to adenocarcinoma cases (Table **1**). Positive SPARC expression in only the tumor stroma was found in 61 (94%) cases, while positive staining in both the stroma and tumor was found in 2 (3%) cases. No cases showed positive staining just in tumor cells while negative staining for both stroma and tumor cells was observed in only 2 (3%) cases. The survival rate of pulmonary squamous cell carcinoma in high SPARC expression group was significantly inferior to that in weak expressing groups (52.5 months, not reached, respectively; P = 0.01; Fig. **5A**). However, because more stage I patients in the weak SPARC expression group may have been associated with a better prognosis, a comparison of survival in stage I squamous patients is needed. In stage I patients, the high expression group also achieved longer survival than the weak expression group (41.2 months, not reached, respectively P = 0.07; Fig. **5B**). Consequently, the high expression of SPARC was considered to be related to a bad prognosis after surgery in squamous cell carcinoma cases.

Table 3. Characteristics of Cases with Squamous Cell Carcinoma.

		SPARC		
Clinical Parameters		**Weak**	**Strong**	**P value**
Age				0.057
	<65	3	7	
	≧65	23	32	
Sex				0.871
	Male	23	35	
	Female	3	4	
Smoking history				0.772
	No	1	1	
	Yes	25	38	
Pathological staging				<0.001
	I	20	11	
	II-IV	6	28	

DISCUSSION

In this study, the association between the expression of SPARC in adenocarcinoma and squamous cell carcinoma and

the prognosis of postoperative patients was evaluated. SPARC is thought to be involved in development, repair of impairment, and remodeling of tissue; however, its function has never been clear. This current study showed that SPARC was more highly expressed in squamous cell carcinoma than in adenocarcinoma and that expression of SPARC in squamous cell carcinoma was more associated with longer survival than in adenocarcinoma.

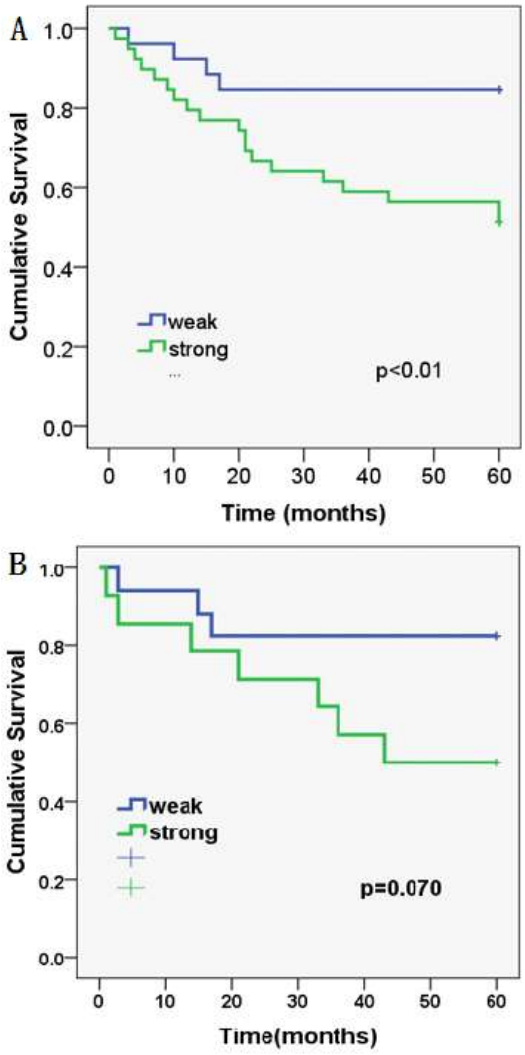


Fig. (5). Kaplan–Meier curves of survival for all patients (A) and stage I patients (B) with squamous cell carcinoma showed longer survival in the weak SPARC expression group (blue) compared to the high SPARC expression group (green). P values were determined using the log-rank test.

In the present study, the expression rates of SPARC in squamous cell carcinoma and adenocarcinoma were found to be 97% and 62%, respectively.

Previous studies showed that the positive rate of SPARC expression in pulmonary squamous cell carcinoma was 75% while that in pulmonary adenocarcinoma was 43% [11]. Our present study was thus consistent with previous studies.

Regarding the distribution of SPARC expression, previous studies reported that SPARC was predominantly expressed in the tumor stroma compared to tumor cells (72%, 4%, respectively) [12]. In our study, positive staining cells were mostly found in the stroma while positive tumor cells made up only 8% of cells in adenocarcinoma and 3% in squamous cell carcinoma. In terms of clinical characteristics, we found that older and male cases showed a higher expression of SPARC. Zhang et al. examined the correlation of SPARC expression in normal and cancer tissues with age, sex, histopathological type, tumor size, tumor differentiation, T-stage, and N-stage of patients. That study found that SPARC expression was significantly higher in patients at stages III–IV compared with those at stages I–II, but no significant correlation with other characteristics, including age and sex, were found [13]. In our study, higher expression was observed more frequently in cases of stage II–IV squamous cell carcinoma. This result was consistent with the previous study. In another study, SPARC expression was significantly higher in patients with a smoking history [11]; however, our study did not show a significant relationship between SPARC expression and smoking status.

In terms of an association between the expression of SPARC and survival benefit, our study demonstrated that high expression in squamous cell carcinoma was related to a significantly poorer prognosis, while a relationship did not exist in adenocarcinoma. Koukourakis et al. reported a significant correlation between SPARC expression in tumor stroma and a poor prognosis [12]. Similar relationships in other organ malignancies have been reported. In head and neck cancer, which is mostly squamous cell carcinoma, high SPARC expression was associated with a shorter disease-free interval and overall survival [14]. In tongue cancer, SPARC expression was significantly higher in tissues with lymph node metastasis than those without metastasis, and significantly higher in the primary tumor with high recurrence rates than those not showing a recurrence [15]. In esophageal squamous cell carcinoma, high SPARC expression was also correlated with lymph node metastasis and a poor prognosis [16]. Many reports describe a relationship between the high expression of SPARC in a variety of squamous cell carcinoma and poor survival, compatible with our results.

In comparison, it seems that SPARC expression in tumor tissues may not be correlated with the prognosis in cases of pulmonary adenocarcinoma. A retrospective study by de Kruijf et al. analyzing the prognosis based on the examination of surgical specimens from 574 patients of early stage breast cancer showed that SPARC expression was higher in tumor stroma than in normal cells; higher expression of SPARC occurred in metastatic lymph nodes than in non-metastatic lymph nodes. They also reported that the tumor stroma ratio is a negative prognostic factor for triple-negative breast cancer [17, 18]. In pancreatic cancer, SPARC is highly expressed predominantly in the tumor stroma, and its overexpression is associated with a poor prognosis [19]. In gastric cancer, SPARC was highly expressed in diffuse-type gastric cancer. In stages I, II, and III, the 5-year survival rates of cases with high SPARC expression in the tumor were significantly lower than those of low expression. By contrast, SPARC expression did not correlate with a 5-year survival rate in patients at stage IV. Furthermore, upregulation of SPARC was one of the independent prognostic factors for gastric cancer [20]. In the

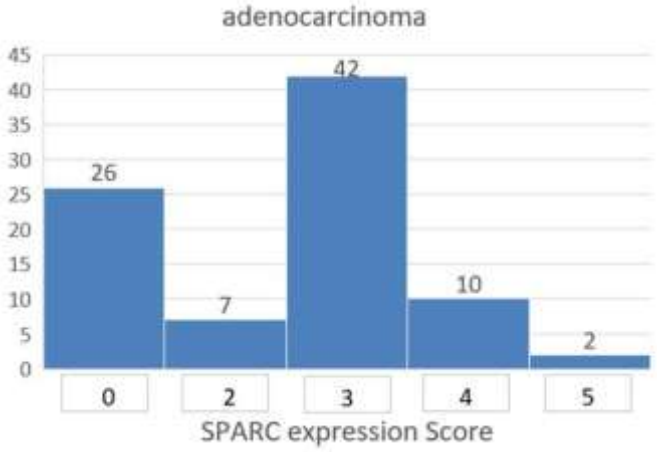
case of adenocarcinoma in a variety of organs, whether high expression of SPARC becomes a poor prognostic factor or not may depend on the primary tumor site and staging. In our study, metastatic cases had been excluded and the prognosis was evaluated only in operable NSCLC cases. Further studies of metastatic stage cases are needed to clarify relationship between the expression of SPARC and prognosis.

In our study, higher expression of SPARC in the stroma was observed in more advanced stages of squamous cell carcinoma. The reactivity of the SPARC antibody to the stroma in vitro has been found not to be related to tumor burden or the proliferation index of Ki 67-positive cancer cells. A significant correlation was confirmed between SPARC expression in the stroma and excessive necrosis. This suggests that the elevation in the level of SPARC expression in fibroblasts is a secondary event due to conditions developed by tumor growth [12] This hypothesis is supported by the association of SPARC expression with hypoxia and acidity in the tissues of cancer cells [12, 21]. SPARC expression in the stroma increases in the presence of hypoxia and oxidative stresses. Consequently, high expression of SPARC promotes the survival of tumor blood vessels even under unfavorable conditions inside the tumor such as hypoxia and high acidity. Thus, it seems that tumor environments as described above are likely to be established in pulmonary squamous cell carcinoma, which tends to form necrotic areas and cavities.

In conclusion, this study showed that SPARC is predominantly expressed in pulmonary squamous cell carcinoma and is an important prognostic factor for operable pulmonary squamous cell carcinoma. This study is a retrospective study that includes various uncontrolled biases. Further prospective studies are warranted.

CONFLICTS OF INTEREST

The authors certify that the work submitted herewith is in full accordance with the editorial policy and ethical considerations set forth in the prerequisites for publication in Current Analysis in Oncology. All authors of this manuscript do not have any conflicts of interest.



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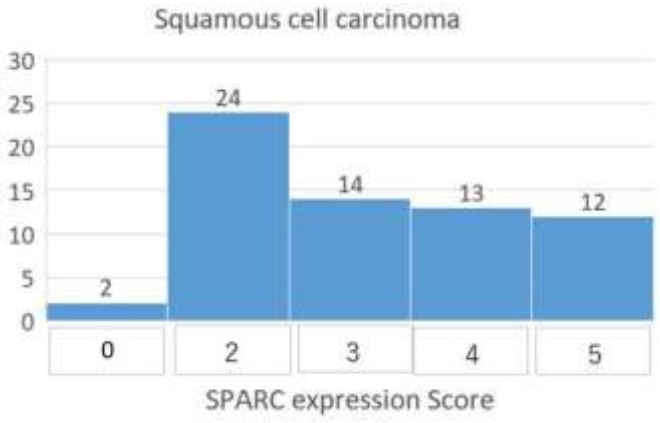

1B

Supplementary figure 1. Histogram of frequency each intensity of immunohistochemistry SPARC in adenocarcinoma (**A**) and squamous cell carcinoma (**B**).

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