Correlation between secreted protein acidic and rich in cysteine protein expression and the prognosis of postoperative patients exhibiting esophageal squamous cell carcinoma

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Received July 22, 2016; Accepted April 27, 2017

DOI: 10.3892/mmr.2017.6959

Abstract. The aim of the present study was to investigate the association between the expression level of secreted protein acidic and rich in cysteine (SPARC) and the prognosis of post-operative patients with esophageal squamous cell carcinoma (ESCC). The expression level of SPARC was detected in the 89 ESCC tissue cases and 100 healthy esophageal mucosa cases, which served as the controls. Immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) were employed to evaluate the SPARC expression in cases with ESCC. RT-PCR demonstrated that the positive rates of SPARC mRNA expression in ESCC were 71.91% (64/89). The positive rates of normal esophageal mucosa mRNA expression were 15.00% (15/100), which were significantly lower than that in the ESCC tissue samples. The difference was statistically significant (P<0.001). Immunohistochemical staining indicated that the positive expression rate of SPARC protein in the ESCC tissue samples was significantly higher than that in the esophageal mucosa tissue samples (65.17 vs. 8.00%; P<0.001). The expression of SPARC protein was negatively correlated with lymph node metastasis (P<0.05), which was not associated with the pathologic gross morphology, tumor differentiation degree or other clinical features. The survival of patients with ESCC was not associated with the expression level of SPARC protein (P>0.05), but was associated with the tumor location (P<0.05), differentiation (P<0.001) and staging (P<0.05). Thus, SPARC mRNA and protein were highly expressed in ESCC, and negatively correlated with lymph node metastasis, which was not associated with postoperative survival of ESCC patients. Thus, detection of SPARC mRNA and protein expression levels may facilitate early diagnosis and prognosis assessment of ESCC.

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Key words: secreted protein acidic and rich in cysteine, esophageal squamous cell carcinoma, immunohistochemistry, prognosis

Introduction

Esophageal cancer is a common type of digestive tract cancer, and the province of Jiangsu is a high incidence area (1-3). Esophageal squamous cell carcinoma (ESCC), esophageal adenocarcinoma (EA) and small cell carcinoma of the esophagus are the most common pathological types of esophageal cancer. The high incidence of ESCC in China is significantly different from that of the European and American countries (4,5). Surgical resection is the first choice of treatment for patients with early esophageal cancer, but the majority of patients experience recurrence or metastasis following surgery; therefore, it is of great significance to investigate the relevant factors that affect the prognosis of postoperative survival (6-8).

Secreted protein acidic and rich in cysteine (SPARC) is a small protein rich in cysteine, which is also known as basement-membrane protein 40 (9-11). As a non structural matrix glycoprotein its function is very complex, and it is involved in many physiological and pathological processes (12,13). In addition, it is significant in the microenvironment of tumor cell activity and tumor growth (14). It was observed that the SPARC protein was highly expressed in the fibrous cells and endothelial cells associated with invasive malignant tumors. The expression level of SPARC was closely associated with the occurrence, development and prognosis of tumors (13,15,16).

To investigate the association between the expression of SPARC and the prognosis of postoperative patients with ESCC, immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) were employed to measure SPARC protein expression levels in cases with ESCC, and in healthy esophageal mucosa samples, which served as the control. In addition, the underlying mechanism of the formation of ESCC was evaluated in an attempt to establish a novel method for its early diagnosis.

Patients and methods

From January 2013 to January 2016, samples of ESCC were collected from 89 patients who underwent surgical resection

Table I. Primer sequences for reverse transcription-polymerase chain reaction analysis.

Primer	Primer sense	Primer sequences 5'-3'	Product size (bp)
Secreted protein acidic and rich in cysteine	Forward	CTGCTGGCAGACAACAGGTA	344
	Reverse	CTGTTTGCTGCTGTGGAAAA	
β-actin	Forward	TGACGTGGACATCCGCAAAG	231
	Reverse	CTGGAAGGTGGACAGCGAGG	

at the First People's Hospital of Yancheng City (Yancheng, China) who had been diagnosed by clinical pathology. Each case had detailed clinical and pathological data and none had received preoperative chemotherapy or radiotherapy. The ESCC patients included 45 males and 44 females (aged 36-73 years; mean age, 53.9±11.6 years). A total of 100 cases with heathy esophageal mucosa were selected from the First People's Hospital of Yancheng City (Yancheng, China) and served as a control group. These included 55 males and 45 females (aged 35-69 years; mean age, 49.5±10.4 years).

No statistically significant differences were detected in age between the ESCC group and the healthy esophageal mucosa group. All specimens were obtained following receipt of informed consent with approval by the Ethics Committee of the First People's Hospital of Yancheng City (Yancheng, China) [ID no. HMU (Ethics) 20131103].

Immunohistochemical staining techniques. The immunohistochemical staining method from Agilent Technologies, Inc. (Santa Clara, CA, USA) was used to detect the distribution of SPARC. Immunohistochemical procedures were performed in strict accordance with the manufacturer's instructions. The EnVision and DAB chromogenic reagent kit (Agilent Technologies, Inc., Santa Clara, CA, USA) were used for immunohistochemical staining. All staining was performed under the same conditions; the tissue samples were sliced to a thickness of 2-3 μm, dehydrated in 80, 90, 95 and 100% ethanol, dewaxed and antigen repair was performed using 0.01 mol/l citric acid (pH 6.0). Normal goat serum (Toyobo Co., Ltd., Osaka, China) was dropped onto the slide and incubated for 10 min at room temperature. Subsequently, the corresponding specific antibody (mouse anti-osteonectin/SPARC; (1:1,000; catalog no. 5420; Cell Signaling Technologies Inc., Danvers, MA, USA) was added to the slide and incubated for 1.5 h at room temperature. The slides were washed with phosphate-buffered saline (PBS) for 3 min three times. The secondary antibody (1:1,000; catalog no. 341200; Cell Signaling Technologies Inc.) was added and incubated for 30 min at room temperature. The slide was stained with DAB, and the nucleus was stained with hematoxylin, dehydrated using a gradient of ethanol, cleared with xylene and sealed using natural gum. SPARC (mouse anti-osteonectin/SPARC; (1:1,000; catalog no. 5420; Cell Signaling Technologies Inc., Danvers, MA, USA) immunoreactivity in the blood vessel walls of ESCC tissues served as a positive control, and the specific antibodies were replaced with PBS to serve as the negative control.

The immunohistochemical results were determined by three pathologists, who observed the positive granule-stained cells in the esophageal cancer tissue samples and the adjacent healthy esophageal mucosa using a BH-2 light microscope (Olympus Corporation, Tokyo, Japcan). The staining score criteria were as follows: 0, 0-15%; 1, >15-30%; 2, >30-45%; 3, >45%. According to the staining intensity for semi-quantitative determination, colorless was 0 and 3 (strong staining) was brown. The final staining score of a sample was determined as the product of the positive cell percentage score and the staining intensity score. Staining score <2, negative (-); staining score 2-4 points, weakly positive (+); staining score, 4-6 points, positive (+ +); staining score ≥6 points, strong positive (+ + +). For the convenience of statistical analysis of the data, the (-) group was defined as the negative expression group (-), and the (+), (+ +) and (+ + +) groups were designated as the positive expression group (+).

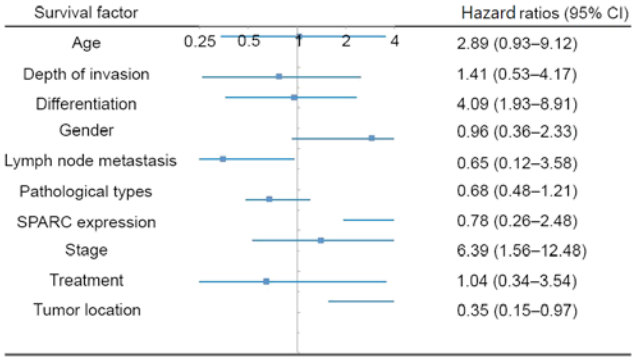

Figure 1. Postoperative survival analysis of esophageal squamous cell carcinoma patients. CI, confidence interval.

Detecting the expression level of SPARC mRNA using RT-PCR. Total RNA was isolated from the tissue samples using TRIzol (Sangon Biotech Co., Ltd., Shanghai, China) and quantified using a Nandrop spectrophotometer. RNA (2 ug) was reverse transcribed to cDNA according to the Titanium® One-Step RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China), and was amplified by semi-quantitative PCR with β-actin serving as the reference. The primer sequences (Sangon Biotech Co., Ltd.) are presented in Table I. The thermal cycling conditions were as follows: Predenaturation at 94˚C for 4 min; 30 cycles of 94˚C for 10 sec, 55˚C for 30 sec and 72˚C for 60 sec.

Amplification of SPARC by PCR was examined by agarose gel electrophoresis and analyzed using Quantity One version 3 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The absorbance value of the belt and the reference were read, and the results were expressed as a ratio (sample value/reference value). When the ratio of the ESCC value and reference value

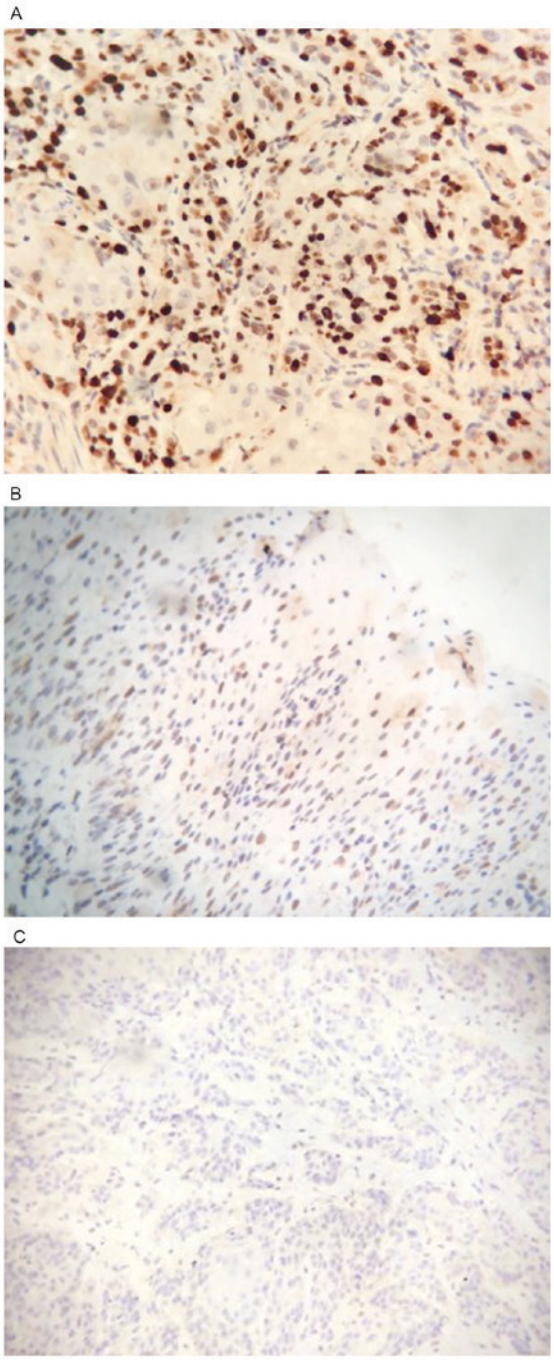

Figure 2. Staining result of immunohistochemistry for SPARC in ESCC and healthy esophageal mucosa tissue samples (magnification, x200). (A) Strongly positive staining of SPARC in the ESCC tissue samples. (B) Weakly positive and (C) negative staining of SPARC in healthy esophageal mucosa tissue samples. SPARC, secreted protein acidic and rich in cysteine; ESCC, esophageal squamous cell carcinoma.

was greater than the β-actin reference value, it was expressed positively. Otherwise, it was considered to be negative.

Statistical analysis. SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The χ^2 test was performed to compare the distribution of SPARC expression levels between the healthy and ESCC tissue samples. Kaplan-Meier survival analysis with the log-rank test was performed to analyze the association between the protein

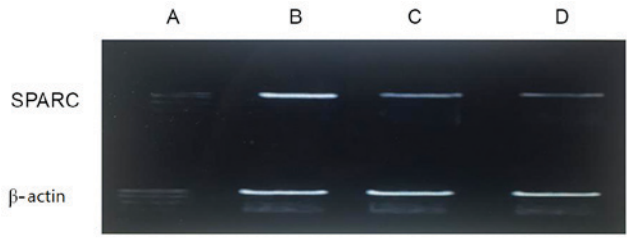

Figure 3. Secreted protein acidic and rich in cysteine mRNA expression levels in the ESCC and healthy esophageal mucosa tissue samples. (A) Negative control group; (B) normal esophageal mucosa group; (C) ESCC samples without lymph node metastasis; (D) ESCC samples exhibiting lymph node metastasis. ESCC, esophageal squamous cell carcinoma.

expression levels in the cancer tissue samples, and multi factor survival stage and independent factor survival stage were used for the other clinicopathologic characteristics and the survival rate of the patients. The hazard ratios were determined using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA) and the 95% confidence intervals (CI) were computed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Association between the expression level of SPARC and the overall survival of postoperative patients with ESCC. The overall survival of patients who were positive for the SPARC protein was 60.92±3.45 months, after a median follow-up time of 61.5 months (6.1-77.3 months). The overall survival of SPARC protein-negative patients was 55.68±5.65 months. Kaplan-Meier survival analysis indicated that there was no significant difference between SPARC-positive and SPARC-negative patients ($P>0.05$). Multi factor survival stage indicated that the tumor location (upper, middle and lower segment), tumor differentiation (high, moderate and poor) and tumor stage (I, II and III) were independent factors affecting the overall survival of the postoperative patients. Additionally, adjuvant therapy, gender, age, gross morphology, tumor invasion depth and lymph node metastasis were not identified as independent factors affecting the overall survival of postoperative patients (Fig. 1).

SPARC mRNA expression in ESCC and healthy esophageal mucosa tissue samples. RT-PCR demonstrated the expression level of SPARC mRNA in ESCC and healthy esophageal mucosa tissue samples. The positive rate of SPARC mRNA in ESCC was 71.91% (64/89), which was significantly higher than that in the healthy esophageal mucosa 15.00% (15/100; $P<0.05$) (Fig. 2).

Expression levels of SPARC protein in ESCC and healthy esophageal mucosa tissue samples. The positive expression rate of SPARC protein in ESCC was 65.17% (58/89) and the positive rate was 8% (8/100) in the normal esophageal mucosa. The expression level of SPARC protein in the ESCC tissue samples was significantly higher than that in the healthy esophageal mucosa samples ($P<0.05$; Fig. 3).

Association between the expression levels of SPARC mRNA and protein in different pathological types of ESCC. The

Table II. Correlation of SPARC mRNA and protein expression levels with clinicopathological features in osteosarcoma.

Characteristic	n	SPARC protein positive rate, n (%)	χ^2	P-value	SPARC mRNA positive rate, n (%)	χ^2	P-value
Gender							
Male	45	30 (66.7)	0.190	0.663	33 (73.3)	0.008	0.927
Female	44	28 (63.6)			31 (70.5)		
Age (years)							
<40	46	31 (67.4)	0.121	0.728	34 (73.9)	0.005	0.945
≥40	43	27 (62.8)			30 (70.0)		
Tumor diameter (cm)							
≥10	35	23 (65.7)	0.351	0.553	26 (74.3)	0.072	0.789
<10	54	35 (64.8)			38 (70.4)		
Lymph node metastasis							
Yes	37	33 (89.2)	7.601	0.006	35 (94.6)	7.411	0.008
No	52	25 (48.1)			29 (55.8)		
Pathologic type							
Ulcer	52	32 (61.54)	0.323	0.125	37 (71.15)	0.332	0.119
Medullary	19	13 (68.42)			14 (73.68)		
Mushroom	11	8 (72.72)			8 (72.72)		
Coarctation	7	5 (71.43)			5 (71.43)		
Degree of tumor differentiation							
High	19	11 (57.89)	0.234	0.512	13 (68.42)	0.276	0.565
Moderate	44	29 (65.91)			32 (72.73)		
Poor	26	18 (69.23)			19 (73.08)		
Tumor stage							
I	43	33 (76.74)	7.231	0.005	38 (88.37)	7.012	0.002
II	28	16 (57.14)			16 (57.14)		
III	18	9 (50.00)			10 (55.56)		

expression levels of SPARC mRNA and protein in ESCC were consistent. SPARC was highly expressed in ESCC tissue samples, and was not associated with sex, age, tumor size, pathologic type or the degree of tumor differentiation, but was associated with staging and metastasis (Table II).

A total of 89 cases of patients with ESCC (according to the pathological morphology) were divided into 52 cases of ulcer type, 19 cases of medullary type, mushroom type in 11 cases and 7 cases of coarctation. The positive expression rates of SPARC protein were as follows: Ulcer type, 61.54% (32/52); medullary type, 68.42% (13/19); mushroom type, 72.72% (8/11); and coarctation type, 71.43% (5/7). Although the results showed that the positive rate of mushroom type was highest, the difference was not statistically significant (P>0.05).

The positive expression rates of SPARC mRNA were as follows: Ulcer type, 71.15% (37/52); medullary type, 73.68% (14/19); mushroom type, 72.72% (8/11); and coarctation type, 71.43% (5/7). Although the results showed that the positive rate of mushroom type was highest, the difference was not statistically significant (P>0.05).

According to the degree of tumor differentiation, the 89 cases of ESCC were divided into 19 cases of high, 44 cases of moderate and 26 cases of poor differentiation. The positive expression rate of SPARC protein was not statistically significant between differentiated samples (P>0.05): High differentiation, 57.89% (11/19); moderate differentiation, 65.91% (29/44); and poor differentiation, 69.23% (18/26).

The positive expression rate of SPARC mRNA was not statistically significant (P>0.05): High differentiation, 68.42% (13/19); moderate differentiation, 72.73% (32/44); and poor differentiation, 73.08% (19/26).

Single factor analysis indicate that tumor stage and lymph node metastasis were negatively associate with SPARC protein and SPARC mRNA expression levels (P<0.05). The SPARC protein and SPARC mRNA expression levels were relatively large in patients with early stage of tumors and no lymph node metastasis. Multi-factor analysis indicated that only lymph node metastasis was negatively correlated with SPARC protein and SPARC mRNA expression levels (P<0.05).

Discussion

Recent studies have demonstrated the particularly complicated processes involved in the occurrence and development of tumors (17,18). It may be caused by the regulation of cell growth and proliferation (19). In addition, abnormal

expression of tumor-associated genes and aberrant activation of cell signal transduction may also be involved (20-21). Cell growth and proliferation in the human body are affected and controlled by numerous factors (22,23). Notably, cell signaling proteins, growth factors and their receptors, apoptotic proteins and transcription factors, and the changes of these factors are closely associated with the occurrence and development of tumors (24).

Previous studies have reported high expression levels of SPARC protein in ESCC (25). Tumor cells that express SPARC in the nucleus are associated with a higher degree of malignancy (26). The present study demonstrated that the SPARC protein was localized in the tumor stroma, which is consistent with the high expression levels of the SPARC protein in fibroblasts and endothelial cells during tissue repair and in aggressive malignant tumors.

The SPARC protein is an important molecule in locally advanced esophageal carcinoma; however, its association with the clinical prognosis of esophageal cancer invasion remains unclear (27,28). The results of the present study indicated that SPARC protein expression in the tumor stroma aided the development of esophageal cancer. A study revealed that SPARC protein expression was not associated with tumor differentiation and the depth of invasion, but was positively correlated with lymph node metastasis, and is associated with poor prognosis (29). Porte *et al* (30) and other studies (31) revealed that the SPARC protein was not associated with tumor size, lymph node status, tumor adjacent tissue invasion, disease recurrence and overall survival. The current study demonstrated that SPARC protein expression in ESCC was not associated with the degree of differentiation and invasion depth, and was not linked to tumor location, gross morphology, sex and age. In contrast to other studies, the current study identified that the SPARC protein was associated with lymph node metastasis and tumor stage in patients with ESCC, but it was negatively correlated. Expression of the SPARC protein in early stage ESCC is highly expressed, and is not associated with lymph node metastasis. This inconsistent result reflects the heterogeneity of patients with ESCC and reveals the complex role of the SPARC protein in the development of ESCC.

Studies have identified that the high expression level of SPARC protein in melanoma and prostate cancer promotes tumor growth and metastasis (32). However, the SPARC protein may act as an antitumor factor in pancreatic and colorectal cancer, resulting in anti-angiogenesis, apoptosis, inhibition of cell proliferation and cell cycle arrest, thus inhibiting tumor growth (33). In the present study, SPARC protein expression in patients with ESCC was associated with the survival prognosis, and the clinical features of the tumor were significantly associated with survival, differentiation and staging.

A limitation of the current study was the relatively small sample size. However, this is one of the larger studies addressing SPARC protein expression in ESCC. The results of the current study demonstrated that the expression levels of SPARC in ESCC tissue samples were significantly higher than those in healthy esophageal mucosa tissue samples, which may indicate the association between the occurrence and development of tumors, and the high expression of SPARC.

In conclusion, the results indicate the potential role of SPARC in the progression of ESCC. Further research on SPARC is required to aid the development of novel therapeutic strategies for ESCC.

Acknowledgements

The present study was supported by the Jiangsu Pharmaceutical Association (grant no. 201542) and the Science and Technology commission of Yancheng City (grant no. YK2015002) and the Youth Medical Talent of Jiangsu Province (grant no. QNRC2016475).

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