Expression and significance of secreted protein acidic and rich in cysteine in human osteosarcoma

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Abstract. Osteosarcoma is the most common primary malignancy of bone, and is a high-grade malignant mesenchymal tumor with high recurrence and metastatic rates. Increased expression of secreted protein, acidic and rich in cysteine (SPARC) indicates poor prognosis in a number of malignances. However, the expression level of SPARC in human osteosarcoma and its associated mechanism remains unclear. To analyze the expression of SPARC in human osteosarcoma and its potential application in the diagnosis and treatment of osteosarcoma, the clinical records and samples of 20 cases of osteosarcoma were collected. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis applied to detect SPARC expression levels in osteosarcoma tissues, with normal bone tissue as control. Immunofluorescence detection was used to examine the distribution of SPARC. The association between SPARC level and clinical factors was analyzed. RT-qPCR (P=0.002) indicated that the SPARC level in osteosarcoma tissues was significantly increased compared with that in normal tissues. Immunofluorescence detection indicated that SPARC was widely distributed in tumor tissues. SPARC protein expression level was positively associated with lung metastasis (P=0.016). The results indicated that SPARC tends to be highly expressed in human osteosarcoma tissues. The expression level of SPARC is associated with lung metastasis, which may be an indicator of prognosis. Thus, SPARC may be a potential tumor marker and therapeutic target in osteosarcoma.

Introduction

Osteosarcoma is the most common primary malignancy of the bone (1), and is a high-grade malignant mesenchymal tumor with high recurrence and metastatic rates (1). Classical chemotherapy drugs include methotrexate, cisplatin, doxorubicin and ifosfamide. However, multidrug resistance is the main problem of chemotherapy, and its associated mechanism is not clear. A number of factors may be associated with tumor resistance to classical chemotherapy (2,3). For patients with drug resistance, effective treatment and tumor markers for prognosis are lacking. Therefore, studies investigating treatments, tumor markers and targets for osteosarcoma treatment are essential. Abraxane® [paclitaxel for injection (albumin-bound)] contains paclitaxel nanoparticles and albumin. As a vector, albumin combines with secreted protein, acidic and rich in cysteine (SPARC), which is expressed in various malignant tumors and is associated with the occurrence and progression of tumors (4-15). Increased expression of SPARC also indicates recurrence and poor prognosis in a number of malignances (4-15). However, the expression level of SPARC in human osteosarcoma and its associated mechanism remains unclear. Therefore, the present study was designed based on our previous study (16) and the hypothesis that there is high SPARC expression in human osteosarcoma, to elucidate the possibility of SPARC as a tumor marker and therapeutic target for osteosarcoma. The present study focused on SPARC protein and gene expression in human osteosarcoma. A selection of clinical factors was analyzed and positive results were demonstrated.

Materials and methods

Tumor sample processing and clinical characteristics. The inclusion criteria for samples were: Specimens which had been preserved well; pathological confirmation of primary malignant osteosarcoma; well-preserved normal tissues 2 cm away from tumor margin; no chemotherapy prior to operation; and complete clinical data. Between January 2013 and September 2013, a total of 20 osteosarcoma specimens and normal tissues in the Department of Orthopedic Oncology Surgery, Beijing Ji Shui Tan Hospital (Beijing, China) matched these conditions. All cases were confirmed as osteosarcoma by a pathologist through post-operative examination. All samples were excised from fresh osteosarcoma tumors and immediately snap-frozen in liquid nitrogen. The frozen samples were stored at -80˚C in tissue bank of the Department of Orthopedic Oncology Surgery, Beijing Ji Shui Tan Hospital. All patient data, including age, sex, tumor site and size, laboratory tests, metastasis and survival were

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collected (Table I). The patients in the present study received direct amputation due to tumor invasion of major vessels, so the potential interruption of chemotherapy drugs on protein expression was avoided. The study was approved by the Institutional Review Board of Beijing Ji Shui Tan Hospital (Beijing, China) and all patients provided written informed consent for the use of their samples.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The reaction was performed with preliminary incubation for 2 min at 95˚C, followed by 45 cycles of denaturation at 95˚C for 20 sec and annealing/extension at 59˚C for 25 sec and 72˚C for 30 sec. The final melting lasted 50 sec from 70 to 95˚C at an interval of 0.5˚C/s. Total RNA was extracted from tumor and normal tissue using TRIzol (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China). The RNA was denatured at 70˚C for 5 min using template RNA and oligo(dT) (both from Beijing Solarbio Science and Technology Co., Ltd.). The reverse transcription reaction solution was incubated at 42˚C for 120 min in 5 μl 5X Moloney murine leukemia virus buffer (Beijing Solarbio Science and Technology Co., Ltd.), 1.25 μl deoxyribonucleoside triphosphate mixture and 25 units RNase inhibitor (Beijing Solarbio Science and Technology Co., Ltd.). Subsequently, qPCR was performed with the following primer sequences of SPARC (GenBank accession no. NM 009242): Forward, 5-CATCAAGGAGCAGGACAT CAAC-3 and reverse, 5-GCAGCAGGAGGCGTGAA-3 (Primer Premier 5.0 and Oligo 6.0). A PCR detection system (Bioer Technology Co., Ltd., Hangzhou, China) was applied to measure the fluorescence emitted with SYBR-Green (Beijing Solarbio Science and Technology Co., Ltd.). Cq was set as the cycle at which fluorescence was significantly increased compared with background groups. GAPDH was used as a control for normalization. The internal control was β-actin (18S RNA) and the external control was the tumor sample. Thus, ΔC_q was C_q (sample) - C_q (external control) and $\Delta\Delta C_q$ was ΔC_q (SPARC gene) - C_q (18S). The relative quantification of SPARC gene was calculated as $2^{-\Delta\Delta Cq}$ (17) and the result was presented as the fold of tumor tissue over normal tissue.

Immunofluorescence detection. Tissues were taken from -80˚C environment and reheated in a Leica CM1850 cryostat (Leica Microsystems GmbH, Wetzlar, Germany) for 30 min. Tissues were embedded with tissue freezing medium (Leica Microsystems GmbH) and placed in the cryostat. The embedded tissues were cut into 6-μm thick slices and fixed in pre-cooling polyformaldehyde for 1 min. They were then immersed in PBS five times and incubated in 5% bovine serum albumin (Beijing Solarbio Science and Technology Co., Ltd.) for 20 min at 37˚C. The slices were incubated with anti-SPARC antibody (cat. no. SC-25574, 1:50; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight. Absorbent paper was used to remove the primary antibody, and the slices were then immersed in PBS several times. Then, the slices were incubated with secondary antibody (dilution 1:100, cat. no. 111-035-003) tagged with fluorescein isothiocyanate (1:100) (both from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at 37˚C and immersed in PBS three times. The nuclei were stained by DAPI (5 μg/ml) for 3 min and immersed in PBS containing Tween-20 three times. The slides were mounted and images were captured using a fluorescence microscope. The staining intensity of SPARC protein was evaluated as following: No fluorescence (-); fluorescence suspicious extremely weak (±); fluorescence is weak but clearly visible (+); bright fluorescence (++); extremely bright fluorescent (+++/++++). The distribution area of SPARC was calculated as the area with intensity: ±, +, ++ and +++/++++ divided by the total area of view under the microscope (green area/total area).

Statistical analysis. All statistical analyses were performed using SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA). SPARC expression level in the tumor and normal control groups were compared with a mean-value paired Student's t-test; correlation tests were performed to analyze the correlation of SPARC and clinical factors. The Pearson method was applied for parametric tests, and the Spearman method was applied for non-parametric tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SPARC expression level. The SPARC gene level was also examined in 20 tumor and normal samples. The gene amplification curve exhibited an exponential growth phase following between 22 and 25 cycles (mean, 23.5 cycles). SPARC RNA expression in the tumor tissues was increased 2.15-fold compared with that in normal tissues (0.676 and 0.314, respectively; P=0.002; Fig. 1).

Immunofluorescence detection. The immunofluorescence detection results suggested that the SPARC protein was widely distributed in tumor tissues. Additionally, SPARC was observed in the tumor stroma, and not confined to the tumor cell or nucleus. These results indicate the distribution characteristics of the secreted protein. The staining intensity of SPARC protein in tumor tissues was increased compared with that in normal tissues. The staining intensity of SPARC protein in tumor tissues was (++) or brighter and the staining intensity of SPARC protein in normal tissues was (-) or (±). The distribution area of SPARC protein in tumor tissues was also increased compared with normal tissues (Figs. 2 and 3).

Correlation analysis. Clinical characteristics including tumor size, tumor site, laboratory tests and metastasis were analyzed (Table II). The SPARC protein expression level was positively associated with lung metastasis (P=0.016). The SPARC protein level was negatively associated with the blood neutrophil level (P=0.003). The Pearson test demonstrated a marginal association between SPARC protein level and tumor site (femur or humerus) (P=0.058). To address whether the tumor site was associated with the SPARC protein level, mean values of SPARC level in femur and humerus groups were compared. The SPARC level in the femur group was significantly decreased compared with that in the humerus group (P=0.005). The distinct tumor sizes in these two groups were also measured: Mean tumor volumes were 1,859 cm^3 in the femur group and 1,143 cm^3 in the humerus group.

Table I. Patient characteristics.

Case	Sex	Age, years	Tumor site	Follow-up time, months	Tumor volume, cm^3	Maximum tumor diameter, cm	Metastasis	Recurrence	Alive
1	M	23	Pelvis	26	1,400	14	N	N	Y
2	M	19	Femur	13	4,056	24	Y	N	N
3	F	9	Femur	27	1,215	15	N	N	Y
4	M	51	Humerus	24	1,980	15	Y	N	Y
5	M	26	Humerus	22	360	12	Y	N	Y
6	M	16	Tibia	19	187	8.5	Y	N	Y
7	M	14	Tibia	19	702	12	N	N	Y
8	M	28	Tibia	18	140	8	N	N	Y
9	F	10	Femur	24	264	8	N	N	Y
10	M	18	Tibia	23	640	10	N	N	Y
11	M	19	Femur	24	885	11	N	N	Y
12	M	21	Femur	21	3,127	22	Y	N	N
13	F	9	Femur	18	1,654	14	N	N	Y
14	M	23	Humerus	18	580	13	N	N	Y
15	M	14	Tibia	22	430	9	Y	N	Y
16	F	17	Humerus	21	1,650	13	Y	N	Y
17	M	21	Tibia	20	260	7	N	N	Y
18	M	28	Tibia	24	346	9	N	N	Y
19	F	10	Femur	12	1,540	11	Y	N	Y
20	F	18	Femur	18	2,132	15	N	N	Y

M, male; F, female; Y, yes; N, no.

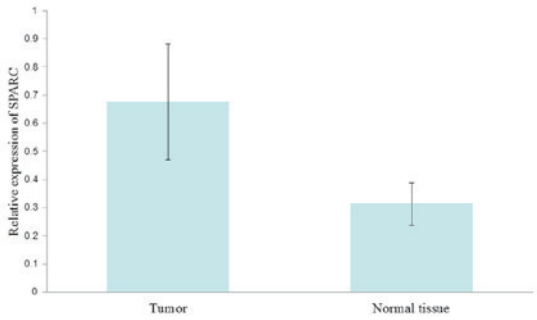

Figure 1. Reverse transcription-quantitative polymerase chain reaction analysis of SPARC. The mean relative expression of SPARC in tumor and normal tissues were 0.676 and 0.314, respectively (P=0.002).

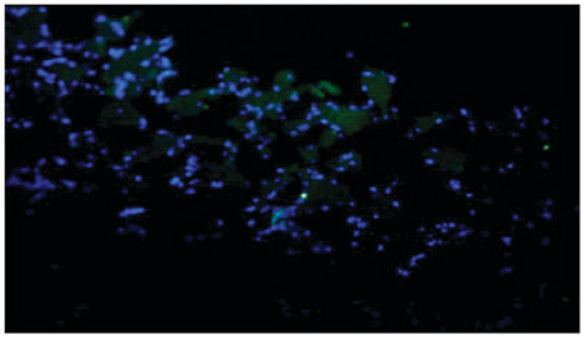

Figure 2. Immunofluorescence staining of SPARC protein in normal controls (magnification, x200). Blue, nuclear staining; green, SPARC protein staining; SPARC, secreted protein, acidic and rich in cysteine.

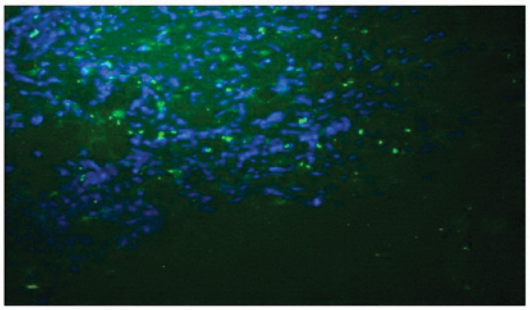

Figure 3. Immunofluorescence staining of SPARC protein in tumor (magnification, x200). Blue, nuclear staining; green, SPARC protein staining; SPARC, secreted protein, acidic and rich in cysteine.

Discussion

Since the 1970s, Jaffe and Cortes began to apply methotrexate and doxorubicin to osteosarcoma chemotherapy (18,19). Rosen first suggested neoadjuvant chemotherapy in the early 1980s (20). Subsequently, the 5-year survival rate of osteosarcoma patients increased to >60% (21,22). At present, the widely used therapeutic module is: Neoadjuvant chemotherapy + surgery + (adjuvant) chemotherapy (18). However, in the previous 20 years, despite concerted efforts, the survival rate of osteosarcoma has not markedly improved (23-25). A number of studies have indicated that the poor response to first-line chemotherapy was not altered by longer durations or higher doses of chemotherapy (26,27). A lack of response

Table II. Association between patient characteristics and secreted protein, acidic and rich in cysteine expression.

Characteristic	Value	Pearson correlation	Spearman correlation	P-value
Sex			-0.348	0.324
Male	14			
Female	6			
Mean age, years (range)	20.5 (9-51)	0.094		0.796
Mean tumor volume, cm^3 (range)	1,185.8 (140-4056)	-0.352		0.319
Maximum tumor diameter, cm (range)	12.5 (7-24)	-0.326		0.357
Tumor site		0.866		0.058
Femur	8			
Humerus	4			
Metastasis			0.709	0.016
Yes	8			
No	12			
Mean alkaline phosphatase level, U/l (range)	202.1 (97-360)	-0.413		0.310
Mean lactate dehydrogenase level, U/l (range)	410.4 (213-954)	-0.665		0.072
Mean hemoglobin level, g/l (range)	124.1 (77-156)	0.359		0.382
Mean erythrocyte number, $x10^{13}$ cells/l (range)	4.1 (2.65-5.08)	0.349		0.397
Mean white blood cell number, $x10^9$ cells/l (range)	7.1 (4.35-12.08)	-0.549		0.159
Mean neutrophilic granulocytes, % (range)	67.7 (46.4-86.0)	-0.885		0.003
Mean erythrocyte sedimentation rate, mm/h (range)	33.9 (8-120)	-0.082		0.862
Mean C-reactive protein level, mg/l (range)	53.5 (2.2-302.0)	-0.434		0.331
Mean D-dimer level, mg/l (range)	1.9 (0.29-4.92)	0.041		0.923

to first-line chemotherapy and metastases are poor prognostic factors affecting long-term survival. Although there are a number of effective drugs for lung and breast cancer, and other malignant tumors, the development of a novel drug for osteosarcoma has proven difficult.

SPARC is a multifunctional glycoprotein. It was identified to be highly expressed in a number of malignant tumors including head and neck cancer, breast cancer, melanoma and colon cancer (4-15). SPARC is associated with tumor development, invasion, metastasis and prognosis (4-15). It is an important protein in the regulation of tumor cell proliferation, invasion and survival, and may interact with vascular endothelial growth factor and basic fibroblast growth factor (10). Paclitaxel for injection (albumin-bound; nab-paclitaxel, Abraxane®) is targeted paclitaxel with the application of albumin nanoparticle technology. Previously, a number of studies (28-30) confirmed that its safety and efficacy are increased compared with paclitaxel, which may enable it to become a novel option for the treatment of osteosarcoma.

The high affinity of SPARC for albumin is a valuable characteristic. Previous studies have indicated that the efficacy of Abraxane® is associated with the expression level of SPARC (31,32). Increased expression of SPARC in metastatic nasopharyngeal carcinoma and breast cancer tissues may improve the distribution concentration of nab-paclitaxel in tumor tissues and the effects of the treatment (33,34), which makes SPARC a potential novel antitumor target and predictive marker. Thus, a series of experiments was performed in the present study. Preclinical studies demonstrated that nab-paclitaxel exerted a significant inhibitory effect on osteosarcoma *in vitro* and *in vivo* (16,35). A tendency of increased expression tendency of SPARC in osteosarcoma was also identified in mice (35), which provided a theoretical basis for the present study.

The SPARC expression level in human osteosarcoma and the associated mechanism remains unclear. The immunohistochemical study of SPARC expression in extraskeletal osteosarcoma by Fanburg-Smith *et al* (36) identified that the SPARC-positive rate in tumor cells was higher compared with that in the tumor matrix. Dalla-Torre *et al* (37) demonstrated a high expression of the *SPARC* gene in osteosarcoma specimens, which is consistent with the hypotheses and results of the present study. However, that study (37) did not detect the protein expression level and the distribution. Certain studies also revealed SPARC expression in osteosarcoma; however, these studies focused only on immunohistochemical tests, not SPARC protein and gene expression, as in the present study (38,39).

In the present study, SPARC protein and gene expression was examined concomitantly in human osteosarcoma tissues and compared with adjacent normal tissues. The RT-qPCR analysis demonstrated a significantly higher expression of SPARC protein in human osteosarcoma samples compared with adjacent normal tissues. To improve the scientific value and decrease the potential effect of various chemotherapeutic

drugs on the expression level of SPARC protein, the present study only enrolled patients who did not receive any chemotherapy prior to surgery. These results provide the basis for additional studies.

To explore the value of SPARC in osteosarcoma, potential factors including clinical features, tumor characteristics and laboratory data were analyzed. The results indicated that the expression level of SPARC was significantly associated with lung metastasis, and these patients exhibited poor prognosis. Therefore, SPARC may be a potential novel marker for the prognosis of osteosarcoma. A number of molecular mechanisms may be involved in tumor progression and metastasis. MicroRNAs exhibit fundamental roles in the regulation of intracellular processes and serve important roles in tumor invasion and metastasis. Epithelial to mesenchymal transition (EMT) allows malignant epithelial cells to become detached from each other and invade the surrounding stroma (40). DNA methylation and histone-tail methylation are also involved in tumor metastasis (36). The potential reversibility of these molecular makes them potential biomarkers and therapeutic targets (41). The present study identified that the expression level of SPARC was negatively associated with the level of blood neutrophils, yet the reason remains unclear. Neutrophil levels represent the degree of immune system activation in the body. It is assumed that activation of the immune system and the resultant number of neutrophils may inhibit the secretion of SPARC in tumor tissues.

There were also differences of SPARC expression in different skeletal sites. The reason that SPARC expression in the humerus was significantly increased compared with in the femur may be that a larger tumor exhibits more necrosis and edema components, which means relatively less tumor cells and protein expression. Therefore, it is hypothesized that the differential expression of SPARC protein in osteosarcoma is associated with intra-tumor heterogeneity, as solid neoplasms are superorganisms with complex compartments and functions. Tumors are highly heterogeneous populations derived from one common progenitor (42). Within a neoplasm, a mosaic of mutant cells competes for space and resources, evades predation by the immune system and may even cooperate to disperse and metastasize to new organs (43). The evolution of tumor cells in a solid tumor may be the most significant obstacle to eliminating them. The understanding of the evolution of neoplastic cells may assist in identifying novel therapeutic targets of tumors. However, the statistical analysis of the present study demonstrated no significant association between SPARC and tumor size or maximum diameter. This is probably due to the small sample size, which is a limitation of the present study. SPARC expression in tumors may be enhanced by intra-tumoral hypoxia and acidity, which indicates poor prognosis (6).

According to previous studies, the role and mechanism of SPARC in the progression of tumors is complicated. It has been suggested that SPARC may exhibit important effects in angiogenesis that are necessary for tumor invasion and metastasis (44,45). SPARC also facilitates tumor invasion and metastasis through disrupting the adhesive interactions between neoplastic cells and the extracellular matrix (5). SPARC may also reduce the adhesion of tumor cells to the extracellular matrix through the degradation of the extracellular matrix and cytoskeletal rearrangement, thereby promoting tumor progression and metastasis (46,47). In melanoma and breast cancer, the association of SPARC expression with the expression, secretion and function of matrix metalloproteinases indicated that SPARC may enhance the invasiveness of tumor cells through the activation of matrix-degrading enzymes (10,48,49).

Although the present study showed some positive and meaningful results on the expression of SPARC in human osteosarcoma, but there is still limitation such as the Western blot test of SPARC expression was not analyzed.

In conclusion, the results of the present study identified increased expression levels of SPARC in human osteosarcoma, and the SPARC expression level was positively associated with lung metastasis. Combined with studies investigating other malignant tumors, SPARC may lead to tumor progression and indicate a poor outcome. Conversely, although patients with increased SPARC expression may be insensitive to conventional therapy, they may be sensitive to Abraxane®, which has not yet been applied to the treatment of osteosarcoma. The value of SPARC in the prognosis and prediction of the treatment outcomes of osteosarcoma by nab-paxlitaxel remains to be evaluated in future studies.

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