The Prognostic Significance of Src and p-Src Expression in Patients with Osteosarcoma

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Background: The expression of Src and phospho-Src (p-Src) is closely related to tumor invasion and metastasis. The aim of the present study was to investigate the expression of these molecules in osteosarcoma and their relationship with each other, to provide a theoretical basis to understand the prognosis of osteosarcoma.

Material/Methods: We selected surgically resected osteosarcoma specimens from 116 patients of Zhongnan Hospital of Wuhan University and Hubei Cancer Hospital, Hubei, China, between January 2000 and January 2010 with detailed follow-up data. Twenty osteochondroma specimens from the corresponding period were used as controls. Expression of Src and p-Src was detected in osteosarcoma and osteochondroma by immunohistochemistry. We analyzed the relationship of the 2 proteins and osteosarcoma patient prognosis.

Results: The expression of Src and p-Src in osteosarcoma was significantly higher than the expression level in osteochondroma ($P<0.05$). The expression levels of the 2 proteins, clinical stage, and tumor metastasis were significantly associated with survival time ($P<0.05$), but there was no correlation between age or sex and survival time. The expression of Src and p-Src in osteosarcoma was positively correlated.

Conclusions: Src and p-Src can be used as an auxiliary indicator to determine a malignant phenotype of bone tumors, and the combined detection of Src and p-Src may indicate the prognosis of osteosarcoma.

MeSH Keywords: Genes, src • Osteosarcoma • Prognosis

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Background

Osteosarcoma is a malignant tumor that originates from mesenchymal cells and is characterized by fusiform stromal cells producing osteoid. It is the most common malignant tumor of the skeletal system [1–3], and mainly occurs in young people aged 15 to 19 years. It usually appears in the metaphysis and has a rich blood supply. The hematogenous metastasis rate is high, with about 85% of metastasis seeding the lung [4]. Due to its rapid progression and poor prognosis, osteosarcoma is a major death-causing disease in adolescence. Sixty to seventy percent of osteosarcoma patients achieve 5-year disease-free survival due to the current treatments, including surgery, chemotherapy, and radiation therapy [5–8]. However, 20–25% of patients have lung metastases when diagnosed [9], and there are still further patients that develop lung metastasis during treatment. The patients with lung metastases have a worse prognosis and the long-term survival rate is only 10–30% [8]. Moreover, patient prognoses remain poor even after receiving systemic treatment. Therefore, it is crucial to choose suitable survival prognostic indicators to estimate the prognosis of patients with osteosarcoma. Recently, some molecules, including Src and p-Src, have been correlated with the genesis and development of osteosarcoma and may be potential prognostic markers.

Src family kinases include Src and another 11 members, and they are jointly involved in intracellular signal transduction processes [10–13]. Src can be activated by multiple signaling pathways to become phospho-Src (p-Src), and p-Src can activate specific signaling pathways by phosphorylating the target proteins [12,14]. Thus, Src and the intracellular signaling pathways constitute an amplification cascade of a network regulation system, and Src is the central hub of a variety of signaling pathways. Recent studies have found that Src family kinases mediate a variety of malignancies [15–19], including osteosarcoma cell signal transduction, and play an important role in promoting the genesis and development of osteosarcoma [20–22].

This study detected the expression of Src and p-Src in osteosarcoma and osteochondroma by immunohistochemistry and explored the proteins' relationship with the clinicopathological features of osteosarcoma patients as well as their correlation in osteosarcoma to evaluate whether Src and p-Src are risk factors for osteosarcoma, and to provide important information for clinical treatment and for a prognosis estimate of osteosarcoma.

Material and Methods

Patients and samples

The local research ethics committee (Zhongnan Hospital of Wuhan University) approved this study, taking out exemption for informed consent procedure. All patients or their carer providers gave informed consent for participation in this study. We selected surgically resected osteosarcoma specimens from 116 patients of Zhongnan Hospital of Wuhan University and the Hubei Cancer Hospital, Hubei, China, between January 2000 and January 2010 with detailed follow-up data (except for the patients that received preoperative radiotherapy and chemotherapy). Twenty osteochondroma specimens from the corresponding period were used as controls. All of the samples were evaluated for diagnosis by 3 experienced pathologists. Of the 116 osteosarcoma patients, 73 cases had metastases and 43 cases did not; 71 were male and 45 were female; patients were aged from 8 to 60 years with an average age of 19.1 years. Lesions were located as follows: 43 femoral, 34 tibial, 21 humerus, 4 upper jaw, 7 ilium, 5 fibula, and 2 withers. According to the Ennecking method, there were 5 cases of stage IB, 17 cases of IIA, 64 cases of IIB, and 30 cases of IIIA. Of the 20 osteochondroma patients, 12 were male and 8 were female, aged from 21 to 78 years with an average age of 60.4 years.

Immunohistochemistry

The streptavidin-peroxidase immunohistochemistry method was used to inspect Src and p-Src expression in specimens. The paraffin sections were routinely dewaxed 3 times with dimethyl benzene, and hydrated with an ethanol gradient. Subsequently, sections were washed with distilled water and phosphate buffered saline (PBS). Dewaxed paraffin sections were treated with 3% hydrogen peroxide for 20 min to block endogenous peroxidases. Then the sections were blocked with 2% goat serum in PBS for 1 h at room temperature and incubated with rat antibody against human Src and p-Src (Cell Signaling Technologies, Danvers, USA). Afterwards, the sections were washed with PBS and subjected to immunohistochemical staining using a PV6000 kit (Bioss Inc., Beijing, China). Then, the sections were washed with PBS and developed with 3,3'-diaminobenzidine (DAB) for 5 min and counterstained with hematoxylin. Finally, the sections were assessed under a light microscope by 2 independent investigators.

Evaluation of immunohistochemistry

We used a semi-quantitative method to calculate an immunohistochemical score (IHS). Five fields were randomly selected under a light microscope (×200) to count the positive cells in each slice and to calculate the percentage of positive cells to total cells. We divided the cell staining density into 4 grades: 1=≤25% positive cells; 2=26–50% positive cells; 3=51–75% positive cells; 4=≥76% positive cells. The cell staining intensities were divided into 4 grades: 0=uncolored; 1=light brown; 2=brown; 3=dark brown. The final IHS was obtained by multiplying the score of density and intensity. The IHS of each

Table 1. Expression of Src and p-Src in osteosarcomas and osteochondromas.

	Cases	Src expression			p-Src expression		
		Expression	Low expression	P	Expression	Low expression	P
OS	116	73 (62.9%)	43 (37.1%)	0.0003*	75 (64.7%)	41 (35.3%)	0.0005*
OC	20	4 (20.0%)	16 (80.0%)		2 (10.0%)	18 (90.0%)	

OS – osteosarcoma; OC – osteochondroma. * Significant p<0.05.

specimen was categorized into 4 groups: 0–2 –; 3–5 +; 6–8 ++, 9–12 +++. Scores of 0–5 were designated as low expression, while scores of 6–12 were designated as high expression. The immunohistochemistry results were tested by 3 of the authors in all specimens using a blinded method.

Statistical analysis

SPSS version 18.0 software (SPSS Inc., Chicago, USA) was used to perform statistical analysis. The semi-quantitative data and sample rate difference were estimated by the chi-square test. Survival rates were assessed by Kaplan-Meier statistics and survival curves were compared by the log-rank test. Multivariate analysis was estimated by the Cox regression method. The correlation between Src and p-Src was assessed by Spearman's correlation analysis. P<0.05 was considered statistically significant for all tests.

Results

Expression of Src and p-Src and their correlation with clinicopathological features of patients with osteosarcoma

The expression levels of Src and p-Src in osteosarcoma and osteochondroma tested by immunohistochemistry are shown in Table 1. The expression of Src was observed in 4 (20.0%) of the total osteochondroma cases, and p-Src in 2 of the total number of cases (10.0%). In the total number of osteosarcoma patients, there was expression of Src and p-Src in 73 cases (62.9%) and 75 cases (64.7%), respectively. The chi-square test confirmed that the difference in the expression level of both markers between osteosarcoma and osteochondroma was statistically significant (p<0.05, Table 1). Typical immunohistochemistry images of Src and p-Src in osteosarcoma and osteochondroma specimens are shown in Figure 1. The positive immunohistochemistry results were the tan particles observed in the cytoplasm. The chi-square test showed that the expression level of Src and p-Src was associated with metastasis and clinical stage (p<0.05), while the expression level of both markers showed no association with age, gender, or histological subtype (Tables 2 and 3).

Prognostic value of Src and p-Src expression

The Kaplan-Meier survival curves show that the expression of the 2 proteins is correlated with shorter survival time (p<0.05, log-rank test, Figures 2 and 3). Based on the expression levels of the 2 proteins, all patients were divided into 3 groups and survival analysis showed that patients that demonstrated expression of p-Src and Src expression were associated with the minimum survival time, whereas patients with low expression of both markers had the longest survival time (p<0.05, Figure 4).

Cox multivariate analysis showed that the expression levels of the 2 proteins, clinical stage, and tumor metastasis were significantly associated with survival, while there was no correlation between age or gender and survival (Table 4).

Correlation of Src and p-Src in osteosarcoma

We used multivariate analysis to determine whether expression of Src and p-Src in osteosarcoma were related. The result showed that the expression of Src and p-Src was positively correlated (r=0.926, p=0.0005).

Discussion

Due to the poor survival rate of osteosarcoma patients, information on the mechanism of osteosarcoma is needed to evaluate the prognosis, so that the physician can decide which treatment will best help the patient. Because the occurrence and evolution of osteosarcoma is the result of oncogene activation or inactivation of tumor suppressor genes, the prognosis of osteosarcoma as judged by clinical features only is not accurate, which was confirmed by modern molecular genetics. The molecular mechanisms leading to the development and progression of osteosarcomas is not yet understood. We notice that the proliferation, invasion, and metastasis of osteosarcoma involve multiple signaling pathways [23–25]. More importantly, these pathways can affect each other by Src through crosstalk, but the clinical significance remains debatable. To confirm our hypothesis that Src and p-Src are potential tumor markers, their protein level and relationship with prognosis were analyzed using immunochemistry.
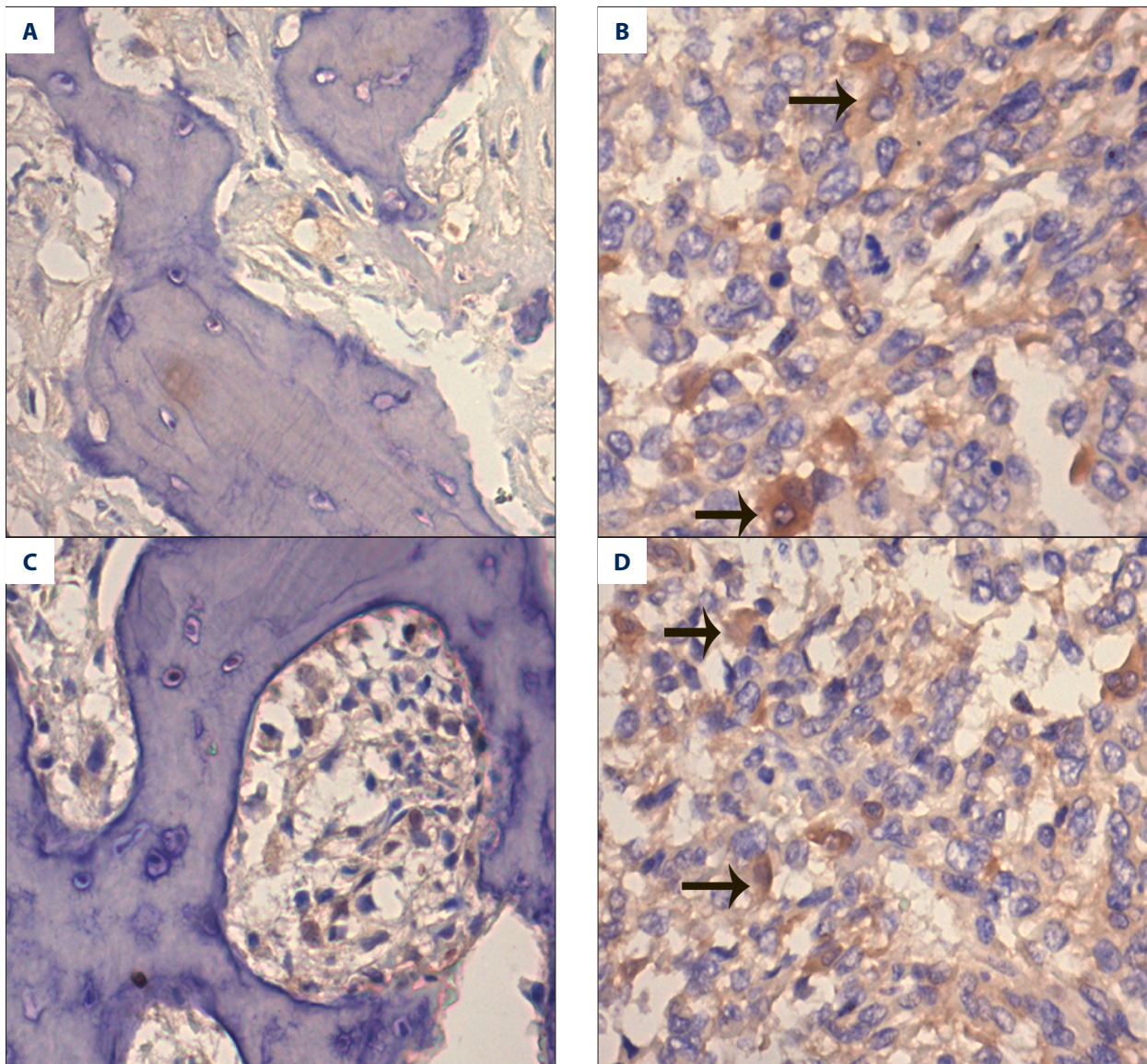
Figure 1. Representative expression of Src and p-Src in osteosarcoma and osteochondroma samples. The sections were counterstained with hematoxylin and the positive staining is brown (indicated by black arrow). (**A**) immunohistochemistry of Src in osteochondroma specimen (×200). (**B**) immunohistochemistry of Src in osteosarcoma specimen (×200). (**C**) immunohistochemistry of p-Src in osteochondroma specimen (×200). (**D**) immunohistochemistry of p-Src in osteosarcoma specimen (×200).

A number of animal and cell experiments have confirmed that Src and p-Src can be detected in osteosarcoma cells [20–22], and in other malignant tumors such as squamous cell carcinoma of the tongue, breast cancer, rhabdomyosarcoma, and Ewing's sarcoma, Src and p-Src also showed high expression [17,18,26–28]. In this study, we used immunohistochemistry to show that the expression percentage of Src and p-Src was 20% and 10% of the 20 osteochondroma cases, respectively, while in 116 cases of osteosarcoma, the expression rate of both proteins was significantly increased, with 62.9% and 64.7% of cases showing high expression of Src and p-Src, respectively. Table 2 shows that the minimum theoretical frequency in osteosarcoma and osteochondroma group was greater than 5, so we chose to use the Pearson chi-square test and these findings are consistent with previous research. In addition, we confirmed that the expression of Src and p-Src in osteosarcoma patients were significantly higher than in the osteochondroma group, but the result may be biased because the 20 cases in the osteochondroma group is obviously far less than the 116 cases in the osteosarcoma group. It was difficult to collect more osteochondroma specimens from the few patients who underwent surgery, and we need more cases for a more reliable result in the next study. Another interesting result

Table 2. Correlation between the expression of Src and p-Src and clinicopathological data.

Clinical feature	Src expression			p-Src expression		
	Expression	Low expression	P	Expression	Low expression	P
Age (years)			0.268			0.310
≤50	42	25		44	23	
>50	31	18		31	18	
Gender			0.949			0.789
Male	41	30		42	29	
Female	32	13		33	12	
Histological type			0.146			0.120
Osteoblastic	35	15		37	13	
Chondroblastic	13	11		14	10	
Fibroblastic	12	8		12	8	
Telangiectatic	7	8		7	8	
Mixed	6	1		5	2	
Primary site			0.889			0.584
Femur	28	15		30	13	
Tibia	19	15		20	14	
Humerus	13	8		13	8	
Fibula	4	1		2	3	
Ilium	5	2		6	1	
Other	4	2		4	2	
Metastasis			0.044*			0.020*
Yes	51	22		53	20	
No	22	21		22	21	
Clinical stage			0.0004*			0.0005*
Stage I B	2	3		2	3	
Stage II A	3	14		4	13	
Stage II B	40	24		40	24	
Stage III	28	2		29	1	

* P<0.05.

is that there is a big mean difference in age between the osteosarcoma (19.1 years) and osteochondroma (60.4 years) group. Although osteosarcoma and osteochondroma both mainly occur in young people, the patients with osteosarcoma seek treatment earlier for the rapid progression, compared to the patients with osteochondroma who seek treatment later for the slow or stationary progression, so the patients with osteochondroma are older than the patients with osteosarcoma in the study.

Src and p-Src promote osteosarcoma invasion, migration, metastasis, proliferation, and other biological effects [20–22]. Therefore, Src and p-Src may be of great relevance to osteosarcoma patients' clinical and pathological features and prognosis. We compared the expression levels of Src and p-Src with patient survival time, pathology, metastasis, and clinical stage, and the results showed that high expression of Src and p-Src was positively correlated with metastasis and clinical stage,

Table 3. Patients characteristic.

Patients characteristic	Src expreossion		P-Src expression		Age		Gender		Metastasis		Clinical stage			
	High	Low	High	Low	≤50	>50	Male	Female	Yes	No	I B	II A	II B	III
Src expreossion														
High			72	1	42	31	42	32	51	22	2	3	40	28
Low			3	40	25	18	30	13	22	21	3	14	24	2
P-Src expression														
High	72	3			44	31	42	33	53	22	2	4	40	29
Low	1	40			23	18	29	12	20	21	3	13	24	1
Age														
≤50	42	25	44	23			36	31	44	23	3	7	38	19
>50	31	18	31	18			35	14	29	30	2	10	26	11
Gender														
Male	40	30	42	29	36	35			45	26	2	9	37	23
Female	32	13	33	12	31	14			28	17	3	8	27	7
Metastasis														
Yes	51	22	53	20	44	29	45	48			2	7	34	30
No	22	21	22	21	23	20	26	17			3	10	30	0
Clinical stage														
I B	2	3	2	3	3	2	2	3	2	3				
II A	3	14	4	13	7	10	9	8	7	10				
II B	40	24	40	24	38	26	37	27	24	30				
III	28	2	29	1	19	11	23	7	30	0				

and was negatively correlated with postoperative survival time. This phenomenon has been confirmed in other malignancies. Li et al. [29] found that breast cancer patients with increased Src expression had a high probability of tumor metastasis. Maślikowski et al. [30] detected the expression of Src and another 42 kinds of tumor factors in breast cancer and lung cancer through immunohistochemistry and confirmed that Src has a negative correlation with prognosis. Singh et al. [31] showed that Src and p-Src could promote colon cancer invasion and metastasis. Donahue et al. [32] found that p-Src was associated with shorter survival time and lower tumor tissue differentiation in pancreatic cancer patients. Tsao et al. [33] demonstrated that, in malignant pleural mesothelioma, higher levels of clinical stage correlated with higher expression of p-Src. In the study, the survival curves showed throughout an "offset" behavior after a lag phase in Figures 2 and 3. The reasons for these phenomena might be the following aspects: although

the statistical analysis methods were appropriate, the result could not fully deduce the real situation due to the small sample size; the expression of Src/p-Src in a single slice might not sufficiently reflect the expression of the 2 proteins in the tumor because the tumor cells were nonuniform distribution in tumor; and the immunohistochemistry results judged by humans might limit the detection accuracy.

The Src inhibitor dasatinib had been demonstrated to inhibit the development of osteosarcoma *in vitro* and *in vivo*. Davis et al. [34] treated a canine osteosarcoma with dasatinib for 26 weeks, and 24 months of follow-up demonstrated no recurrence. Shor et al. [35] confirmed that dasatinib could suppress the expression of Src in osteosarcoma cells, induce osteosarcoma cell apoptosis, and could prevent cell migration and invasion *in vitro*. Moreover, Shor et al. demonstrated that dasatinib may provide a benefit by controlling the growth and
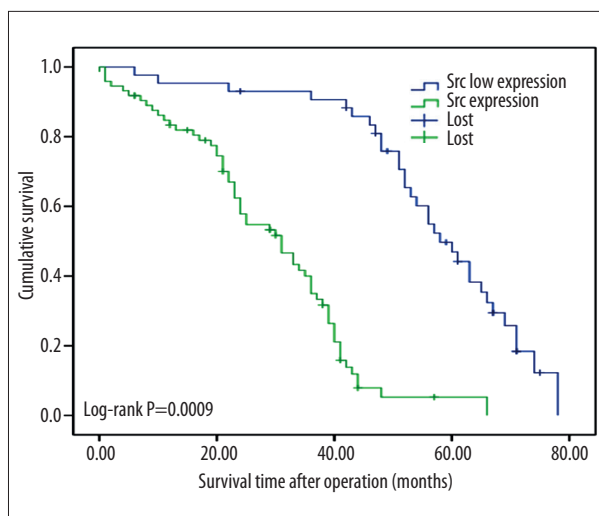
Figure 2. Postoperative survival curves for patients according to the expression of Src.
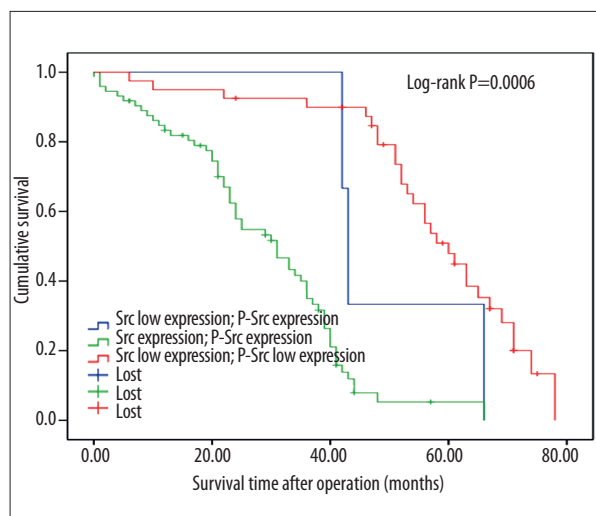

Figure 4. Postoperative survival curves for patients according to the conjoint analysis of Src and p-Src expression.
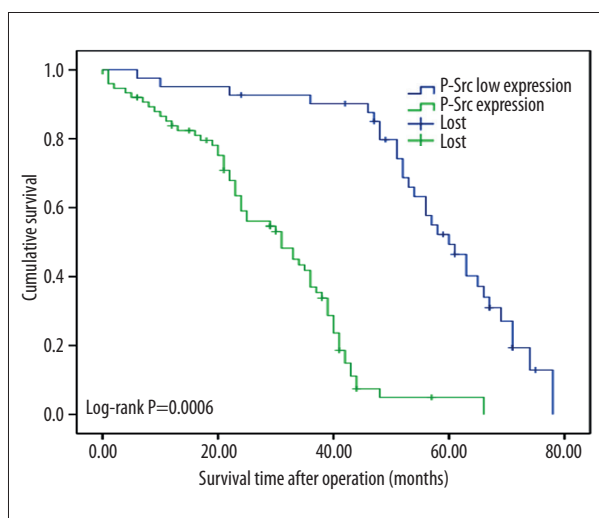

Figure 3. Postoperative survival curves for patients according to the expression of p-Src.

metastasis of sarcomas in patients. However, some of the research findings are not so optimistic. Hingorani et al. [36] demonstrated that dasatinib could completely inhibit Src phosphorylation in primary osteosarcomas in the nude mouse, but could not stop the progression of lung metastases. Therefore, further data on the relationship between Src and lung metastasis of osteosarcoma are required.

Immunohistochemistry is a semi-quantitative technique; hence, we need to be cautious about its results. In addition, osteochondromas were used as a control in this study due to limited conditions, so the results may not be as forceful as a comparison with normal bone specimens. Therefore, this experiment is a preliminary study and requires a larger number of cases for long-term follow-up. In order to provide more valuable experimental data, a multi-unit collaboration and the use of accurate quantitative experimental indicators for statistical analysis are needed.

Table 4. Multivariate survival analysis of overall survival in patients with osteosarcoma.

Variables	P	RR	95% CI for RR
Age	0.065		
Gender	0.820		
Metastasis status	0.014	1.853	1.133–3.032
Clinical stage	0.000	1.852	1.325–2.587
Src	0.029	2.472	1.095–5.582
p-Src	0.005	3.584	1.457–8.816

RR – relative risk; CI – confidence interval.

Conclusions

In this study we have preliminarily identified Src/p-Src as potential biomarkers of osteosarcoma in clinic patients, which could promote osteosarcoma progression or metastasis and may indicate a poorer prognosis.

Acknowledgments

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