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Research Article

Overexpression of SP6 Correlates with Osteosarcoma Metastasis and Poor Prognosis

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ABSTRACT

Background: SP6 (Specificity protein 6) has been explored as a prospective biomarker in several cancers. In this research, the prognostic value of SP6 expression in osteosarcoma was predicted by bioinformatics analysis. Data were obtained from the Gene Expression Omnibus (GEO) database.

Methods: Gene expression data and clinical materials were downloaded from the GSE21257 dataset. The mRNA expression of SP6 was compared between metastatic and non-metastatic tissues with the Wilcoxon rank-sum test, and the relationship between SP6 and clinicopathological characters was analysed using logistic regression. In addition, the correlation between SP6 and survival rate was assessed using Kaplan-Meier and Cox regression. Moreover, receiver operating characteristic (ROC) curve analysis was conducted to determine the prognostic merit of SP6 for osteosarcoma. The biological functions of SP6 were annotated and evaluated through gene set enrichment analysis (GSEA) and gene set variation analysis (GSVA).

Results: SP6 was significantly highly expressed in metastatic osteosarcoma tissues ($p = 0.002$). High SP6 expression showed a positive correlation with Huvos grade (OR = 6.60 for I vs. II, $p = 0.028$). The overall survival (OS) of the patients with high SP6 expression was significantly poorer than the low SP6 expression group ($p = 0.027$). The multivariate analysis revealed that SP6 expression ($p = 0.002$, HR = 15.40 (95% CI [2.84–83.44])) was independently correlated with OS. GSEA and GSVA showed that "spliceosome" and "base excision repair" were significantly upregulated in the high expression group of SP6.

Conclusion: SP6 may serve an independent prognostic biomarker in osteosarcoma.

Introduction

Osteosarcoma is a primary bone malignancy that affects adolescents and young adults [1]. Although the combination of chemotherapy and surgery improve its prognosis, many patients still experience recurrence or metastasis [2]. Pulmonary metastasis is the most common cause of death for osteosarcoma patients [3]. Therefore, identifying reliable biomarkers for osteosarcoma metastasis and prognosis can provide attractive potential targets and prognostic evaluation.

The Krüppel-like family of transcription factors (KLFs) are involved in human various developmental processes. In mammals, 17 KLF family members have been identified, and both oncogenic and tumor-suppressive properties have been documented for KLF genes. In recent

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years, KLF14 (also called SP6) has elicited significant attention. SP6, also known as KLF14, is an important regulator of a wide range of biological processes. A number of studies have indicated that SP6 transcription factors comprise a set of new proteins that may play an inhibitory role in tumors [4, 5]. A previous study has reported that SP6 depletion enhances AOM/DSS-induced colon tumorigenesis, which demonstrates that KLF14 serves as a novel tumor suppressor [6]. A recent study revealed that HAND2-AS1 suppressed colorectal cancer (CRC) progression by upregulating SP6 expression [7]. SP6 has been explored as a prospective biomarker in several cancers. However, one study showed that SP6 promoted cell growth via positive regulation of the antioxidant response under androgen-depleted conditions in prostate cancer cells [8]. Thus, SP6 may have dual roles in the regulation of tumorigenesis, either inhibitory or promoting, which may depend upon the specific tissues [4-10]. Nevertheless, the relative roles of SP6 in osteosarcoma remain undefined.

In the current study, we compared the mRNA expression of SP6 between metastatic and non-metastatic osteosarcoma tissues. Moreover, we analysed the prognostic value of SP6 and searched for the relationship between SP6 and clinicopathological characters as well as overall survival (OS). Furthermore, the biological pathways related to the regulatory mechanism of SP6 were explored by gene set enrichment analysis (GSEA) and gene set variation analysis (GSVA). This study revealed the role of SP6 in osteosarcoma.

Materials and Methods

I Data Mining

The analysis was conducted on the raw gene expressions of the osteosarcoma datasets and corresponding clinical data obtained from the Gene Expression Omnibus data repository (Link 1). GSE21257 contained a total of 53 pre-chemotherapy biopsies of osteosarcoma patients with relatively complete clinical data, including age, gender, subtype, grade, and tumor location. The data set was based on Platforms GPL10295 (Illumina human-6 v2.0 expression bead chip). We downloaded the signal values of the probes from the data set and mapped microarray probes to Gene IDs by annotation information. Afterwards, we screened out the probes matching more than one gene. Finally, we calculated the average expression value for genes measured by multiple probes and obtained 24973 gene expression values.

The inclusion criteria of study patients were as follows: (a) diagnosis of patients with osteosarcoma and (b) detection of gene-level in tissue samples; (c) complete survival data available. Patients were excluded if data were missing on any of the following baseline variables: age, gender, subtype, grade, and tumor location. Finally, 46 samples remained for further analysis after the exclusion of 7 samples without adequate clinical information. We focused on the expression differences of SP6 between metastatic and non-metastatic samples in osteosarcoma.

II Gene Set Enrichment Analysis (GSEA) and Gene Set Variation Analysis (GSVA)

GSEA is a computational method for exploring whether an a priori defined set of genes shows statistically significant, concordant differences between two biological states. It has also been applied to analyze the slight changes in the expressions of genes that belong to a key pathway [11]. We divided 46 samples into high (n=23) and low (n=23) expression groups based on the median expression of SP6. GSEA was carried out to analyze significant survival differences between SP6 high and low groups on GSEA software [12]. By running GSEA, normalized enrichment scores (NES) and nominal *p*-value (NOM *p*-value) were generated for the pathway enrichment analysis in each phenotype. The default-weighted enrichment statistic was adapted to conduct the permutation for 1000 times with normalized $p < 0.05$ considered significantly enriched.

Additionally, the "GSVA" R package was utilized to detect the pathways that are related to each phenotype [13]. *p* less than 0.01 was considered statistically significant. We downloaded the reference gene set “c2.cp.kegg.v6.2.symbols.gmt” from the Molecular Signature Database (MSigDB, Link 2).

III Statistical Analysis

Statistical analysis was conducted using R (version.3.6.0). Wilcoxon test was performed to validate expression levels SP6 between metastatic and non-metastatic samples. A *p*-value < 0.05 was to be considered statistically significant. To validate the possibility of SP6 as a prognostic biomarker, we outlined the receiver operating characteristic (ROC) curves and calculated area under the ROC curve (AUC) with the "survivalROC" R package. We used a logistic regression to analyze the association between clinical characteristics and SP6 expression. Cox regression was used for the correlation between clinical characteristics and survival. Multivariate Cox analysis was performed to find independent risk factors for survival.

Results

I Clinical Characteristics

The clinical characteristics of 46 patients were obtained from GSE21257, including patients' gender, age, subtype, Huvos grade, tumor location, survival status, and metastasis of osteosarcoma (Table 1).

Table 1: Clinical characteristics of the osteosarcoma patients.

Characteristic	n (%)
Age (years)	
<12	11(23.91)
12-20	28(60.87)
≥20	7(15.22)
Gender	
Male	16(34.78)
Female	30(65.22)
Tumor location	
Femur	25(54.35)
Fibula	2(4.35)
Humerus	5(10.87)
Tibia	14(30.43)
Histological subtype	
Giant cell rich	1(2.17)
Chondroblastic	5(10.87)

Fibroblastic	4(8.70)
Osteoblastic	29(63.04)
Sclerosing	2(4.35)
Telangiectatic	2(4.35)
Anaplastic	2(4.35)
Pleomorphic	1(2.17)
Huvos grade	
I	12(26.09)
II	16(34.78)
III	13(28.26)
IV	5(10.87)

II SP6 was Highly Expressed in Tissues from Patients with Metastasis

The mRNA expression of SP6 was then compared between 29 osteosarcoma tissues with metastasis and 17 osteosarcoma tissues without metastasis using the Wilcoxon rank-sum test. We found the expression of SP6 was significantly increased in tissues with metastasis (p = 0.002) (Figure 1), indicating SP6 may have prognostic values for osteosarcoma patients.

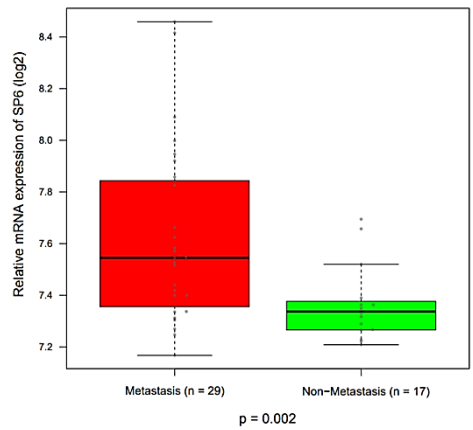

Figure 1: Association with SP6 expression and osteosarcoma metastasis. SP6 is higher in osteosarcoma with metastasis than without metastasis.

III Association with SP6 Expression and Clinicopathologic Characteristics

We explored the relationship between SP6 expression and clinical characteristics (Table 2). High SP6 expression showed a positive correlation with Huvos grade (OR = 6.60 for I vs. II, p = 0.028). Univariate analysis suggested that high SP6 expression was associated with poor prognostic features. The results showed that high expression of SP6 predicts poor prognosis in osteosarcoma.

Table 2: SP6 expression associated with clinical characteristics (logistic regression).

Clinical characteristics	Odds ratio in SP6 expression	p -Value
Age (12-20 vs. <12)	2.16 (0.52-9.33)	0.287
Gender (male vs. female)	3.30 (0.95–12.82)	0.069
Tumor location (tibia vs. femur)	0.37 (0.09-1.40)	0.151
Histological subtype (Osteoblastic vs. Chondroblastic)	4.29 (0.55-83.39)	0.217
Huvos grade (I vs. II)	6.60 (1.34-41.18)	**0.028**

Bold values indicate $p < 0.05$.

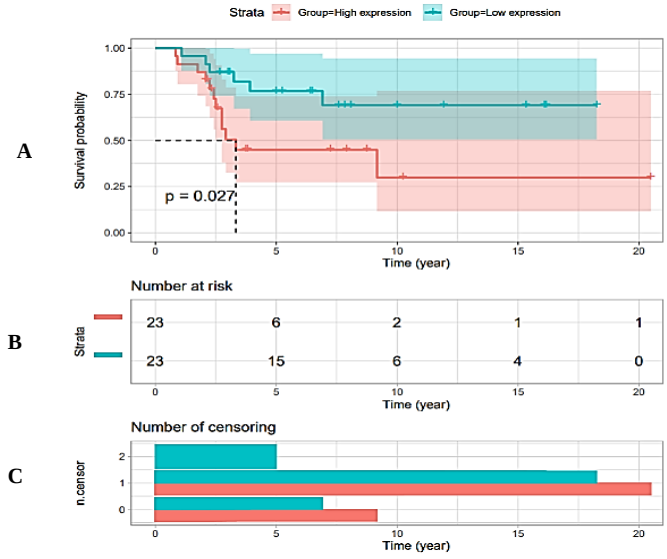

Figure 2: High expression of SP6 is associated with poor OS in patients with osteosarcoma. **A)** The Kaplan–Meier curves, **B)** number at risk, and **C)** number of censoring of OS in osteosarcoma.

IV Prognostic Role of SP6 Expression in Osteosarcoma

Forty-six osteosarcoma patients were included in the current study, and the median follow-up time was 71.2 months. Overall survival of the patients with high SP6 expression was significantly poorer than the low SP6 expression group (p = 0.027) (Figures 2A & 2C). Time-dependent ROC curve analysis was used to evaluate the discriminative ability of SP6 at five years of follow-up (Figure 3; AUC: 0.746). Univariate analysis of prognostic factors for OS was performed with the Cox regression model (Table 3). High SP6 expression was correlated with worse OS (p = 0.002, hazard ratio [HR] = 8.41 (95% CI [2.19–32.37])). High Huvos grade correlated with better OS (p = 0.025, hazard ratio [HR] = 0.52 (95% CI [0.30–0.92])). Results of multivariate analysis showed that SP6 expression (p = 0.002, HR = 15.40 (95% CI [2.84–83.44])) and Huvos grade (p = 0.019, HR = 0.51 (95% CI [0.29–0.90])) were independently associated with OS (Table 3). In addition, the forest plot of multivariable analysis for survival was shown in (Figure 4). The above results indicated that SP6 was a prognostic biomarker, and high levels of SP6 predicted poor prognosis.

Table 3: Univariate analysis and multivariate analysis of the correlation of SP6 expression with survival.

Parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age	0.88	0.43-1.79	0.719			
Gender	1.41	0.52-3.79	0.500			
Tumor location	1.07	0.76-1.50	0.701			
Histological subtype	1.15	0.84-1.59	0.385			
Huvos grade	0.52	0.30-0.92	**0.025**	0.51	0.29-0.90	**0.019**
SP6	8.41	2.19-32.37	**0.002**	15.40	2.84-83.44	**0.002**

Bold values indicate $p < 0.05$; HR: Hazard Ratio; CI: Confidence Interval.

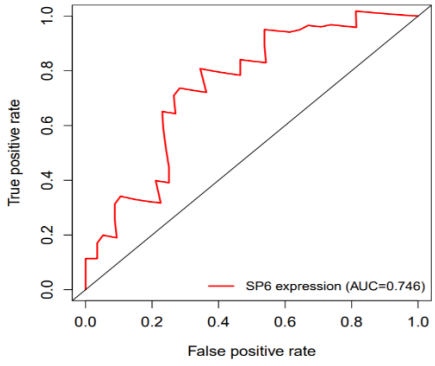

Figure 3: Time-dependent ROC analysis for SP6 to predict 5-year survival. The ROC curve has an AUC of 0.746.

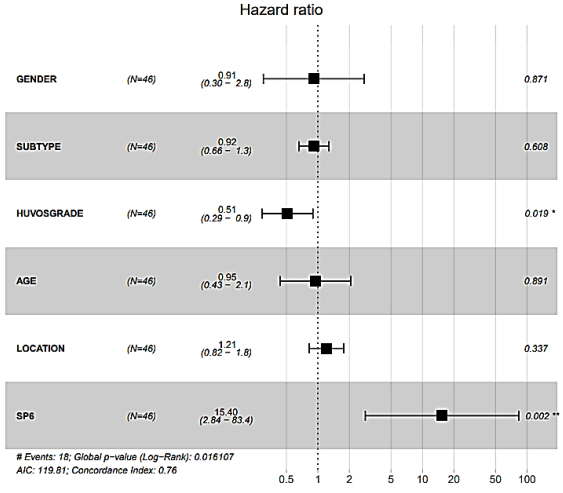

Figure 4: Forest plot of the multivariable analysis showed that SP6 was an independent risk factor for survival.

V SP6-Related Signaling Pathways Based on GSEA and GSVA

We performed GSEA to search signaling pathways activated in osteosarcoma enriched in the high SP6 expression data sets. As shown in (Figure 5), we focused on the top five significantly enriched KEGG pathways, including "propanoate metabolism", "spliceosome", "pyruvate metabolism", "base excision repair" and "RNA degradation". Furthermore, GSVA confirmed that "spliceosome" and "base excision repair" were significantly upregulated in high expression group, further suggesting their importance in the progression of osteosarcoma (Figure 6).

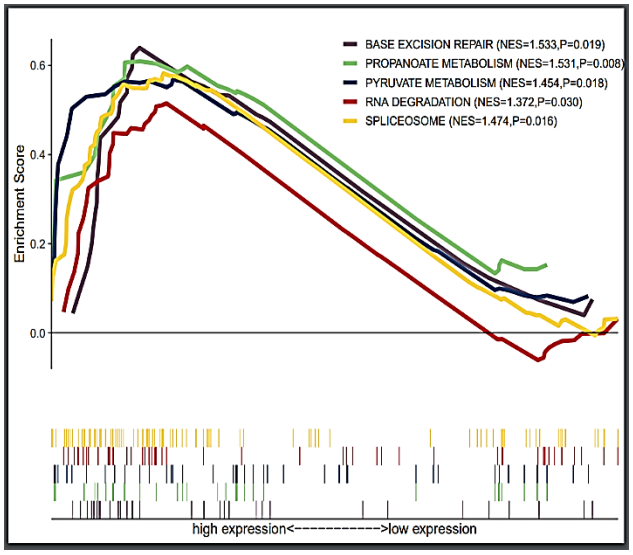

Figure 5: Gene set enrichment analysis (GSEA) of SP6. The top five significantly enriched pathways in high expression phenotype were plotted.

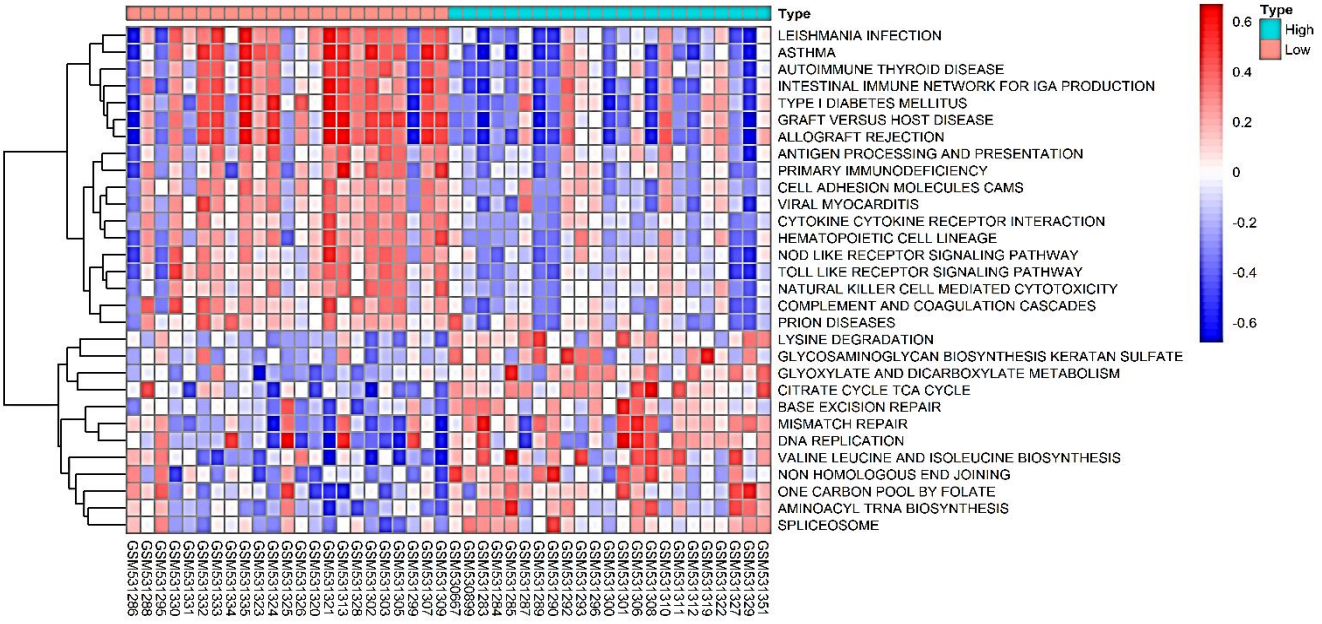

Figure 6: Gene set variation analysis (GSVA) of SP6. GSVA confirmed that "spliceosome" and "base excision repair" were significantly upregulated in the high expression group.

Discussion

Osteosarcoma is the most common primary malignant tumor of bone. High local aggressiveness, rapid recurrence and metastasis, considerable histological heterogeneity, widespread genetic instability, lack of specific biomarkers bring great challenges for the treatment of osteosarcoma [14]. Despite intense research efforts, the molecular mechanism underlying osteosarcoma metastasis and prognosis remains elusive [15, 16]. The search for prognostic biomarkers for cancer is a major challenge [17]. Therefore, the present study focused on the potential prognostic role of SP6 in osteosarcoma.

In this study, bioinformatics analysis of expression microarray data sets demonstrated that high SP6 expression was correlated with poor survival status and metastasis in osteosarcoma. Additionally, increased SP6 in osteosarcoma tissues was positively correlated with clinical Huvos grade. And the results of univariate and multivariate Cox analysis suggested that SP6 may be an independent biomarker for osteosarcoma prognosis. Moreover, ROC analysis confirmed the prognostic value of SP6 in osteosarcoma. Previous studies have revealed that SP6 plays an important role in various cancer types. SP6 reduction serves as a mechanism leading to tumorigenesis in human breast cancers [6]. Wu *et al*. found that the downregulation of SP6 could prompt glycolysis by target glycolytic enzyme LDHB, and SP6 could constitute potential prognostic predictors in colorectal cancer [10]. Additionally, Kanamori *et al*. found SP6 was overexpressed in the metastasis through analysis of the ratio of DNA copy number alterations between the primary and metastatic bone tumors [18].

Many studies have reported that overexpression of TGF-β is a hallmark of osteosarcoma [19-21]. TGF-β pathway plays an extremely important role in the interaction between osteosarcoma cells and their microenvironment and activation of the TGF-β pathway promotes osteosarcoma cell metastasis [20, 21]. Studies show that SP6 is closely linked to the TGF-β pathway [22, 23]. A previous study revealed that activation of the TGF-β pathway caused induction of SP6 expression. Subsequently, SP6 combined with competitive to the TGF-βRII promoter and blocked TGF-βRII expression [22]. SP6 mediates a negative feedback loop to reduce the expression of TGF-βRII upon TGF-β stimulation. The SP6 transcriptional pathway becomes an important process for regulating the activity of TGF-β signaling. SP6 is also involved in many different physiological and pathological processes. SP6 and TGF-β pathway may play an essential role in the pathogenesis of osteosarcoma, which deserves further study.

We further explored the function of SP6 in osteosarcoma by GSEA, and the results revealed that genes in high expression group of SP6 were significantly enriched in KEGG pathways, including "propanoate metabolism", "spliceosome", "pyruvate metabolism", "base excision repair" and "RNA degradation". Moreover, GSVA confirmed that "spliceosome" and "base excision repair" were significantly upregulated in the high expression group of SP6. Some previous studies have reported that "spliceosome" plays a significant role in osteosarcoma. Zhang *et al*. reported "spliceosome" was markedly associated with osteosarcoma through GSEA analysis of the competing endogenous RNA regulatory network [24]. Li *et al*. identified differentially expressed genes (DEGs) in the progression of fluoride-affected osteosarcoma and subsequently found the DEGs were enriched in "spliceosome" [25].

DNA repair systems play critical roles in the prevention of tumorigenesis, and genetic defects in DNA repair systems could cause human tumors. DNA base damage is mainly repaired by the base excision repair (BER) pathway. The clinical data suggest that BER factors have a vital role in development and prognosis in cancer [26-28]. In addition, a large number of DNA BER proteins have been investigated as a potential biomarker and therapeutic target [29-31].

Limitations still existed in the current study. First, the dataset of GSE21257 has provided detailed clinical information, but the sample size was relatively small. Second, future studies, both *in vitro* and *in vivo*, would be conducted to identify the biological roles of SP6 in osteosarcoma. Consequently, there is a considerable need for further research.

Conclusion

Our study demonstrated that the expression of SP6 was significantly increased in metastatic osteosarcoma tissues, and elevated SP6 expression served as an independent risk factor for survival in osteosarcoma. Further experimental validation, both *in vitro* and *in vivo* will be required to verify the above results based on bioinformatics analysis. SP6 may be a potential biomarker for the prognosis of osteosarcoma.

Author Contributions

DMG and JLY designed the experiment. LLZ undertook data acquisition. SCW was involved in the interpretation of data. CPL and SCW analysed and visualized the data. CPL drafted the manuscript. The final manuscript was read and approved by all authors.

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Conflicts of Interest

None.

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