

Increased Six1 expression is associated with poor prognosis in patients with osteosarcoma

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Abstract. *Sine oculis homeobox homolog 1 (Six1)* is an evolutionarily conserved transcription factor that acts as master regulator of development and is frequently dysregulated in various types of cancer. *Six1* has been demonstrated to be upregulated in human osteosarcoma cell lines compared with osteoblastic cell lines. However, the association of *Six1* expression with the progression and prognosis of osteosarcoma patients remains unclear. The purpose of the present study was to investigate the association between *Six1* expression and the clinicopathological characteristics and prognosis of osteosarcoma. *Six1* protein was detected by immunohistochemistry in a series of 100 osteosarcoma patients, and Kaplan-Meier survival analysis was performed to assess prognosis. The results revealed that increased *Six1* protein expression was prevalent in osteosarcoma and was significantly associated with Enneking stage ($P=0.002$) and tumor size ($P=0.010$). Additionally, according to the log-rank test and Cox regression model, expression of *Six1* is indicated to be an independent prognostic factor in osteosarcoma patients. In summary, positive expression of *Six1* protein is closely associated with the tumor progression and poor survival of osteosarcoma patients. The results suggest that *Six1* is overexpressed in individuals with poor prognosis, and may thus be used as a prognostic biomarker in patients with osteosarcoma.

Introduction

Osteosarcoma is the most common primary malignant bone tumor, accounting for ~20% of primary malignancies of the bone among children and adolescents. It frequently leads to

lung metastasis and often ultimately results in mortality (1,2). Osteosarcoma is highly aggressive and has a wide range of histological appearances. Conventional osteosarcoma may be classified as osteoblastic, chondroblastic or fibroblastic, depending on the predominant type of extracellular matrix present (3). However, no statistically significant differences have been identified between these three histological types with regard to the survival of patients with high-grade tumors. Furthermore, the treatment for all types is similar (4). The prognosis of this disease is poor despite the use of multimodal therapy, including surgery, radiotherapy and chemotherapy (5,6). Progression to metastatic disease is the predominant cause of treatment failure, while the cellular and molecular mechanisms defining the biological behavior of osteosarcoma cells are poorly understood (6). Therefore, numerous studies have been conducted to broaden the understanding of factors governing the behavior of osteosarcoma cells in order to improve treatment results by identifying potential therapeutic targets (6-8).

Sine oculis homeobox homolog 1 (Six1) is a mammalian homolog of the *Drosophila sine oculis* gene; it is highly conserved between *Drosophila* and humans (9,10). The appropriate expression of this gene is crucial for the development of multiple organs, including the brain, eyes, ears, craniofacial structures and kidney sensory structures (11-13). Aside from its involvement in early organ development, *Six1* is often aberrantly expressed in a variety of tumor types, including breast cancer (14), ovarian cancer (15,16), cervical cancer (17,18), Wilms' tumors (19), rhabdomyosarcomas (20) and hepatocellular carcinoma (21). Notably, the aberrant expression of *Six1* in cancer can induce developmental programs out of context, which contributes to tumor onset and progression (22,23).

The association between *Six1* and the prognosis of osteosarcoma remains unknown. The present study was conducted to investigate the correlation of *Six1* with clinicopathological features in osteosarcoma and its effect on the prognosis of osteosarcoma patients.

Materials and methods

Patients and tissues. In the present study, resected specimens from 100 osteosarcoma patients who had been

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clinically and pathologically diagnosed between January 2006 and September 2013 were retrieved from the Department of Orthopaedics of Inner Mongolia People's Hospital (Hohhot, Inner Mongolia Autonomous Region, China) for tissue microarray (TMA) construction and immunohistochemical (IHC) analysis. The included patients had undergone preoperative treatment according to a standard protocol, consisting of neoadjuvant chemotherapy [six cycles of cisplatin (300–400 mg/m² on days 1–5 per month) and methotrexate (1 g/6 h on days 1–3 per month) were administered with a minimum of a 21-day interval], followed by appropriate surgical management and postoperative adjuvant chemotherapy. Detailed pathological and clinical data were collected by reviewing medical records; collected data included age, gender, tumor site, histological type, Enneking stage (24) and tumor size. Two investigators (L.C. and D.Z.) who were blinded to the clinical information confirmed all histological diagnoses and judged staining scores independently. The study protocol was performed under the approval of the Ethics Committee of the Inner Mongolia People's Hospital and informed consent was obtained from all patients. Follow-up was terminated on 31 December 2013. Overall survival (OS) was defined as the interval between surgery and mortality or the last observation taken. The data were censored at the last follow-up period for living patients. Disease-free survival (DFS) was defined as the interval between the date of surgery and the date of diagnosis of any type of relapse or the last follow-up assessment.

TMA construction and IHC analysis. Representative sections of osteosarcoma tissues in the pre-existing paraffin-embedded tissue blocks were determined according to the overlaid hematoxylin and eosin-stained slides. The TMA was constructed using a needle to punch cylinders of 1.5 mm diameter from the representative section of each block, and placing the cylinders into an array on a recipient paraffin block. Multiple sections (5.0- μ m-thick) were cut from the TMA block and mounted on microscope slides for IHC analysis. The TMA consisted of a total of 100 cases of paraffin-embedded osteosarcoma tissue. The clinical characteristics of the patients are summarized in Table I. The TMA slide was dried overnight at 37°C, deparaffinized in xylene, rehydrated through a graded alcohol series, and then immersed in 3% hydrogen peroxide for 10 min to inactivate peroxidase activity. Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) at 100°C for 20 min, then cooling for 20 min at room temperature to expose antigenic epitopes. The slides were pre-incubated with 5% normal goat serum (Guangzhou Jetway Biotech Co., Ltd., Guangzhou, China) at room temperature for 30 min to reduce non-specific reactions. A primary rabbit polyclonal antibody against Six1 (HPA001893; Atlas Antibodies AB, Stockholm, Sweden) was diluted (1:1,000) with 1X PBS and applied overnight in a humidified chamber at 4°C. The slides were sequentially incubated with a goat anti-rabbit polymer peroxidase-labeled secondary antibody (1:500; ZDR-5306; ZSGB-Bio, Beijing, China) for 20 min at room temperature, and then visualized using 3,3'-diaminobenzidine (Beyotime Institute of Biotechnology, Haimen, China) as a chromogen. Finally, the sections were counterstained with hematoxylin.

A known Six1-positive slide was used as a positive control, and PBS replaced anti-Six1 primary antibody as the negative control.

Evaluation of IHC staining. Immunoreactivity for the Six1 protein was scored according to the staining intensity and positive percentage. Sections were considered positive if they exhibited immunoreactivity in the nucleus and/or cytoplasm when observed by an evaluator who was blinded to the clinical history and outcome of the case. Positive percentage scores were assigned according to the following scale: 0, 0% positively stained cells; 1, 1–25% positively stained cells; 2, 26–50% positively stained cells; and 3, >50% positively stained cells. Staining intensity was also scored semi-quantitatively as follows: 0, none; 1, mild; 2, moderate; and 3, intense. A total score was obtained (ranging from 0 to 9) by multiplying the positive percentage score and the intensity score for each section. From the total scores, 0, 1–3, 4–6, and 7–9 were recorded as –, +, ++, and +++, respectively. Negative expression was defined as a score of 0 or 1; positive expression was defined as a score of >1. The scores were accepted if the two investigators (L.C. and D.Z.) agreed with the values. Otherwise, the values were re-estimated until a consensus was reached. The conclusions were in complete agreement in 80% of the cases, which indicated that the scoring method was highly reproducible.

Statistical analysis. Statistical analysis was performed using the SPSS statistical software program (version 18.0; SPSS, Inc., Chicago, IL, USA). The associations between Six1 protein expression and the clinicopathological data of osteosarcoma patients were analyzed using the χ^2 test. The association between survival and each variable was determined with the Kaplan-Meier method. Differences between survival rates were analyzed with the log-rank test and Cox regression analysis. $P < 0.05$ (two-tailed) denotes the presence of a statistically significant difference.

Results

Clinicopathological data of the patients. As shown in Table I, among the 100 studied cases, 35 (35%) were female and 65 (65%) were male, equating to a female:male ratio of ~1:2. The age ranged from 7 to 69 years, with a mean $\pm$ standard deviation of 24.70 $\pm$ 13.66 years; 62 patients (62%) were aged $\leq$ 25 years and 38 patients (38%) were aged >25 years. Regarding the anatomical site of the tumor, 39 tumors (39%) were in the femur, 15 (15%) were located in the tibia, and 5 tumors (5%) were located in the humerus; the remaining 41 tumors (41%) were located in other sites. The size of the tumors ranged from 2 to 17 cm, with a mean of 8.49 $\pm$ 3.54 cm.

Six1 expression in the osteosarcoma tissues. IHC staining was performed to investigate Six1 protein expression levels. The analysis revealed that the expression of Six1 was predominantly localized in the cytoplasm and the distribution of positive cells was primarily located in peripheral portion of the osteosarcoma; a number of osteosarcoma tissues exhibited strong staining, while others exhibited weak or absent

Table I. Six1 expression in relation to clinical and pathological factors in a series of 100 patients with osteosarcoma.

Variable	Number	Six1 expression		χ^2	P-value
		Negative	Positive		
Age (years)					0.798
≤25	62	31	31	0.065	
>25	38	20	18		
Gender					0.367
Female	35	20	15	0.813	
Male	65	31	34		
Tumor site					0.085
Femur	60	32	28	4.924	
Tibia	27	16	11		
Humerus	13	3	10		
Histological type					0.181
Osteoblastic	49	20	29	4.875	
Chondroblastic	28	16	12		
Fibroblastic	18	11	7		
Telangiectatic	5	4	1		
Enneking stage					0.002
I	18	15	3	9.183	
II	82	36	46		
Tumor size (cm)					0.002
≤5	21	17	4	9.543	
>5	79	34	45		

Six1, sine oculis homeobox homolog 1.

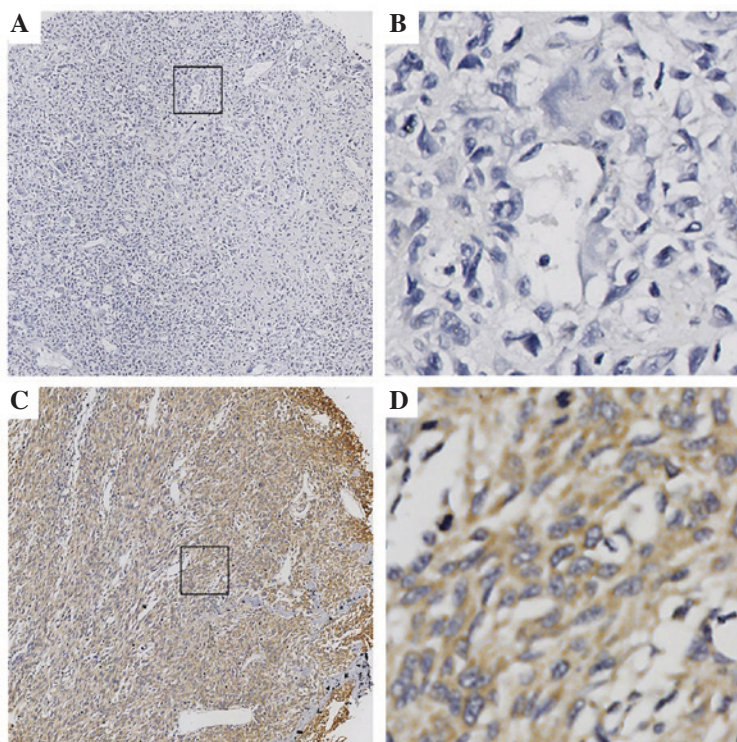


Figure 1. Immunohistochemical staining of Six1 protein in osteosarcoma tissues. Six1 was predominantly expressed in the cytoplasm of osteosarcoma cells. (A and B) Negative Six1 expression: (A) magnification, x100; (B) magnification, x400. (C and D) Positive expression of Six1: (C) magnification, x100; (D) magnification, x400. Six1, sine oculis homeobox homolog 1.

Table II. Univariate survival analysis of osteosarcoma according to immunohistochemistry scores for Six1 and other clinicopathological factors.

Variable	Overall survival time (months)			Disease-free survival time (months)		
	Mean	95% CI	P-value	Mean	95% CI	P-value
Six1			<0.001			<0.001
Negative	50.395	43.425-57.336		46.644	40.289-52.999	
Positive	29.668	25.286-34.050		28.006	24.272-31.739	
Age (years)			0.639			0.938
≤25	42.371	35.656-49.085		38.016	32.222-43.809	
>25	38.910	32.278-45.542		37.312	31.406-43.218	
Gender			0.273			0.540
Male	39.297	33.346-45.248		36.902	31.554-42.249	
Female	43.978	35.821-52.135		39.065	32.221-45.909	
Tumor site			0.681			0.223
Femur	39.627	34.000-45.254		36.434	31.723-41.145	
Tibia	46.253	36.284-56.222		43.268	34.076-52.460	
Humerus	31.856	22.043-41.133		29.558	20.185-38.931	
Histologic type			0.790			0.505
Osteoblastic	40.786	33.788-47.784		37.098	31.145-43.051	
Chondroblastic	43.759	35.156-52.362		41.415	33.524-49.307	
Fibroblastic	37.944	26.547-49.342		36.132	25.878-46.386	
Telangiectatic	31.800	16.850-46.750		28.000	15.298-40.702	
Enneking stage			<0.001			<0.001
I	66.750	56.684-76.816		61.185	51.637-70.733	
II	34.340	30.053-38.627		32.157	28.454-35.859	
Tumor size (cm)			<0.001			0.001
≤5	65.059	53.404-76.714		56.588	45.940-67.237	
>5	35.314	30.938-39.690		33.529	29.524-37.535	

P<0.05 was considered to indicate a statistically significant difference. Six1, sine oculis homeobox homolog 1; CI, confidence interval.

Table III. Multivariate analysis of factors associated with OS and DFS.

Variable	OS			DFS		
	HR	95% CI	P-value	HR	95% CI	P-value
Six1 (negative vs. positive)	1.757	1.089-2.835	0.021	1.848	1.165-2.931	0.009
Gender (female vs. male)	0.953	0.579-1.568	0.849	0.885	0.554-1.411	0.607
Enneking stage (I vs. II)	3.126	1.432-6.826	0.004	2.783	1.399-5.537	0.004
Tumor size (≤5 vs. >5 cm)	2.744	1.190-6.328	0.018	1.854	0.932-3.686	0.078

P<0.05 was considered to indicate a statistically significant difference. OS, overall survival; DFS, disease free survival; HR, hazard ratio; CI, confidence interval; Six1, sine oculis homeobox homolog 1.

staining (Fig. 1). Positive Six1 expression was observed in 49.0% of the 100 osteosarcoma tissues. A significant differences in the distribution of IHC scores between the different Enneking stages (P=0.002) was noted: Positive Six1 expression was observed significantly more often in cases with high-stage disease compared with cases with a low-stage. A highly significant association was also identified between Six1

expression and tumor size (P=0.010). However, the other clinicopathological parameters, including age, gender, tumor site and histological type were not significantly associated with Six1 expression (Table I).

Six1 expression is associated with patient prognosis. To evaluate the correlation of Six1 protein expression with

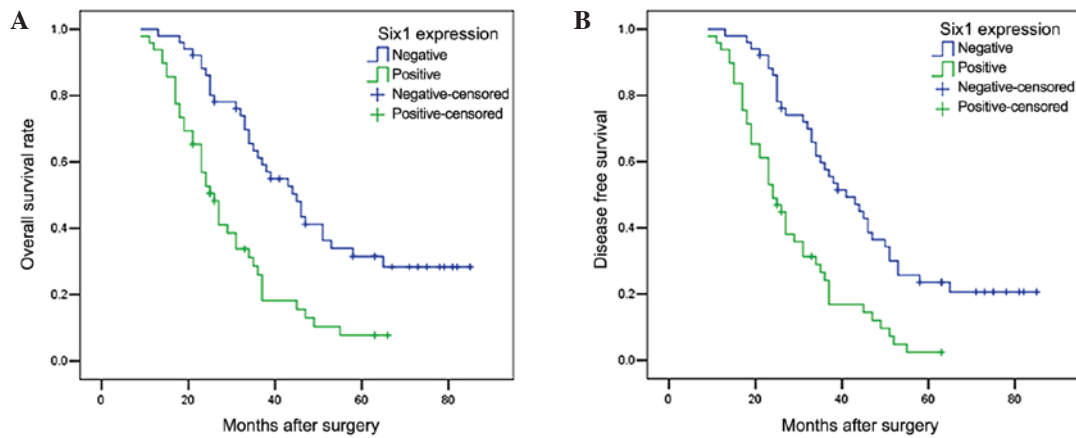


Figure 2. Kaplan-Meier analysis of (A) OS and (B) DFS of patients with osteosarcoma based on positive or negative Six1 expression. Osteosarcoma patients with Six1-positive expression exhibited significantly poorer OS and DFS rates compared with those with Six1-negative expression. OS, overall survival; DFS, disease-free survival; Six1, sine oculis homeobox homolog 1.

patient prognosis, the osteosarcoma patients were divided into two groups: Negative Six1 expression ($n=51$) and positive Six1 expression ($n=49$). A log-rank test and Kaplan-Meier analysis were performed to assess the effect of Six1 expression on patient survival. The log-rank test revealed that the OS time of osteosarcoma patients with Six1-positive expression was markedly shorter than those with Six1-negative expression ($P<0.001$; Fig. 2A; Table II). Similar results were also observed in the DFS analysis ($P<0.001$; Fig. 2B; Table II). At time of last follow-up, 41 patients in the Six1-positive group had succumbed to disease, with a mean OS time of 29.67 months. Of these, 3 patients developed local recurrence and 6 had lung metastasis. Comparatively, 33 patients succumbed to disease in the Six1-negative group and the mean OS time was 50.40 months. Furthermore, as shown in Table III, multivariate Cox analysis indicated that Six1 expression was one of the independent prognostic factors, along with Enneking stage and tumor size.

Discussion

Homeobox genes encode transcription factors that are essential for the development of numerous organs and the control of various processes, including cell proliferation, apoptosis, migration and invasion (11,23,25,26). The Six1 homeoprotein, a member of the Six family of homeodomain transcription factors, has been found to be aberrantly expressed in multiple types of cancer, including breast cancer (14,22,27), hepatocellular carcinomas (21), ovarian cancer (15), Wilms' tumors (19) and rhabdomyosarcomas (20,28,29). Six1 is also involved in cellular migration and invasion during embryogenesis (25,30-33) and in breast cancer (34,35). A previous study demonstrated that Six1 was able to promote metastasis by coordinating with transforming growth factor β (TGF- β) to increase the expression of vascular endothelial growth factor C (VEGF-C) through two pathways. Firstly, Six1 is able to enhance TGF- β signaling by upregulating TGF- β receptor 1 expression, which promotes the activation of SMAD2/3 and its binding to the VEGF-C promoter, thus increasing the expression of VEGF-C. Secondly, Six1 can cooperate with SMAD2/3 to

bind to the VEGF-C promoter and modulate VEGF-C expression. In tumor cells lacking Six1 expression, the expression of VEGF-C was slightly influenced by TGF- β stimulation; however, SMAD2/3 could be phosphorylated and bind to the VEGF-C promoter. Therefore, Six1 is necessary for TGF- β to induce the increased expression of the VEGF-C gene (36). Overexpression of Six1 also significantly enhances the activation of the cyclin D1 promoter in pancreatic cancer and promotes cell cycle progression and proliferation (37). Six1 overexpression is positively correlated with OS and DFS rates of patients with breast cancer (38).

It has been reported that Six1 expression levels are upregulated in osteosarcoma cell lines compared with noncancerous human osteoblastic cell lines, and Six1 was able to increase the growth, proliferation and migration of U2OS cells, as well as suppress apoptosis (39). Therefore, we hypothesized that Six1 may be associated with the progression and prognosis of osteosarcoma.

In the present study, the expression of Six1 protein was detected in osteosarcoma samples from cases with various clinicopathological features. Six1 expression was identified to be positively associated with Enneking stage and tumor size, but not age, gender, tumor site or histological type. These results indicate that Six1 expression in osteosarcoma is associated with osteosarcoma progression. In order to investigate the influence of Six1 on the prognosis of osteosarcoma patients, 100 patients were followed up after surgery. Six1 was identified as an independent factor able to predict the OS time of osteosarcoma patients. Firstly, the log-rank test revealed that patients with Six1-positive expression tended to have a significantly shorter OS time (mean, 29.67 months) compared with the Six1-negative group (mean, 50.40 months). Secondly, Cox regression analysis identified that Six1 may act as an independent prognostic factor, in addition to Enneking stage and tumor size.

In summary, Six1 is differently expressed in cases of osteosarcomas with different clinicopathological features, showing positive correlations with Enneking stage and tumor size, which indicates poor prognosis in patients with positive Six1 expression. Six1 protein may therefore be suggested as a useful prognostic biomarker for osteosarcoma.

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