Expression of high mobility group box 1 protein predicts a poorer prognosis for patients with osteosarcoma

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Abstract. The high mobility group box 1 (HMGB1) protein functions as an extracellular signaling molecule that is critical in inflammation and carcinogenesis. The HMGB1 protein is actively secreted by natural killer cells, monocytes and macrophages, and acts as an inflammatory cytokine. The present study enrolled 174 patients that underwent a tumorectomy between 2006 and 2013 in Shandong Provincial Hospital. The age of the patients ranged between 13 and 74 years, with a median age of 27 years. The tumors of the patients were staged according to the Union for International Cancer Control 2009 tumor-node-metastasis tumor staging system. Nuclear grading was based on the Fuhrman grading system. In the osteosarcoma tissue samples, HMGB1 expression was detected in 84 samples (48.3%) with a low immunoreactivity and in 90 samples (51.7%) with a high immunoreactivity. The association between clinicopathological characteristics and tumor cell HMGB1 expression (low vs. high) was summarized. The association between HMGB1 expression and tumor size, tumor stage and nuclear grade was statistically significant (P=0.034, 0.008 and 0.019, respectively). There was no significant association between HMGB1 expression and the age of the patients (P=0.335; Table I). The current study demonstrated that patients with a high HMGB1 expression (>50% cells expressing HMGB1) had poorer survival rates, and therefore a poorer prognosis, compared with patients with low HMGB1 immunostaining (10-50% cells expressing HMGB1). The results of the present study suggest that higher expression levels of HMGB1 are significantly associated with a poorer prognosis and may act as a marker for prognosis in osteosarcoma, particularly osteosarcoma recurrence. Additional studies investigating the biological features of HMGB1 may confirm the potential role of HMGB1 as a novel target for anticancer therapy in osteosarcoma.

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Introduction

Osteosarcoma accounts for ~20% of pediatric, solid and malignant tumors, and it is the most common malignant bone tumor in adolescents and young adults (1). In total, ~40% of osteosarcoma tumors metastasize and patients possess a poor overall prognosis (2-4). The 5-year survival rate is reported to be 37% in osteosarcoma patients with tumor metastasis and 19% for patients with >5 metastatic lung lesions (5). Uncontrolled cell proliferation and robust tumor angiogenesis are characteristics that contribute to the poor prognosis of osteosarcoma patients (6). Aggressive osteosarcoma cells demonstrate increased angiogenesis and vasculogenic mimicry (7). Several studies have revealed that tumor cells may directly form tumor blood vessels through vasculogenic mimicry (8-13). This is closely associated with tumor metastasis and the poor prognosis of various cancers, including osteosarcoma (11,12). Consequently, highly proliferative and vasculogenic osteosarcoma cells are a clear target for novel anti-osteosarcoma drug identification.

The high mobility group box 1 (HMGB1) protein was originally described as a non-histone DNA binding protein that is highly conserved, with a high acidic and basic amino acid content (14). The HMGB1 protein binds to minor grooves in DNA and promotes the assembly of site-specific transcriptional proteins (15). In addition to its well-established nuclear functions, the HMGB1 protein acts as an extracellular signaling molecule that is critical in inflammation and carcinogenesis (16,17). The HMGB1 protein is actively secreted by natural killer cells, monocytes and macrophages, and functions as an inflammatory cytokine (18,19). Necrotic cells, particularly those derived from cancer tissues, passively release the HMGB1 protein, which mediates local inflammation and the development of cancer (16,20). Extracellular HMGB1 protein interacts with several receptors, including the receptor for advanced glycation end products (RAGE), Toll-like receptors and cluster of differentiation (CD)24 (21). It has been reported that HMGB1 and RAGE are overexpressed in clear cell renal cell carcinoma (ccRCC). In addition, HMGB1 promotes the development and progression of ccRCC via extracellular-signal-regulated kinase 1/2 activation, which is partially mediated by RAGE (22).

Although the expression of HMGB1 has been associated with the prognosis of urinary tumors, including bladder

urothelial carcinoma (23), no studies have investigated the prognostic role of HMGB1 in osteosarcoma, such as the association between HMGB1 expression and the prognosis of osteosarcoma. In the present study, the expression of HMGB1 in clinical osteosarcoma samples, prognostic role of HMGB1, and the association between HMGB1 expression and clinicopathological features were investigated, with the aim of identifying HMGB1 as a prognostic marker in patients with osteosarcoma.

Materials and methods

Patients and tissue specimens. The present study assessed 174 patients who underwent tumorectomies between 2006 and 2013 at Shandong Provincial Hospital (Jinan, Shandong, China). The age of patients ranged between 13 and 74 years, with a median age of 27 years. The tumors of the patients were staged according to the Union for International Cancer Control 2009 tumor-node-metastasis (TNM) tumor staging system (24), and nuclear grading was based on the Fuhrman grading system (25). The follow-up time was calculated from the date of surgery to the date of the patient's last follow-up or mortality. Patients were excluded if they had incomplete medical records or inadequate follow-up. Tissue samples were obtained from the surgical samples of patients with osteosarcoma. The specimens were fixed in 4% buffered formalin and embedded in paraffin for analysis. The present study was approved by the ethical committees of Shandong Provincial Hospital.

Immunohistochemistry (IHC). Sections of formalin-fixed, paraffin-embedded osteosarcoma tissue samples were cut to 4 μm thick slides in the Department of Pathology of Shandong Provincial Hospital. The slides were deparaffinized in an oven at 60˚C for 2 h and maintained in xylene (Jining Huakai Resin Co., Ltd., Jining, China) for 20 min. The slides were then rehydrated in graded ethanol (100% for 5 min, 95% for 5 min and 75% for 5 min; (Jining Huakai Resin Co., Ltd.) and washed with 0.01 M phosphate-buffered saline (PBS; pH 7; Boster Biologics, Pleasanton, CA, USA) three times for 5 min each. Antigen retrieval was performed using high-pressure heating of the samples in a 0.01 M sodium citrate buffer at 95˚C for 10 min. The slides were washed three times with PBS, and 3% H_2O_2 (Jiangmen Hengjian Pharmaceutical Co., Ltd., Jiangmen, China) was added for 30 min at 37˚C to quench endogenous peroxidase activity. Subsequently, the slides were blocked with 10% goat serum (Beijing Jorferin Biotechnology Co., Ltd., Beijing, China) for 30 min at 37˚C, followed by incubation with the rabbit anti-human polyclonal primary antibody targeting HMGB1 (cat no. ab18256; Abcam, Cambridge, UK), which was diluted to 1:100 with PBS, at 4˚C overnight. Subsequent to being washed three times with PBS, the slides were incubated with biotinylated goat anti-rabbit secondary antibody (1:200 dilution; cat no. ZDR-5118; Histostain-Plus kit; ZSGB-Bio, Beijing, China) and horseradish peroxidase (Roche, Basel, Switzerland) for 30 min. The slides were then stained with 5% diaminobenzidine (Beijing Jorferin Biotechnology Co., Ltd.) for 1 min. The slides were counterstained with hematoxylin (Shanghai Bogoo Biotechnology Co., Ltd., Shanghai, China) for 2 min and washed with distilled water for 5 min. Subsequently, the slides were differentiated in 1% acid alcohol for ~10 sec, dehydrated, and cleared in graded alcohol and xylene (75% alcohol for 5 min, 95% alcohol for 5 min, 100% alcohol for 5 min and xylene for 20 min). Incubation of a tissue sample with PBS and without the primary antibody formed a negative control. The slides were routinely stained with hematoxylin and eosin (Shanghai Bogoo Biotechnology Co., Ltd.) to observe the nucleus and the cytoplasm of the cells.

Scoring of immunohistochemical staining. For the evaluation of the immunoreactivity of HMGB1 staining, the images of HMGB1 staining were independently analyzed by 2 pathologists (Department of Pathology, Shandong Provincial Hospital Affiliated to Shandong University), who were blinded to the clinicopathological data and prognosis of the patients. The fractions of HMGB1 staining reactivity were assessed by positive cell proportion analysis; ≥50 tumor cells were counted in 4 randomly selected regions of each section of the tissue and the mean percentage of stained cells was evaluated. The staining intensity of HMGB1 expression in tumor cells was estimated per section and classified as low reactivity (10-50% cells expressing HMGB1) and high reactivity (>50% cells expressing HMGB1).

Statistical analysis. Statistical data was analyzed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). The association between HMGB1 expression and clinicopathological parameters was explored with Pearson's χ^2 test or Fisher's exact test. The Kaplan-Meier method was applied to calculate survival curves for the recurrence-free survival rate, and the log-rank test assessed the significance of the observed differences. The patients that succumbed during the follow-up period were censored. For multivariate analysis, Cox's proportional hazards regression model was performed to assess the risk factors for tumor recurrence. $P<0.05$ was regarded as statistically significant. Two-tailed tests were applied to all the analyses.

Results

Immunohistochemical expression and the association with clinicopathological features. Osteosarcoma cell membrane and cytoplasm staining is exhibited in Fig. 1A and B. In osteosarcoma tissue samples, HMGB1 expression was detected in 84 samples (48.3%) with low immunoreactivity and in 90 samples (51.7%) with high immunoreactivity. The association between clinicopathological characteristics and tumor cell HMGB1 expression (low vs. high) was summarized in Table I. The association between HMGB1 expression and tumor size, stage and nuclear grade was statistically significant (P=0.034, 0.008 and 0.019, respectively). There was no significant association between HMGB1 expression and the age of the patients (P=0.335; Table I). The current study concluded that patients with high HMGB1 expression (>50% cells expressing HMGB1) had poorer survival rates, and therefore a poorer prognosis, compared with patients with low HMGB1 immunostaining (10-50% cells expressing HMGB1).

Survival analysis. In total, 174 patients were analyzed in the current study. The follow-up time for the patients

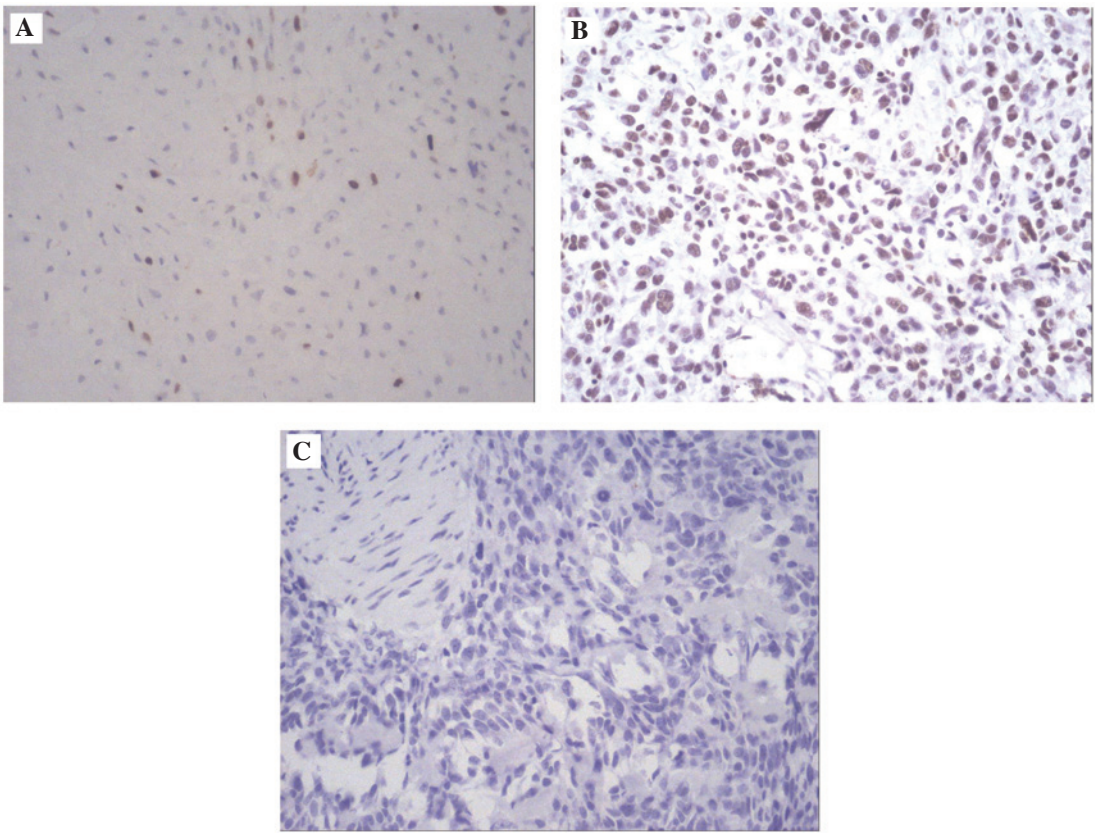

Figure 1. Representative immunohistochemistry samples of osteosarcoma tissues demonstrating HMGB1 expression: (A) Low HMGB1 staining reactivity. (B) High HMGB1 staining reactivity. Nuclear counterstaining with hematoxylin. (C) Negative control, primary antibody omitted during procedures. Magnification, x200. HMGB1, high mobility group box 1.

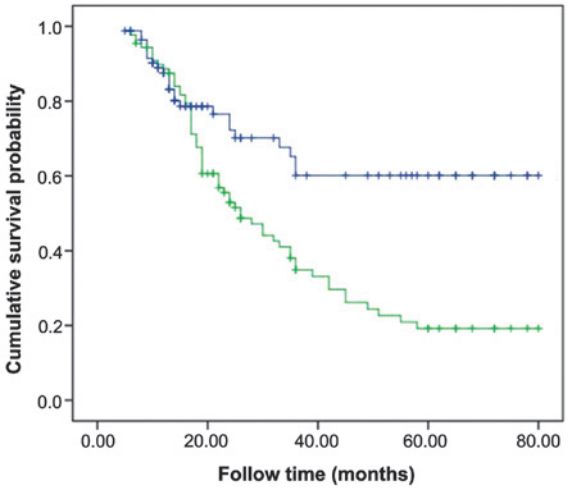

Figure 2. Kaplan-Meier survival curves demonstrating the recurrence-free survival rate of 174 patients with osteosarcoma, according to HMGB1 staining reactivity (low, 10-50%; high, >50%) and log-rank test, P=0.002. Blue and green curves indicate low and high reactivity of HMGB1, respectively. HMGB1, high mobility group box 1.

ranged between 5 and 80 months (median, 32.5 months). The Kaplan-Meier survival curves demonstrated that high HMGB1 expression was associated with a significantly lower recurrence-free survival rate compared with low HMGB1 expression (P=0.003; Fig. 2; Table II). In addition, the recurrence-free survival rate was significantly associated with tumor size (P<0.001), clinical stage (P<0.001) and tumor grade (P<0.001). Furthermore, Cox's proportional hazard model revealed that tumor stage, tumor grade and HMGB1 expression were independent predictors of a poorer prognosis in patients with osteosarcoma (P<0.001, 0.002 and 0.033, respectively; Table III), while the age of patients and tumor size were excluded as independent prognostic predictors (P=0.146 and 0.083, respectively; Table III).

Discussion

Osteosarcoma accounts for ~20% of pediatric, solid and malignant tumors, and is the most common malignant bone tumor in adolescents and young adults (26). In total, ~40% of osteosarcomas metastasize and patients possess a poor overall prognosis (27,28). The 5-year survival rate is reported to be 37% for osteosarcoma patients with tumor metastasis and 19% for patients with >5 metastatic lung lesions (5). Uncontrolled cell proliferation and robust tumor angiogenesis are prominent characteristics that contribute to the poor prognosis of osteosarcoma patients (6). Although there has been considerable progress in the diagnosis, therapeutic strategies and understanding of the biological behaviors of osteosarcoma, certain aspects of prognosis remain unclear, particularly concerning the prognostic markers for tumor recurrence and the patient survival rate. The most concerning problems in osteosarcoma are the inability to identify patients that will or will not respond to standard therapy, and the lack of a marker that may predict

Table I. Expression of HMGB1 in relation to clinicopathological characteristics of patients with osteosarcoma.

Variables	HMGB1 staining		P-value
	Low reactivity, n (%)	High reactivity, n (%)	
Age, years			
<60	50 (47.5)	61 (58.1)	
≥60	34 (56.6)	29 (49.5)	0.332
TNM stage			
I	23 (63.1)	16 (42.9)	
II	35 (55.6)	32 (50.5)	
III	15 (42.4)	22 (63.7)	
IV	11 (36.6)	20 (69.5)	0.008[a]
Nuclear grade			
1	20 (66.3)	13 (39.8)	
2	46 (51.2)	49 (54.9)	
3-4	17 (39.5)	29 (66.6)	0.017[a]
Tumor size			
<7.0cm	42 (56.6)	37 (49.5)	
>7.0cm	41 (46.5)	54 (59.6)	0.023[a]

[a]P<0.05. TNM, tumor-node-metastasis; HMGB1, high mobility group box 1.

Table II. Univariate analyses of the recurrence-free survival in 174 osteosarcoma cases.

Univariate analysis	P-value
Age <30 vs. ≥30 years	0.750
TNM stage I vs. II vs. III vs. IV	<0.001[a]
Nuclear grade 1 vs. 2 vs. 3-4	<0.001[a]
Tumor size	<0.001[a]
HMGB1 reactivity Low vs. high	0.002[a]

Univariate analyses performed using Kaplan-Meier method and log-rank test. [a]P<0.05. HMGB1, high mobility group box 1; TNM, tumor-node-metastasis.

Table III. Multivariate analysis for predictors of survival.

Covariate analysis	P-value
Age	0.246
TNM stage	<0.001[a]
Nuclear grade	0.001[a]
Tumor size	0.083
HMGB1 reactivity	0.025[a]

Mulivariate analyses performed using Cox's proportional hazards regression model. [a]P<0.05. HMGB1, high mobility group box 1; TNM, tumor-node-metastasis.

the prognosis of an osteosarcoma patient (29). Consequently, significant attention has been directed to the elucidation of novel prognostic markers that may be associated with tumor recurrence and the progression of osteosarcoma, in order to predict the prognosis of patients with osteosarcoma (30-32).

Evidence supporting the role of HMGB1 in cancer progression, angiogenesis, invasion and metastasis development has been steadily accumulating (33). Existing studies suggest that HMGB1 may demonstrate an important role in tumor progression beyond cancer development, including the association between HMGB1 overexpression and the presence of lymph node metastasis and advanced-stage hepatocellular, head and neck, esophageal squamous cell, cervical and ovarian carcinoma (34-38). The present study demonstrates that HMGB1 overexpression, as determined by immunohistochemistry, is an important prognostic factor in osteosarcoma. To the best of our knowledge, the present study is the first to demonstrate that HMGB1 expression is associated with clinical prognosis in osteosarcoma. This observation provides the opportunity to consider potential clinical applications of HMGB1 as a prognostic marker. IHC staining for HMGB1 expression revealed that the immunoreactivity was localized to the tubular epithelium and osteosarcoma cytoplasm, and there was a significantly higher staining intensity associated with a more advanced tumor differentiation grade.

HMGB1 was initially defined as a chromatin-associated protein with high acidic and basic amino acid content (14). HMGB1 is a nuclear protein that acts as a chromatin-binding factor and exists in the nuclei of cancerous and normal cells (17). HMGB1 modifies the interaction of DNA with transcription factors, including p53 steroid hormone receptors, by non-specifically binding to the minor groove of DNA, thereby playing a role in DNA repair, transcription, differentiation, extracellular signalization, and somatic recombination (39).

HMGB1 demonstrates affinity for various DNA structures, including supercoiled and single-stranded DNA, B- and Z-DNA, DNA mini-circles, 4-way junctions, looped structures, hemicatenated DNA, and triplex DNA (39). Native HMGB1 released from tumor cells inhibits DNA replication. However, this effect decreases following acetylation, and in addition, recombinant HMGB1 is phosphorylated by the *in vitro* protein kinase C (40); therefore, HMGB1 cannot inhibit the replication of DNA (23).

Rapid tumor growth causes a decrease in the intensity of chronic hypoxia, and the formation of microvessels and necrotic foci (41). Antigenic factors are released from hypoxic and necrotic regions and inflammatory cells, such as macrophages, which are stimulated to release angiogenic cytokines and growth factors, migrate to necrotic foci (41). HMGB1 activation results in NF-κB activation, and this stimulates the release of leucocyte adhesion molecules and pro-inflammatory cytokines, leading to the enhancement of inflammation and angiogenesis (41). HMGB1 also stimulates angiogenesis by activating factors, including vascular endothelial growth factor (VEGF) (42).

Wang *et al* investigated the association between HMGB1 expression and angiogenesis in bladder cancer samples. The authors reported that HMGB1 is associated with CD34 and VEGF, which are angiogenesis indicators (43). HMGB1 is associated with the pathological stage of tumors, as quantitative polymerase chain reaction has revealed an increase in HMGB1 mRNA expression as the tumor stage progresses (43-45).

However, there is little data concerning the prognostic role of HMGB1 in osteosarcoma. The results of the current study demonstrated that HMGB1 expression is negatively associated with the clinical prognosis of patients with osteosarcoma. The survival curves demonstrated that, in patients with osteosarcoma, increased HMGB1 expression is associated with a poorer recurrence-free survival rate. The present study confirmed that the TNM stage and tumor size were predictive prognostic markers for the recurrence-free survival rate in patients with osteosarcoma, which was consistent with the findings of previous studies (6,46,47). Cox's multivariate regression revealed that independent prognostic factors for osteosarcoma consisted of tumor stage, tumor grade and HMGB1 reactivity, while the age and tumor size were not prognostic factors.

In summary, the results of the present study revealed that increased expression levels of HMGB1 were significantly associated with a poorer clinical prognosis, and therefore may act as a marker for prognosis, particularly in osteosarcoma recurrence. Additional studies concerning the biological features of HMGB1 are required to confirm the potential role of HMGB1 as a novel target for anticancer therapy in osteosarcoma.

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