Original Article
Bone morphogenetic protein 9 is a potential tumor suppressor in osteosarcoma

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Abstract: Transforming growth factor-β (TGF-β) is known to promote tumor migration and invasion. Bone morphogenetic proteins (BMPs) are members of the TGF-β family expressed in a variety of human carcinoma cell lines. Although accumulating evidence has shown that BMP9 plays important roles in the regulation of various cellular processes, the function of BMP9 in clinical osteosarcoma remains to be explored. In this study, BMP9 expression was analyzed in 55 osteosarcoma patient samples and their matching, distant non-cancerous tissues. And the roles of BMP9 in osteosarcoma cell proliferation, apoptosis and cell cycle were also examined. Our results showed that different expression level of BMP9 was detected in all osteosarcoma samples while no expression in normal tissues. Surprisingly, there was a negative association between the expression level of BMP9 and osteosarcoma grade, with low level of BMP9 being found in high histological grade osteosarcoma. Knockdown of BMP9 accelerated the proliferation of MG63, SaOS-2, and U2OS cells. BMP9 overexpression, however, induced cell apoptosis in U2OS cells. Together, these results indicated that BMP9 plays a pivotal role in osteosarcoma. Future studies defining the mechanism of BMP9 effect may lead to novel therapeutic approaches for osteosarcoma.

Keywords: BMP9, osteosarcoma, tumor suppressor

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in children and young adults. Conventional OS is classified into osteoblastic, chondroblastic and fibroblastic OS, according to its histological features [1]. OS is highly aggressive and it metastasizes mostly to the lungs and bones [2]. However, despite the use of aggressive chemotherapeutic treatment strategies, the survival of OS patients has shown limited improvement. The prognosis is very poor, particularly in patients with clinically detectable metastasis at diagnosis or relapsed disease [3]. Thus, a novel strategy to efficiently inhibit metastasis is highly desirable.

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily and play a critical role in skeletal development, bone formation and stem cell differentiation [4, 5]. At least 15 different BMPs have been identified in humans and disruptions in BMP signaling result in a variety of skeletal and extra skeletal anomalies [6-8]. BMP9, also known as growth differentiation factor 2 or GDF-2, is a relatively poorly characterized member of the BMP family [9]. It has been shown to function as a pleiotropic cytokine and is implicated in the bone morphogenesis, functional differentiation, glucose homeostasis, iron homeostasis, and angiogenesis [10-14]. Recently, accumulating evidence shows that BMP9 plays important roles in the regulation of various cellular processes, including cell proliferation, migration, differentiation and apoptosis [9, 13, 15-19]. However, the expression pattern of BMP9 and the correlation of expression level of BMP9 and other clinicopathological parameters in clinical osteosarcoma remain to be explored.

In our study, 55 osteosarcoma patient samples were collected for analysis of the expression level of BMP9 and its correlation with osteosarcoma grade. Then the potential function of BMP9 was further determined in OS cell lines.

Materials and methods

Materials

Fresh biopsy specimens of osteosarcoma tissue and corresponding noncancerous bone tis-

sues were collected from 55 patients with osteosarcoma who underwent radical surgery at Urumqi General Hospital of Lanzhou Military Area Command of Chinese PLA. None of the patients had received any chemotherapy, radiotherapy or other adjuvant therapy before the operation. This study was approved by The Ethics Committee of Urumqi General Hospital of Lanzhou Military Area Command of Chinese PLA and all patients provided informed consent. Tumors were diagnosed and classified according to the American Joint Committee on Cancer breast cancer TNM staging system and the World Health Organization breast cancer histology classifications [20, 21].

Immunohistochemical SP method

All fresh specimens were fixed with formalin and embedded in paraffin according to the standard protocol. Tissue sections were deparaffinized and rehydrated routinely and then subjected to antigen retrieval by placing slides in 1× citrate buffer for 15 min at 100°C in a microwave oven. After treatment with 3% H_2O_2 for 30 minutes, the sections were incubated with 20% normal serum for 50 minutes and then with the BMP9 primary antibody (ab35088, Abcam) overnight at 4°C. On the following day, the sections were washed with PBS thrice and then processed using an ultrasensitive TM S-P kit (Maixin Biotechnology, Fuzhou, China). After the washes in PBS, the color reaction was conducted using a 3,3'-diaminobenzidine kit (Maixin Biotechnology). The sections were counter-stained with hematoxylin and covered with a coverslip. The stained tissue sections were reviewed and scored independently by two pathologists (Dr. Jiang Wang and Min Liu). The percentage of BMP9 positive cells was rated as follows: -, ≤5% positive tumor cells; +, 5-30% positive cells; ++, 30-55% positive cells; and +++, >55% positive cells.

Cell culture and small interfering RNA knockdowns

The human OS cell lines, MG63, SaOS-2, and U2OS were cultured in DMEM-HG supplemented with 10% FBS and 100 U/ml penicillin G/streptomycin at 37°C in a humidified atmosphere of 5% CO_2/95% air. Small interfering RNA (siRNA) oligonucleotides for BMP9 (Invitrogen) were introduced into cells using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol, and cells were analyzed for 5 days after transfection.

Cell proliferation analysis

The cells were seeded at after 48 hours of transfection and collected in 1D/2D/3D/4D/5D for cell proliferation analysis using CCK-8 assay (YEASEN) following the manufacturer's instructions. Cell Counting Kit-8 (CCK-8) allows very convenient assays by utilizing Dojindo's highly water-soluble tetrazolium salt. WST-8 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) produces a water-soluble formazan dye upon reduction by dehydrogenase in mitochondria in the presence of an electron carrier. Color depth is proportional to the cell proliferation. The absorbance value at 450 nm wavelength by enzyme standard instrument reflects the number of living cells indirectly.

Real-time-PCR

Total RNA was extracted from collected samples 48 hours after transfection using RNeasy mini kit (Qiagen). Reverse transcription was performed using PrimeScript® RT Reagent Kit (Perfect Real Time, TaKaRa). For quantitative real-time PCR, reactions were performed using SYBR® Premix ExTaqTM II (Perfect Real Time, TaKaRa) and 7500 Real-Time PCR System (Applied Biosystems). For each sample, the cycle threshold (CT) values were obtained from three replicates. Primer sequences are the following: GAPDH forward: 5'-AGGTCGGTGTGAA-CGGATTTG-3', reverse: 5'-GGGGTCGTTGATGGC-AACA-3'; BMP9 forward: 5'-CTGCCCTTCTTTG-TTGTCTT-3', reverse: 5'-CCTTACACTCGTAGGC-TTCATA-3'. The relative expression levels of target genes were analyzed using the 2-ΔΔCT method.

Western blot

Cell extracts were prepared at 48 hours after transfection as previously described. Proteins were electrophoresed in 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and incubated overnight at 4°C in PBS buffer containing 5% BSA. β-actin (ab8227) expression levels were determined with a monoclonal antibody to monitor protein loading and retention.

Cell cycle analysis

After 48 hours of transfection, cells were trypsinized and washed in ice-cold PBS. Cells were fixed by adding them dropwise into ice-cold

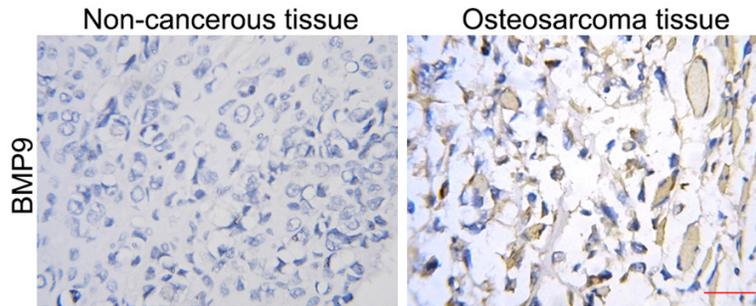Non-cancerous tissue   Osteosarcoma tissue

Figure 1. Expression of BMP9 in osteosarcoma and its distant noncancerous tissues. The representative images of non-cancerous samples and osteosarcoma samples were shown. Bar, 200 μm.

Table 1. The expression level of BMP9 determined by IHC in osteosarcoma samples

Class	No.	BMP9 grade (No./%)			
		-	+	++	+++
Osteosarcoma	55	0/0.00	6/10.91	29/52.73	20/36.36

Table 2. Association between BMP9 expression and clinicopathological factors from osteosarcoma

Clinicopathological features	Variable	N (%)	Intensity of BMP9 staining (mean ± SD)	P value
Age (years)	≥30	26 (47.27)	0.443 ± 0.225	0.845
	< 30	29 (52.73)	0.462 ± 0.264	
Gender	Male	30 (54.55)	0.436 ± 0.216	0.823
	Female	25 (45.45)	0.476 ± 0.287	
Tumor size (cm)	< 5.00	23 (41.82)	0.388 ± 0.259	0.786
	≥5.00	32 (58.18)	0.354 ± 0.341	
Histology grade	IIA	37 (67.27)	0.628 ± 0.135	0.027
	IIB/III	18 (32.73)	0.319 ± 0.204	
TNM stage	I	24 (43.64)	0.366 ± 0.320	0.758
	II	31 (56.36)	0.454 ± 0.386	
Survival (months)	< 6	33 (60.00)	0.466 ± 0.216	0.624
	>6	22 (40.00)	0.408 ± 0.322	

80% ethanol while vortexing, followed by incubation on ice for 60 min. The fixed cells were washed with cold PBS and incubated at 37°C for 30 min in 0.5 ml of PBS containing 10 μg/ml propidium iodine (Sigma) and 5 μg/ml RNase A (New England Biolabs). DNA content was determined by FACS scan analysis (Becton Dickinson).

Statistical analysis

Data were analyzed by SPSS 19.0 statistical software. Measurement data were analyzed by Student's *t*-test, while categorical data were analyzed by the chi-square test. $P < 0.05$ was considered as significant.

Results

Differential expression level of BMP9 protein in osteosarcoma and its distant noncancerous tissues

We first detected the expression level of BMP9 by immunohistochemical staining in osteosarcoma and the matching distant normal tissue samples from 55 patients. The results of BMP9 staining were scored as none (-), weak (+), moderate (++), and strong (+++) according to the assessment of two independent pathologists. No BMP9 expression was detected in the distant non-cancerous cells (**Figure 1**). In contrast, BMP9 protein was found in all osteosarcoma samples at varying degrees (**Table 1**). In most cases, BMP9 protein was expressed moderately or strongly (++, 29/55, 52.73%; +++, 20/55, 36.36%). Overall, the results demonstrated that BMP9 is overexpressed in osteosarcoma compared with normal tissues

Correlation between BMP9 expression and clinicopathological parameters

We then conducted the association analysis between the expression level of BMP9 protein and clinicopathological parameters from osteosarcoma patients (**Table 2**). The results showed that higher level of BMP9 was found at grade IIA osteosarcoma than that at grade IIB/III ($P < 0.05$), which suggested that the expression level of BMP9 was negatively correlated with osteosarcoma grade. However, there was no association between expression level of BMP9 and other clinicopathological parameters, such as age, gender, tumor size, TNM stage and survival time (**Table 2**).
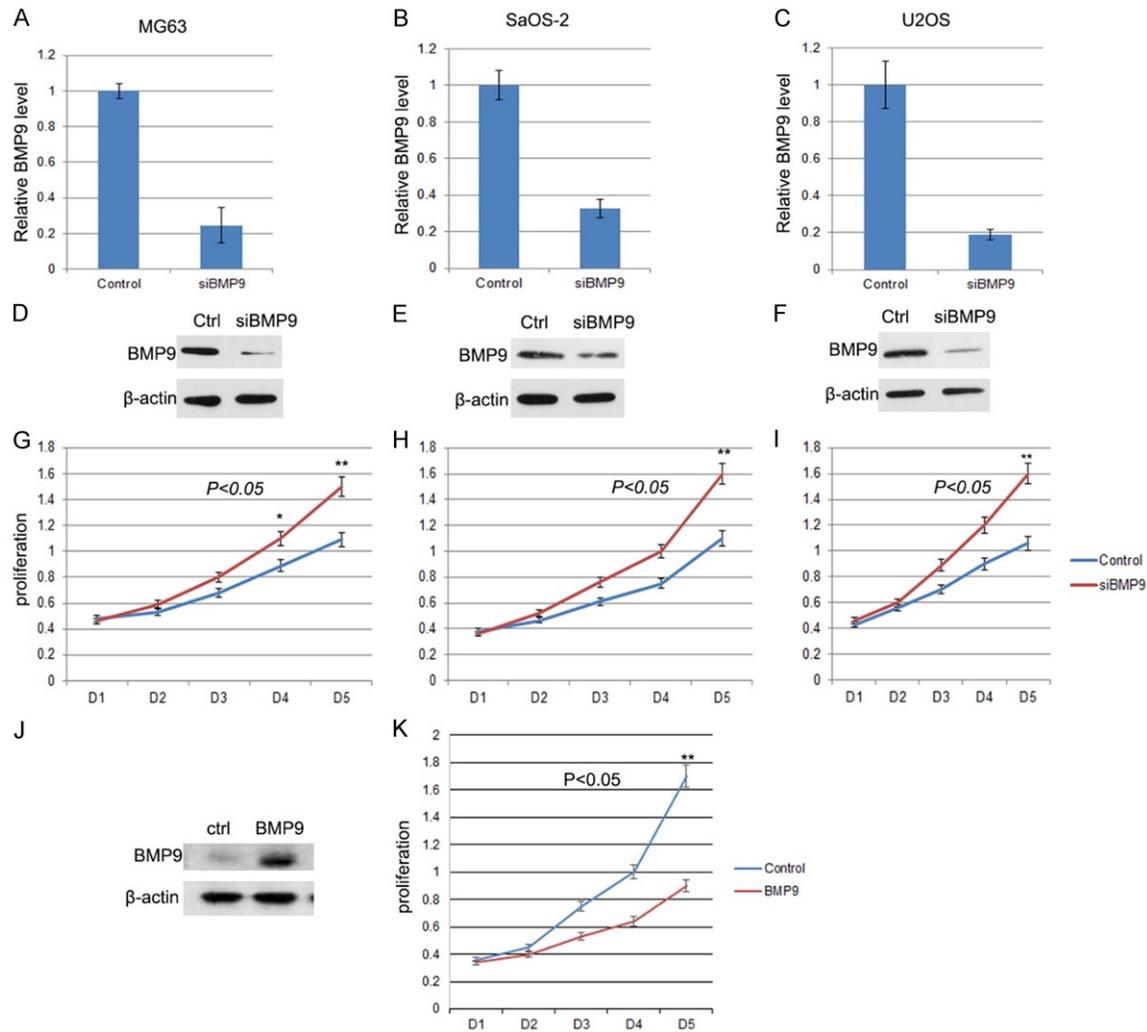
Figure 2. BMP9 expression negatively correlated with the proliferation of osteosarcoma cells. A-C. The mRNA level of BMP9 in three human OS cell lines, MG63, SaOS-2, and U2OS were detected after 48 hours of RNA interference. D-F. The protein level of BMP9 in MG63, SaOS-2, and U2OS were examined after RNA interference to confirm the knockdown effect. G-I. The proliferation of MG63, SaOS-2, and U2OS cells after RNA interference. The experiments were repeated thrice, and the representative results were shown. J, K. Rescue BMP9 expression in BMP9 knockdown U2OS cells inhibited cell proliferation.

BMP9 knockdown accelerated osteosarcoma cell proliferation

To investigate the role of BMP9 in OS cell proliferation, we knock downed the expression of BMP9 in three human OS cell lines, MG63, SaOS-2, and U2OS, using siRNA. The results showed that the RNA level and protein level were both reduced significantly in the three cell lines after RNA interference (**Figure 2A-F**). And the proliferation rate was accelerated in all three cell lines (**Figure 2G-I**). We also did a rescue experiment that we overexpressed BMP9 in BMP9 knockdown cells using a siRNA resistant BMP9 expression vector. The BMP9 pro-

tein level was checked by Western blot and cell proliferation was analyzed as above. The results were showed in **Figure 2J** and **2K** that BMP9 overexpression was able to decrease the cell proliferation rate. Thus BMP9 negatively regulate cell proliferation in OS cell lines.

Overexpress BMP9 induced cell apoptosis in U2OS cells but did not affect cell cycle

To further study the function of BMP9 in osteosarcoma development, we overexpressed BMP9 in U2OS cells, which is the most commonly used cell line in studying osteosarcoma. As show in **Figure 3A**, BMP9 overexpression
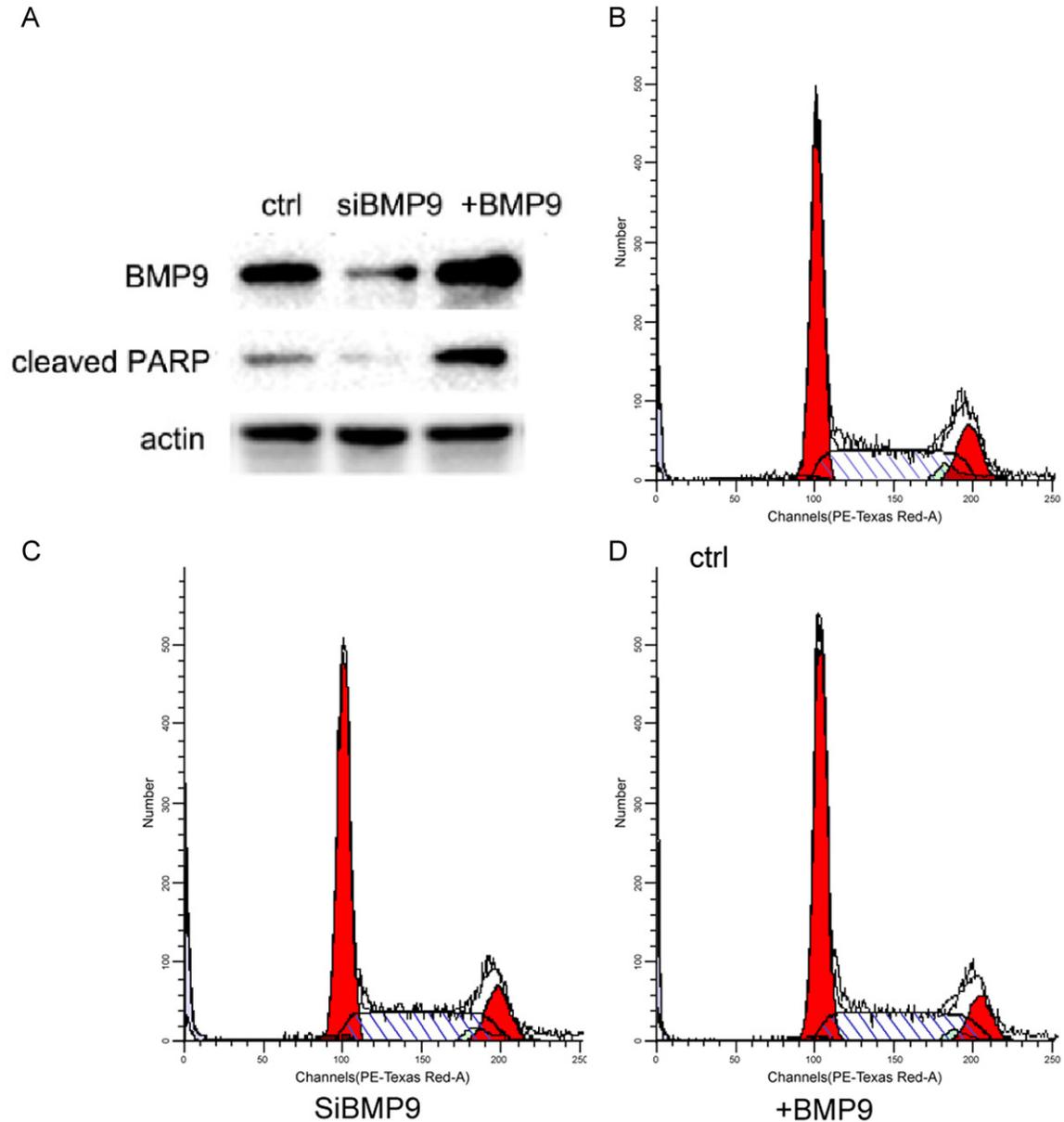
Figure 3. BMP9 induced apoptosis in osteosarcoma cells but did not affect cell cycle. A. Apoptosis marker, cleaved PARP, was examined in control, BMP9 knockdown and BMP9 overexpressed U2OS cells. B-D. Cell cycle was determined by Flow cytometry in control, BMP9 knockdown or BMP9 overexpressed cells.

induced cleaved PARP level, while knockdown BMP9 decrease the background level of cleaved PARP. These results indicated that BMP9 promoted cell apoptosis. We also compared cell cycle profiles between control, BMP9 knockdown and BMP9 overexpressed cells. BMP9 expression level did not affect cell cycle (**Figure 3B-D**).

Discussion

It is reported that BMP9 positively regulates the osteogenic differentiation of mesenchymal

stem cells [14]. BMP9 has also been found to regulate the proliferation and invasion of osteosarcoma, breast cancer and ovarian cancer cells [17, 18]. These studies indicate that BMP9 plays an important role in a multitude of cellular processes under normal physiological conditions and in the development of many cancers. In the current study, we detected the expression level of BMP9 protein in osteosarcoma and distant normal tissue samples. It is interesting that no expression or very low level of BMP9 expression was detected in non-tumor tissues while BMP9 protein staining was found

to be positive in all of the osteosarcoma samples. However, we found a negative association between the expression level of BMP9 and osteosarcoma grade. We found that, compared with low grade tumors, BMP9 expression level was lower in high histological grade osteosarcoma. This result indicated that BMP9 could have a pivotal role during osteosarcoma development.

In the present study, we also investigated the effect of BMP9 on human OS cell lines, MG63, SaOS-2, and U2OS. We observed that BMP9 knockdown had a significant facilitative effect on MG63, SaOS-2, and U2OS cell proliferation. These results are consistent with previous studies that have shown an inhibitory effect of BMP9 on the migration of prostate cancer and breast cancer cells [18, 22]. The response to BMP9, however, is not uniform among all cancers. BMP9 has been shown to trigger epithelial-mesenchymal transition of hepatocellular carcinoma cells [23]. The pro-tumorigenic or anti-tumorigenic effect of BMP9 on different cancer cells might be caused by its complicate interactions with other proteins that exist in different cells or different developmental stage. Therefore, the biological effect of BMP9 on different cells may depend not only on BMP9 expression level but also on cell type, microenvironment and the presence of other factors that are not yet defined.

In conclusion, we found that BMP9 was overexpressed in osteosarcoma, and the expression level of BMP9 was negatively correlated with osteosarcoma grade. And BMP9 silencing accelerated the proliferation of MG63, SaOS-2, and U2OS cells. Thus, BMP9 may serve as a biological marker for osteosarcoma grading. Future studies should focus on defining the mechanism of BMP9 function, which may lead to novel therapeutic approaches for osteosarcoma.

Disclosure of conflict of interest

None.

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