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PPM1D accelerates proliferation and metastasis of osteosarcoma by activating PKP2

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Abstract. – OBJECTIVE: This project aims to elucidate the diagnostic and prognostic values of PPM1D in osteosarcoma and the molecular mechanism.

PATIENTS AND METHODS: PPM1D levels in osteosarcoma and adjacent tissues were detected. Pathological information of included osteosarcoma patients was collected for analyzing the relationship between PPM1D and prognosis of osteosarcoma. Regulatory effects of PPM1D on in vivo and in vitro progressions of osteosarcoma were assessed by generating xenograft model in nude mice and PPM1D knockdown models in MG63 and U2OS cells, respectively. The involvement of PKP2, the target gene of PPM1D in osteosarcoma progression was finally evaluated.

RESULTS: PPM1D was upregulated in osteosarcoma tissues than adjacent ones. High level of PPM1D indicated higher risks of distant metastasis and worse prognosis in osteosarcoma. In vivo knockdown of PPM1D contributed to a delay in tumor growth of osteosarcoma in nude mice. PKP2, as the downstream gene targeting PPM1D, was highly expressed in osteosarcoma tissues and positively correlated to PPM1D level. The overexpression of PKP2 was able to abolish the inhibited proliferative and migratory abilities in osteosarcoma cells with PPM1D knockdown.

CONCLUSIONS: PPM1D triggers proliferative and migratory abilities of osteosarcoma by positively regulating PKP2, which can be served as an effective diagnostic marker for osteosarcoma in the early phase.

Key Words:
PPM1D, PKP2, Osteosarcoma, Proliferation, Metastasis.

Introduction

Osteosarcoma is a common primary malignant tumor in humans, and the incidence of osteosarcoma has gradually increased, accounting for about 20% of primary malignant tumors, in recent years[1,2]. Osteosarcoma mainly affects adolescents between 15-19 years, which covers 5% of childhood tumors[2]. Surgery with adjuvant radiotherapy or chemotherapy is preferred to osteosarcoma patients[2-4]. However, the prognosis of osteosarcoma is relatively poor due to the high malignancy, and high rates of hematogenous and lymphatic metastases[5-8]. Therefore, the search for effective and reliable biomarkers is of significance for enhancing the clinical outcomes of osteosarcoma[9,10].

PPM1D (WIP1) is a proto-oncogene, which is a phosphatase induced by wild-type p53. It exerts serine/threonine activity, and participates in the growth, cell cycle regulation, and DNA damage repair of tumor cells[11,12]. Previous studies[13-17] have focused on the tumor-associated characteristics of PPM1D. In this study, the clinical samples of osteosarcoma were firstly collected to detect the differential expression of PPM1D. Subsequently, by generating *in vivo* and *in vitro* osteosarcoma cell lines, its molecular mechanism on regulating the proliferation and metastasis of osteosarcoma were mainly explored in this study.

Patients and Methods

Clinical Samples of Osteosarcoma

The clinical samples of cancer tissues and adjacent normal bone tissues from osteosarcoma patients were collected from 45 patients diagnosed as osteosarcoma by pathology, and stored at -80°C after labeling. None of included osteosarcoma patients had chemotherapy or radiotherapy. In addition, these patients were followed up after discharge for general conditions, clinical symptoms, and imaging examinations through telephone and outpatient review. Tumor node metastasis (TNM) staging of osteosarcoma was

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diagnosed based on the Union for International Cancer Control (UICC) criteria. This study was approved by the research Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University and complied with the Helsinki Declaration. Informed consent was obtained from the osteosarcoma patients or their families.

Cell Lines and Reagents

Osteosarcoma cell lines (143B, HOS, U2OS, MG63, SaOS-2, SOSP-9607) and the osteoblast cell line (hFOB) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) and 1% streptomycin at 37°C with 5% CO_2.

Transfection

Cells in the 6-well plate were cultured to 40-60% density. Transfection of sh-PPM1D, pcDNA-PKP2 or negative control (GenePharma, Shanghai, China) was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h cell transfection, the cells were collected for verifying transfection efficacy and functional experiments.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells/well. At 24, 48, 72 and 96 h, optical density at 450 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8) kit (Dojindo Molecular Technologies, Kumamoto, Japan) for plotting the viability curves.

Transwell Migration Assay

Cell suspension was prepared at 5×10^5 cells/mL. 200 μL of suspension and 700 μL of medium containing 20% FBS was respectively added on the top and bottom of a transwell insert, and cultured for 48 h. The migratory cells on the bottom were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Migratory cells were counted in 5 randomly selected fields per sample.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The cells were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolating RNAs, and purified by DNase I treatment. Qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan), followed by qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. PPM1D: forward: 5'-GGAGCACTTGTGGGGTTTCA-3', reverse: 5'-TTTGGCCATTCCGCCAGTTT-3'; PKP2: forward: 5'-TAGTGCAGGCGATGCCTATG-3', reverse: 5'-GAGTGGTAGGCTTTGGCAGT-3'; GAPDH: forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3', reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA) on ice for 15 min (Beyotime, Shanghai, China), and the mixture was centrifuged at 14000×g, 4°C for 15 min. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples with the adjusted same concentration were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and loaded on polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). The membrane was cut into small pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure and grey value analyses.

Dual-Luciferase Reporter Assay

The cells were pre-seeded in a 24-well plate and co-transfected with pcDNA-PKP2/pcDNA-NC and PPM1D-WT/PPM1D-MUT, respectively. Luciferase activity was measured after 48 h of co-transfection.

In Vivo Xenograft Model

This study was approved by the Animal Ethics Committee of Jinzhou Medical University Animal Center. Ten male nude mice with 8 weeks old were administrated with MG63 cells transfected with sh-NC (n=5) or sh-PPM1D (n=5) in the armpit. Tumor width and length were recorded every 5 days. Mice were sacrificed at 30 days for collecting tumor tissues. Tumor volume was calculated using the formula: Tumor width^2×tumor length/2. The positive expression of PPM1D was detected by immunoprecipitant staining.

Statistical Analysis

GraphPad Prism 6 V6.01 (La Jolla, CA, USA) was used for statistical analyses. The differences between the groups were compared by the *t*-test. The influence of PPM1D on clinical data of osteosarcoma patients was analyzed by Chi-square test. Kaplan-Meier survival curves were depicted, followed by log-rank test for comparing differences between curves. Data were expressed as mean ± standard deviation. $p<0.05$ was considered as statistically significant.

Results

PPM1D Was Highly Expressed in Osteosarcoma Samples

QRT-PCR was conducted to examine PPM1D levels in 45 clinical samples of osteosarcoma and adjacent tissues. PPM1D was highly expressed in osteosarcoma tissues than controls (Figure 1A). By analyzing clinical records of included osteosarcoma patients, we found that PPM1D was correlated to the incidence of distant metastasis of osteosarcoma (Table I). Higher level of PPM1D was detected in osteosarcoma patients with distant metastasis than those non-metastatic patients (Figure 1B). In addition, PPM1D was upregulated in osteosarcoma cell lines in comparison to osteoblasts (Figure 1C). According to the plotted Kaplan-Meier survival curves, PPM1D was an unfavorable factor to the prognosis of osteosarcoma (Figure 1D).

Knockdown of PPM1D Inhibited Proliferative and Migratory Abilities in Osteosarcoma

Protein level of PPM1D was markedly downregulated in MG63 and U2OS cells transfected with sh-PPM1D, suggesting an effective transfection (Figure 2A). Cell viability was markedly declined after transfection of sh-PPM1D in MG63 and U2OS cells (Figure 2B). In addition, migratory cell number was lower in osteosarcoma cells with PPM1D knockdown in comparison to those of controls, indicating the inhibited metastasis (Figure 2C).

Interaction Between PKP2 and PPM1D

Based on miRDB database, the results of bioinformatics analysis suggested that PKP2 was predicted to be a candidate of PPM1D targets. The knockdown of PPM1D significantly downregulated protein level of PKP2 in osteosarcoma

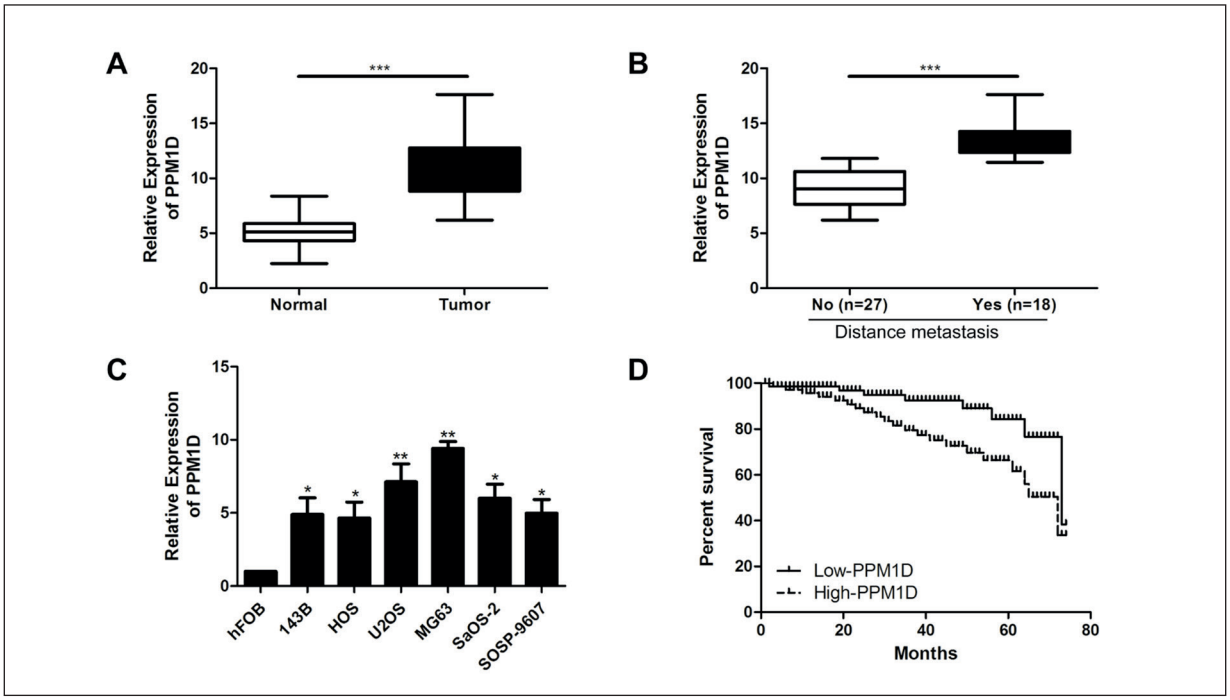

Figure 1. PPM1D was highly expressed in osteosarcoma samples. **A,** PPM1D levels in osteosarcoma tissues and normal ones. **B,** PPM1D levels in osteosarcoma patients either with distant metastasis or not. **C,** PPM1D levels in osteosarcoma cell lines. **D,** Overall survival in osteosarcoma patients based on PPM1D levels. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table I. Association of PPM1D expression with clinicopathologic characteristics of osteosarcoma.

Parameters	Number of cases	PPM1D expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.302
< 21	19	8	11	
≥ 21	26	15	11	
Gender				0.848
Male	28	14	14	
Female	17	9	8	
Enneking stage				0.958
IA	6	3	3	
IIA	12	6	6	
IIB	18	10	8	
III	9	4	5	
Distance metastasis				0.011
No	27	18	9	
Yes	18	5	13	

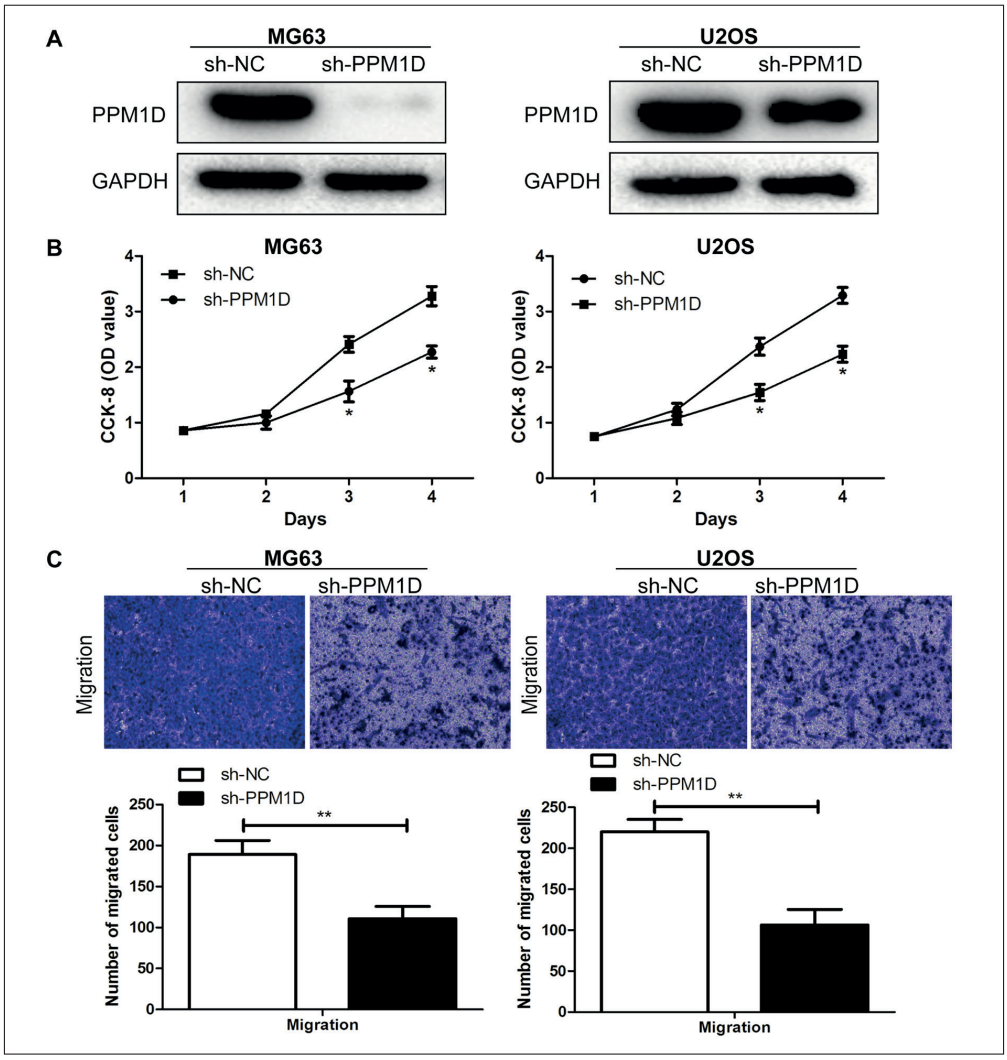

Figure 2. Knockdown of PPM1D inhibited proliferative and migratory abilities in osteosarcoma. **A,** Transfection efficacy of sh-PPM1D in MG63 and U2OS cells. **B,** Viability in MG63 and U2OS cells with PPM1D knockdown. **C,** Migration in MG63 and U2OS cells with PPM1D knockdown (magnification: 40×). *$p < 0.05$, **$p < 0.01$.

cell lines (Figure 3A). Subsequently, Dual-Luciferase reporter assay demonstrated that PPM1D could target PKP2 *via* the predicted binding sites in their seed sequences (Figure 3B). Compared with normal bone tissues, PKP2 was upregulated in osteosarcoma tissues (Figure 3C), and its level was positively correlated to that of PPM1D (Figure 3D).

PKP2 Was Responsible for PPM1D-Regulated Osteosarcoma Progression

To further elucidate the co-regulation of PKP2 and PPM1D on osteosarcoma progression, cell co-transfection was conducted. Co-transfection of sh-PPM1D and pcDNA-PKP2 in MG63 and U2OS cells resulted in higher protein level of PKP2 compared with those co-transfected with sh-PPM1D and pcDNA-NC (Figure 4A). In comparison to those with PPM1D knockdown, viability and migratory cell number were higher in osteosarcoma cells co-transfected with sh-PPM1D and pcDNA-PKP2 (Figure 4B, 4C).

In Vivo Knockdown Of PPM1D Suppressed the Tumorigenicity of Osteosarcoma

MG63 cells transfected with sh-NC or sh-PPM1D were administrated to nude mice. The

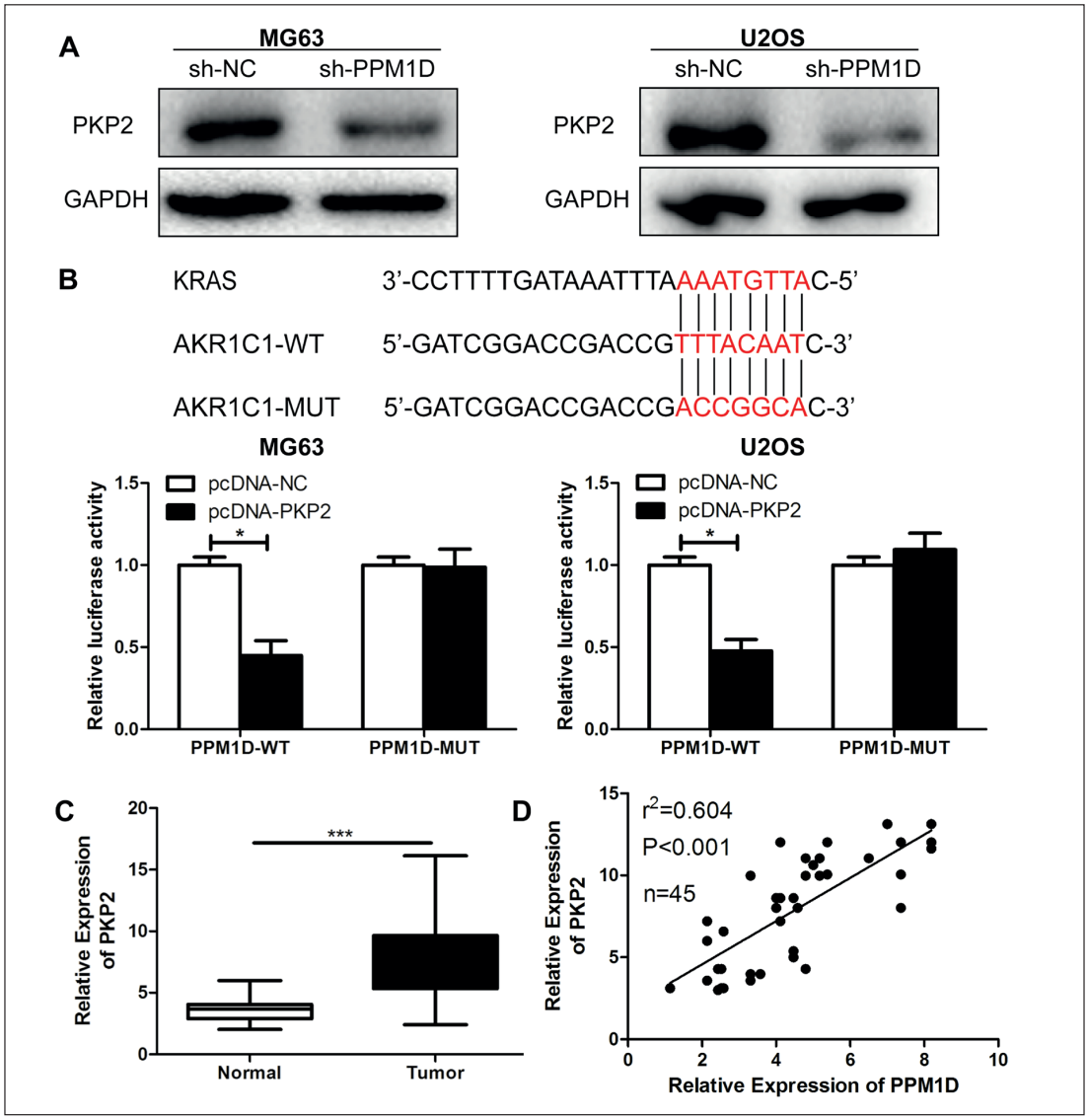

Figure 3. Interaction between PKP2 and PPM1D. **A,** Protein level of PKP2 in MG63 and U2OS cells with PPM1D knockdown. **B,** Target binding between PPM1D and PKP2. **C,** PKP2 levels in osteosarcoma tissues and normal ones. **D,** A positive correlation between PKP2 and PPM1D levels in osteosarcoma tissues. *$p < 0.05$, ***$p < 0.001$.

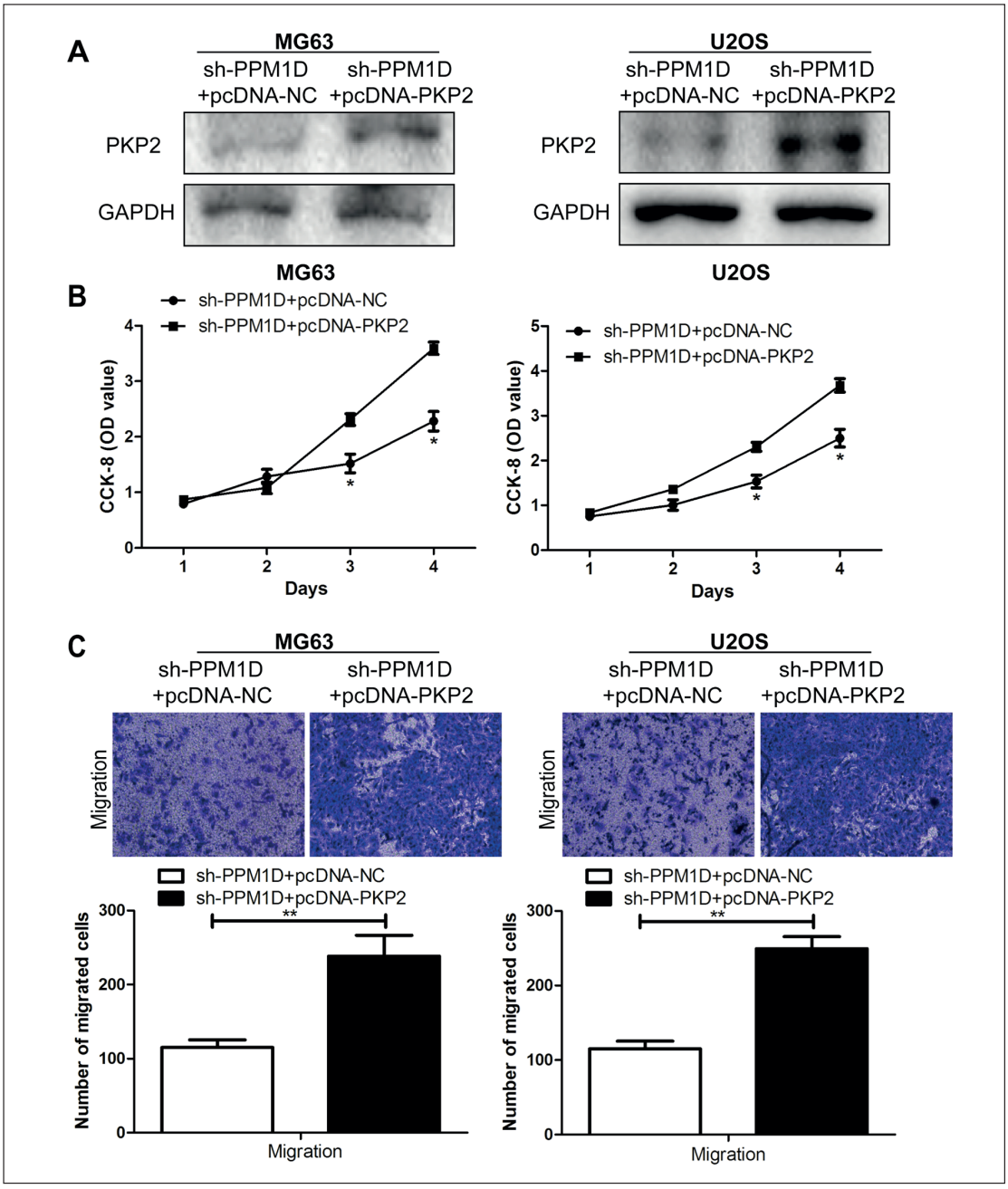

Figure 4. PKP2 was responsible for PPM1D-regulated osteosarcoma progression. **A,** Transfection of pcDNA-PKP2 in MG63 and U2OS cells with PPM1D knockdown. **B,** Viability in MG63 and U2OS cells co-regulated by PPM1D and PKP2. **C,** Migration in MG63 and U2OS cells co-regulated by PPM1D and PKP2 (magnification: 40×). *$p < 0.05$, **$p < 0.01$.

tumor growth rate of osteosarcoma was much lower in mice with *in vivo* knockdown of PPM1D, as well as tumor weight (Figure 5A, 5B). Positive level of PPM1D was lower in mice administrated with MG63 cells transfected with sh-PPM1D, confirming the *in vivo* transfection efficacy (Figure 5C). It is concluded that PPM1D accelerated tumorigenesis of osteosarcoma in nude mice.

Discussion

Osteosarcoma is a highly heterogeneous tumor, and it mostly affects adolescents and children[1,2]. Tumors mainly involve long bones, such as femur, tibia, fibula, and ilium. The postoperative prognosis and survival of osteosarcoma are unsatisfactory[2-4]. At present, surgery and multidrug-based

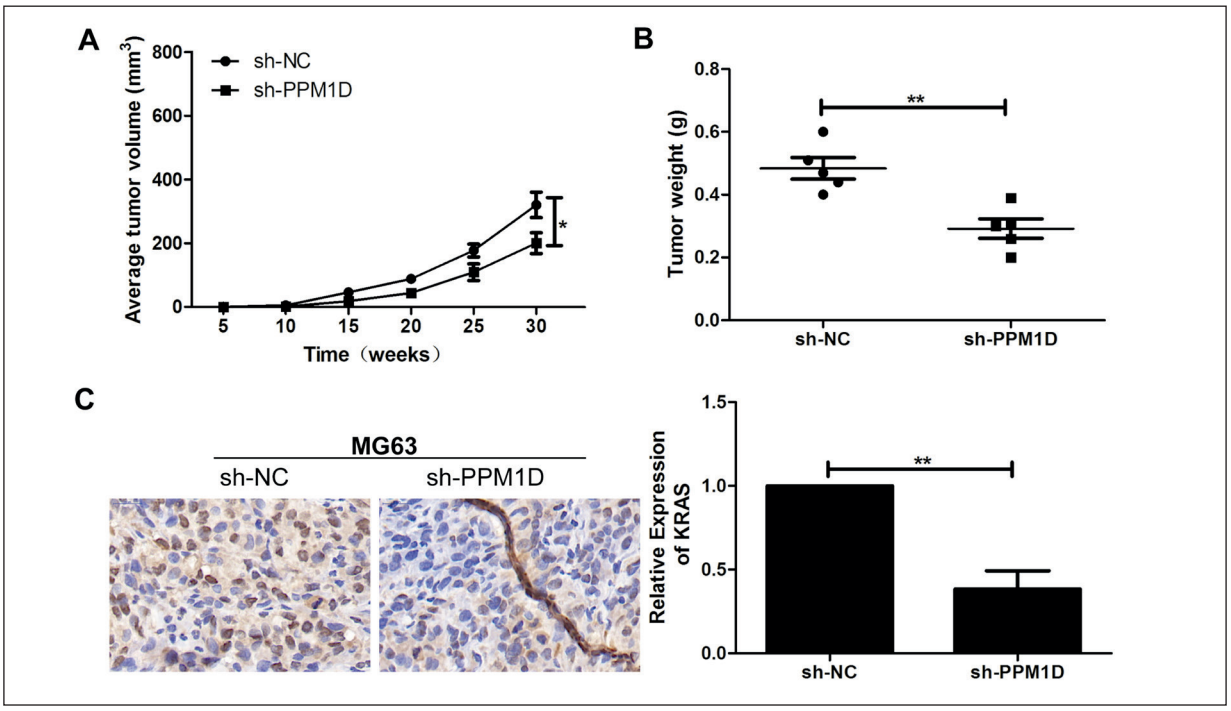

Figure 5. *In vivo* knockdown of PPM1D suppressed the tumorigenicity of osteosarcoma. **A,** Average tumor volume of nude mice administrated with transfected MG63 cells. **B,** Tumor weight of nude mice administrated with transfected MG63 cells. **C,** Positive expression of PPM1D in osteosarcoma tissues collected from nude mice administrated with transfected MG63 cells (magnification: 200×). *$p < 0.05$, **$p < 0.01$.

chemotherapy are widely applied in clinical treatment of osteosarcoma[3-5]. Drug resistance and side effects of chemotherapy largely limit therapeutic efficacy of osteosarcoma, and seeking for effective biomarkers and developing targeted therapy of osteosarcoma are now well concerned[4-7].

PPM1D is a phosphatase located on chromosome 17q23.2, which was originally identified to have an inhibitory effect on growth. Later, some studies[11,12] have overturned the previous finding that PPM1D is able to stimulate growth. Increased copy number of PPM1D and overexpressed mRNA level of PPM1D have been detected in multiple types of tumor cells[13-17]. However, the function and regulatory mechanism of PPM1D in osteosarcoma are not clear. Consistently, we have found that PPM1D was highly expressed in clinical samples and cell lines of osteosarcoma. The incidence of distant metastasis in osteosarcoma patients had a close relation to high expression level of PPM1D, suggesting an oncogenic characteristic of PPM1D in osteosarcoma patients. In addition, Kaplan-Meier survival curves showed that the strong tendency of metastasis attributed to the poor prognosis of osteosarcoma. After knockdown of PPM1D in MG63 and U2OS cells, the proliferative and migratory abilities were significantly attenuated. In addition, a xenograft model was generated in nude mice by administrating transfected MG63 cells. As the data revealed, *in vivo* knockdown of PPM1D not only slowed down the tumor growth, but also reduced tumor weight of nude mice.

To further clarify the molecular mechanism of PPM1D on aggravating the malignant progression of osteosarcoma, we predicted potential candidate of PPM1D targets. Based on the predicted binding sites, Dual-Luciferase reporter assay confirmed the binding relationship between PPM1D and PKP2. Compared with normal tissues, PKP2 was highly expressed in osteosarcoma tissues and positively linked to PPM1D level. Interestingly, the overexpression of PKP2 was capable of reversing the inhibited proliferative and migratory abilities in osteosarcoma cells with PPM1D knockdown. Our findings suggested that there might be a feedback loop in which PPM1D contributed to the deterioration of osteosarcoma by positively regulating PKP2, which suggested that PPM1D as a target for developing targeted therapy of osteosarcoma was a promising strategy of osteosarcoma.

Conclusions

These data showed that PPM1D triggers proliferative and migratory abilities of osteosarcoma by positively regulating PKP2, which can be served as an effectively diagnostic marker for osteosarcoma in the early phase.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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