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Knockdown of pyruvate kinase type M2 suppresses tumor survival and invasion in osteosarcoma cells both *in vitro* and *in vivo*

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ABSTRACT

Osteosarcoma (OS) is the mostly diagnosed primary bone malignancy. Emerging evidence indicates that the activity of pyruvate kinase M2 (PKM2) isoform is crucial for the survival of tumor cells. In the present study, the effect of *PKM2* knockdown on the proliferation and migration of OS cells were assessed both *in vitro* and *in vivo*. Small hairpin RNA (shRNA) technology were employed to suppress the expression of PKM2 in MG-63 and Saos-2 cell lines. *In vitro*, shRNA-mediated knockdown of *PKM2* efficiently inhibited cell proliferation, and induced G1 cell cycle arrest and apoptosis in both cell lines, which was associated with decreased expressions of cyclin D1 and Bcl-2 as well as increased expressions of Bax, cleaved-caspase-3, and cleaved-PARP. The invasion and migration potential of OS cell lines were also inhibited by *PKM2* knockdown through the regulating effect of PKM2 on MMP-2 and VEGF signaling. *In vivo*, knockdown of *PKM2* decelerated tumor growth rate and induced structure deterioration in tumor tissues. The current study for the first time showed that the activity of PKM2 was indispensable for the development and metastasis of OS, thereby providing the basic information for the future development of PKM2-based anti-OS therapies.

1. Introduction

Osteosarcoma (OS) is the mostly diagnosed type of primary bone malignancies and commonly affects children, adolescents, and young adults [1,2]. The origin of OS is characterized by the osteogenesis of malignant cells [3], which majorly occurs in the distal femur and is followed by the oncogenesis in proximal tibias and humerus [1]. Current OS treating regimes depend on the concatenated application of surgeries and intensive multi-agent chemotherapies [4] and have increased the overall survival rate of OS patients to 70–80% [5,6]. However, even with the improvement of treating methods, there are still 20–30% OS patients being impaired by the pulmonary metastasis and relapse, resulting in an overall 20% five-year survival rate in those patients [7]. Moreover, given the invasiveness of surgery methods and side effects of chemotherapies, effective and mild approaches for OS treatment are still required.

Mutation of several molecular groups have been reported to be associated with the pathogenesis of OS, including retinoblastoma 1 (*RB1*), tumor protein *p53* (*TP53*), Apurinic/Apyrimidinic exonuclease 1 (*APEX1*) [8–12]. The inactivation or loss of tumor repressor such as *RB1* or *TP53* is proved to induce the extensive proliferation and the resistance to DNA damage in OS cells [10,11]. The overexpression of *APEX1* is also correlated with the recurrence, metastasis, and survival in OS patients [12]. Based on these findings, emerging studies are being performed to develop novel and specific molecular targeted therapies to improve the prognosis in OS patients. Pyruvate kinase (PK) is a rate-limiting enzyme of the glycolytic process and catalyzes the dephosphorylation of phosphoenolpyruvate to pyruvate [13]. Four isoenzymes of PK are currently identified, including L, R, M1, and M2, of which PKM2 is founded to be involved in the onset and development of cancer cells [14]. The current theory indicates that the activation of PKM2 results in the reprogramming of metabolism in cancers [15,16], creating the needed balance to support energy requirements of multiple cancer types [17]. For instance, the suppression of PKM2 inhibits the tumor growth and invasion in lung adenocarcinoma [14]. The up-regulation of PKM2 is also found to promote the tumor invasion in breast and intestinal cancers [18–20]. Regarding OS, study of Liu et al. reports that the overexpression of PKM2 predicts a poor prognosis for patients with OS [21]. However, the detail function of PKM2 in the occurrence and development of OS remains unrevealed.

Therefore, the major purpose of the current study was to elaborate the function of PKM2 in the survival and metastasis of OS cells. The

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expression of *PKM2* gene was knocked down in OS cell lines and the effect of PKM2 suppression on the cell proliferation, invasion, migration, and apoptosis was determined. *In vivo* OS orthotopic model was induced to validate the results derived from *in vitro* assays. Findings outlined in the current study demonstrated that the suppression of PKM2 could inhibited the OS cells growth and invasion both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemical and agents

Antibodies against PKM2 (PB0418), cyclin D1 (BA0770), and vascular endothelial growth factor (VEGF) (BA0407) were purchased from Boster (China). Antibodies against Bcl-2 (D160117), Bax (D120073), and matrix metalloproteinase-2 (MMP-2) (D198344) were purchased from Sango Biotech (China). Antibodies against cleaved-caspase-3 (ab2302) and cleaved-PARP (ab32561) were purchased from Abcam (UK). Secondary goat anti-rabbit (A0208) and goat anti-mouse (A0216) IgG-HRP antibodies were purchased from Beyotime Biotechnology (China). Antibodies against β-actin (bsm-33139 M) were purchased from Bioss (China). Polybrene (H9268) were purchased from Sigma (USA). RNA Purified Total RNA Extraction Kit (RP1201) and super M-MLV reverse transcriptase (RP6502) were purchased from BioTeke (Beijing, China). PMSF lysis (P0013B), Protein concentration determination kit (P0012) using BCA method, Cell cycle detection kit (C1052), CCK-8 detection kit (C0037), and Hoechst staining kit (C0003) were purchased from Beyotime Biotechnology (China). Cell apoptosis detection kit (KGA106) was purchased from KeyGen BioTECH (China). In Situ Cell Death Detection Kit (11684817910) was purchased from Roche Group (Switzerland).

2.2. Cell culture and animals

Human OS cell lines Saos-2 (ZQ0447) and MG-63 (ZQ0403) were purchased from Zhongqiaoxinzhou Cell Research (China) and maintained in 10% DMEM supplemented with 10 fetal bovine serum (FBS) and 1% (v/v) antibiotics mixture at 37 °C in an atmosphere consisting of 5% CO_2 and 100% humidity. Cells from three to five passages were collected and preserved for subsequent assays. BALB/c mice were provided by Changsheng Biotechnology (Liaoning, China) and housed in cages at room temperature (20–25 °C) with a constant humidity (55 ± 5%) with food and water available *ad libitum*. All the assays with animals were performed following the Institutional Animal Ethics Committee and Animal Care Guidelines for the Care and Use of The First Affiliated Hospital of China Medical University.

2.3. Construction of lentivirus plasmid and infection

Specific shRNA targeting PKM2 (5′-GTGGAGACGTTGAAGGAGA-3′) and non-targeting shRNA (5′-TTCTCCGAACGTGTCACGT-3′) were packaged as lentivirus particles by GenePharma (Shanghai, China). For transfection, OS cells and different lentivirus particles were co-plated in to one well of six-well plates in a ratio of 1:50 and polybrene were added to promote the infection. Cells with stable knockdown of *PKM2* were selected by culturing the cells in DMEM supplemented with 3 μg/ml puromycin for two weeks. The knockdown efficiency of *PKM2* was validated with reverse transcription real time polymerase chain reaction (RT^2-PCR) and western blotting assay as described following.

2.4. RT^2-PCR

Total RNA in different groups was extracted using RNA Purified Total RNA Extraction Kit. cDNA templates was achieved using super M-MLV reverse transcriptase from total RNA. The reaction mixture of real time PCR contained 10 μl SYBR GREEN matermix, 0.5 μl of each primer (PKM2, forward: 5′-CGCTGGATAACGCCTACAT-3′, backward: 5′-CCA TTTTCCACCTCCGTC-3′; β-actin, forward: 5′-CCATCGTCCACCGCA AAT-3′, backward: 5′-GCTGTCACCTTCACCGTTC-3′), 1 μl cDNA template, and 8 μl ddH_2O. Thermal cycling parameters were set as followings: a denaturation step at 94 °C for 10 min, followed by 40 cycles of amplification at 94 °C for 10 s, 60 °C for 20 s and 72 °C for 30 s, then the reaction was stopped at 25 °C for 1 min. The signal was detected after the step at 72 °C for 30 s. The relative expression levels of the *PKM2* were calculated using ExicyclerTM 96 (BIONEER, South Korea) according to the formula of $2^{-\triangle\triangle ct}$.

2.5. Western blotting

Cells in different groups were lysated using 1% PMSF and total cellular protein was collected by centrifugation at 10005 × *g* for 4 min β-actin were used as internal reference protein. The concentrations of the protein samples were determined using Protein Concentration Determination Kit according to the manufacturers' instruction. 30 μg protein from different samples were subjected to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 80 V for 2.5 h. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. After being rinsed with TTBS, the membranes were blocked with skimmed milk solution for 1 h. Then the membranes were incubated with the primary antibodies against PKM2 (1:400), cyclinD (1:400), Bcl-2 (1:500), Bax (1:500), cleaved-caspase-3 (1:1000), cleaved-PARP (1:1000), MMP-2 (1:500), VEGF (1:400), and β-actin (1:5000) at 4 °C overnight. Secondary HRP-conjugated IgG antibodies (1:5000) was added onto the membranes after four washes with TTBS and incubated for 45 min at 37 °C. After final six washes with TTBS, the blots were developed using the Beyo ECL Plus reagent and the images were recorded in the Gel Imaging System. The relative expression levels of proteins were calculated by the Gel-Pro-Analyzer (Media Cybernetics, USA).

2.6. CCK-8 assay

Cell viability was determined using CCK-8 assay: briefly, exponentially growing OS cells were seeded into 96-well plates (3×10^3/well) and incubated for 96 h (25 replicates for each group). Every 24 h, CCK-8 solution (10 μl) was added to five randomly selected wells of each group and the cultures were incubated at 37 °C for 1 h. The OD values in different wells were detected with a Microplate Reader (ELX-800, BIOTEK, USA) at 450 nm.

2.7. Flow cytometry

Cell cycle distribution of OS cells was determined with Cell cycle detection kit using a FACS flow cytometer (Accuri C6, BD, USA). Apoptotic process in OS cells was determined using Cell apoptosis detection kit according to the manufacturer's instructions with a FACScan flow cytometer (Accuri C6, BD, USA). The total apoptotic rate was equal to the sum of the late apoptotic rate (UR, upper right quadrant-advanced stage apoptosis) and the early apoptotic rate (LR, lower right quadrant-prophase apoptosis).

2.8. Hoechst staining

OS cells from different groups were firstly cultured in 12 well plates (5×10^4/well) for 24 h at 37 °C. Then the morphological changes of cell nuclei of OS cells were detected by adding 0.5 ml Hoechst into the wells and incubated for 5 min. The results were observed using a fluorescence microscope (IX53, Olympus, Japan) at 400× magnification.