**Original Article**

**Dipalmitoylphosphatidic acid reduces osteosarcoma growth by activation of AKT signaling pathway**

Wei Liu1*, Shuo Wang2*, Ying-Jian Zhang1, Guang-Wen Fang1, Yuan Xue2

1Department of Orthopaedic Surgery, Tianjin Baodi Hospital, Tianjin 301800, P.R. China; 2Department of Orthopaedic Surgery, Tianjin Medical University General Hospital, Tianjin 300052, P.R. China. *Equal contributors.

Received June 26, 2017; Accepted December 31, 2017; Epub February 15, 2018; Published February 28, 2018

**Abstract:** Aim: Osteosarcoma is one of the most common bone tumors. It has high malignant degree and tends to occur in adolescents. In this study, we aim to investigate the effects of dipalmitoylphosphatidic acid (DPPA) in osteosarcoma and its underlying mechanism. Methods: BALB/c mice were used for the establishment of osteosarcoma model and the KRIB cell line was purchase from America ATCC. 2×105 KRIB cells (10 µl) were injected to tibias of BALB/c mice. The osteosarcoma models were successfully established after 8 weeks. When the mice grew up to 12 weeks, the treatment group were injected intraperitoneally with DPPA. While the control group were injected intraperitoneally with saline. Detection of the histological sections to make sure the establishment of osteosarcoma models. The mice in treatment and control groups were harvested and tissue samples were taken. The histological changes were detected by Hematoxylin and Eosin Staining (H&E staining); the expression of fibronectin was detected by immunohistochemistry and western blot was applied to determine the changes in (serine/threonine kinase) AKT related pathway. The variation of cell cycles and apoptosis were detected by Flow cytometric assays. Results: The osteosarcoma models were successfully established in 48 mice which were separated into two groups: the treatment and control group. The two groups were treated with DPPA and saline respectively. The histological sections demonstrated severe tissue erosion and increased infiltration of inflammatory cells in the control group, however, the inflammatory cells were significantly reduced in the treatment group with DPPA. In addition, compared to the control group with increased expression of fibronectin, decreased expression of fibronectin was detected by immunohistochemistry in the treatment group (p<0.01 compared with the control group). Flow cytometric assays showed that DPPA arrested KRIB cells at the G1 phase and increased the cell number of apoptosis (p<0.05 compared with the control group). And western blot results exhibited a higher p-AKT expression in the treatment group (p<0.001 compared with the control group). Furthermore, there was an increased survival rate after DPPA treatment. Conclusion: DPPA reduces osteosarcoma growth by activation of AKT signaling pathway.

**Keywords:** DPPA, osteosarcoma, AKT

**Introduction**

Osteosarcoma is characterized by the spindle matrix in the osteoid tissue and amputation is the main method for osteosarcoma therapy. However, the five-year survival rate of osteosarcoma only has 10% to 20%. Recently, the application of neoadjuvant therapy was used, which combined chemotherapy with surgery. This novel method significantly reduced the disability rate of patients from 15% to 5% and increased the 5-year survival rate to 60%-70% and prolonged the survival time [1-3]. Despite the favorable results achieved by combination therapy, chemotherapy is still failed to improve outcomes of osteosarcoma. Therefore, the more advanced therapies are urgently needed. In recent years, biologic targeted therapy for osteosarcoma achieved some promising results. Thus, it is interesting to find novel target for biologic targeted therapy for osteosarcoma.

The serine/threonine kinase (Akt), also known as Protein Kinase B (PKB), plays an important role in cellular signal transduction. It combines Phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K) forming PI3K/Akt signaling pathway. Akt is an important downstream molecule of PI3K, playing a key role in the development and invasion of multiple cancers. In addition, many
researches indicate that dysregulation of the PI3K/Akt signaling pathway has relationship with a variety of cancers such as lung, breast, prostate and bladder cancer.

It has been reported that the phosphorylation of the Forkhead Transcription Factor Forkhead Like1 (FKHRL1) is directly regulated by the PI3K/Akt phosphorylation cascade. It is known that FKHRL1 regulates genes promoted cell apoptosis and activates caspases through regulation of Fas and Bcl-2. Dipalmitoyl phosphatidic acid (DPPA) is an intermediate produced by the hydrolysis of glycerophospholipid by phospholipase D. It inhibits angiogenesis and plays a key role in regulation of Raf-1 translocation and activation of mitogen-activated protein kinase (MAPK) cascade II and III [4].

Studies have demonstrated that DPPA promotes the penetration of drugs into the cells [5, 6]. In addition, its metabolite lyso-PA enhances the expression of Bcl-2 in the Hela cells [7]. However, DPPA has no impact on the expression of Bcl-2 in osteosarcoma [8], implying that DPPA has different regulation effects in the different pathological stages. Besides, it is still unclear the role of DPPA in the occurrence and development of osteosarcoma. Fibronectin distributes on the cell surface, which is a glycoprotein with high-molecular weight. It promotes cell adhesion and acts as a marker for evaluation of damage degree.

Materials and methods

Establishment of osteosarcoma model

BALB/c mice were used for the establishment of osteosarcoma model and the KRIB cell line was purchased from America ATCC. $2 \times 10^5$ KRIB cells (10 µl) were injected to tibias of BALB/c mice. The osteosarcoma models were successfully established in 48 mice after 8 weeks. The 48 mice were then separated into two groups: the treatment and control group. When the mice grew up to 12 weeks, the treatment group (n=24) were injected intraperitoneally with DPPA (0.1 mg/kg) [9] every 48 h and lasted for 4 weeks. In the control group (n=24), mice were injected intraperitoneally with saline (0.1 mg/kg) every 48 h and lasted for 4 weeks. This study was approved by the Institute Research Ethics Committee of Tianjin Baodi Hospital.

Cell lines and treatments

Osteosarcoma cell line KRIB was purchased from America ATCC. These cells were maintained in Dulbecco’s Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a 5% CO$_2$ humidified incubator.

Hematoxylin and eosin staining (H&E)

The mice were harvested and tissue samples were taken. The tissue samples were pre-treated and sectioned. Placing the sections on slides, stained with hematoxylin and eosin, dehydrated and covered. The slides were observed under the microscope and pictures were taken for analysis.
DPPA reduces osteosarcoma growth

Figure 2. Expressions of fibronectin in osteosarcoma after DPPA treatment for different weeks including 16 weeks, 17 weeks and 18 weeks. A. Immunostaining for fibronectin in osteosarcoma in mice with or without DPPA treatment at different time points. B. Expressions of fibronectin in osteosarcoma in mice with or without DPPA treatment. **p<0.01 compared with the control group.

were chosen in the middle of the visual field, then invited three different pathology doctors for diagnosis.

**Immunohistochemistry**

The mice were harvested and tissue samples were taken. The tissue samples were pre-treated and sectioned. The sections were deparaffinized and treated with 0.3% H$_2$O$_2$ solution. After that, the sections were blocked with 10% FBS and incubated with primary antibody overnight. Washing the sections with PBS, incubated with secondly antibody and then colored by DAB solution. Removing the coloring solution, PBS washed and stained with hematoxylin. The pictures were taken under microscope.

And the morphological data were handled with Image-Pro-Plus6.0.

**Western blot**

The protein was extracted from tissue using commercial kit (Nuclear and Cytoplasmic Protein Extraction Kit, Beyotime Biotechnology) and protein concentration was measured by BCA assay (BCA Protein Assay Kit, Beyotime Biotechnology). The protein samples and marker were running on the PAGE gel and then transferred to the PVDF membrane. Block the membrane with 5% non-fat milk, and wash the membrane with Tris Buffered Saline (TBST). And the membrane was incubated with primary antibody overnight. The primary antibody was
DPPA reduces osteosarcoma growth

**Flow cytometric assays**

KRIB cells were plated at a density of $5 \times 10^5$ cells/dish in 60-mm dishes and incubated for 24 hours. Next, the cells were serum-deprived for 24 hours, followed by the re-addition of DPPA (100 uM) in DPPA group or DMEM in control group; the cells were then harvested 20 hours later. The cells were stained using BD PharmingenTM PI/RNase staining buffer (#550825), and the analysis was conducted using a flow cytometer (Becton Dickinson). Cell cycle modeling was performed using the Modfit LT software, version 3.2 (Verity Software House).

**Statistical analysis**

The data are processed by Graph Pad Prism 5.0 and the graphic data are dealt with Pro-Plus6.0. The data are presented as the means ± standard deviation from at least 3 separate experiments. One-way anova was applied for multiple groups’ comparison and two groups were compared with t-test analyses. And the significance level was set at $p<0.05$.

**Results**

DPPA attenuates the development of osteosarcoma

The histopathological evaluation of tumor tissues showed that treatment with DPPA for four weeks showed the darker red in cytoplasm with less infiltration of inflammation cells in the tumor tissue. In addition, DPPA-treated group showed higher numbers of osteofibrous cells with better integrity. These results demonstrated that the DPPA-treated group showed less malignant degree when compared that in the control group, indicating that the DPPA may attenuate the development of osteosarcoma (Figure 1A). The Ki67 immunohistochemistry staining showed that the control group had a higher cell proliferation. However, the cell proliferation was attenuated in the DPPA-treated group, indicating that DPPA could attenuate the pathological development of osteosarcoma.
DPPA reduces osteosarcoma growth

Fibronectin is responsible for the cell adhesion and has a close relationship with cell growth. Thus, we further detected the expression of fibronectin in the osteosarcoma tumor tissue using immunohistochemistry. To monitor dynamical results, the expression of fibronectin was observed at the 16th, 17th, and 18th week, respectively. In the control group, higher expression of fibronectin was detected. However, the DPPA-treated group had lower levels of expression of fibronectin. In addition, the structures of osteosarcoma were normal in the treatment group when compared that in the control group. The expression of fibronectin reflected the structural changes in the osteosarcoma also proved that the osteosarcoma models were successfully established. As shown in Figure 2, the immunohistochemistry results demonstrated a statistical difference between DPPA-treated group and control group for expression of fibronectin (p<0.01 compared with the control group).

DPPA treatment affected the cell-cycle osteosarcoma cells

As shown in Figure 3A and 3B, compared with control group the KRIB cells were arrested in the G1 phase after treatment of DPPA. At the same time, the cells in S phase were reduced. From the Figure 3C, it is obvious that the cell number of apoptosis in DPPA group were much more, compared to control group (p<0.05). These data revealed that DPPA suppressed cell cycle progression.

DPPA activates the Akt pathway and remits the development of osteosarcoma

Fibronectin acts as the adhesion molecule and plays an important role in the regulation of cell adhesion in the extracellular matrix. Besides, the Akt signal pathway also plays an important role in the regulation of cell adhesion in the extracellular matrix. By extraction of protein from the tumor tissue, the levels of Akt and p-Akt were further investigated using western blotting. As shown in Figure 4, the results sh-
DPPA reduces osteosarcoma growth

owed that expression of p-Akt in the tumor tissues from 16 weeks old mice was significantly increased when compared that in the DPPA-treated group (p<0.001 compared with the control group) indicating that DPPA could activate the Akt pathway and attenuate the development of osteosarcoma.

*Increased survival rate after DPPA treatment*

In the present study, we found that DPPA attenuates the development of osteosarcoma by activation of Akt pathway. However, it is still unknown that DPPA treatment could increase the survival rate, which we will further investigate. As shown in Figure 5, DPPA treatment significantly increased the survival rate in comparison to the control group (p<0.01).

**Discussion**

DPPA is an important intermediate in the progress of metabolism. Recent research demonstrated that DPPA exhibits significant inhibitory effect in tumor proliferation by silencing UspA2 gene expression in rat. It is known that UspA2 is an important gene for generation of fibronectin and silencing UspA2 leads to the decrease of fibronectin [10-15]. In the present study, we infer that DPPA could affect the development of osteosarcoma. Therefore, the osteosarcoma model was established by using mice. The results demonstrated that DPPA attenuated the pathological development of osteosarcoma, which in turn confirmed our hypothesis. However, further studies are still needed to investigate the mechanism of DPPA for inhibition of osteosarcoma development. Researchers have found that the abnormal expressions of some molecules are one of the main reasons leading to infinite osteoblast proliferation [16]. Besides, in the early stage, the expressions of some molecules were abnormal [17, 18]. Akt is an important gene and plays a key role in the regulation of cell proliferation. It inhibits cell proliferation by silencing CDK expression. Fibronectin acts as the adhesion molecule and plays an important role in cell adhesion in extracellular matrix. Additionally, Akt signaling pathway significantly affects cell adhesion in the extracellular matrix. Some studies have demonstrated that fibronectin changes the cell polarity and then disturbs mitosis by regulation of the Akt signaling pathway [19]. As shown in Figure 2, fibronectin expression was significantly changed after DPPA treatment. We infer that DPPA could affect Akt signaling and then attenuate the pathological progress of osteosarcoma in rats. The immunohistochemistry results have showed higher expression of fibronectin in the control group in comparison to the DPPA treatment group. In addition, studies also have demonstrated that Akt knock out mice induced by agents had no osteoblast proliferation [20-22].

Therefore, it is important to investigate the mechanism related to the osteosarcoma, which further contribute to better understanding of the occurrence and development of osteosarcoma. Some studies have found that fibronectin plays a dominant role in the fibrosis and the expression of fibronectin is related to fibrosis. In the present study, we found that DPPA treatment suppresses the development of osteosarcoma by decreasing expression of fibronectin and activation of Akt signaling pathway. This study, for the first time, reveals that DPPA could impact the Akt signaling pathway and increase the survival rate of mice.

**Acknowledgements**

The study was supported by the Tianjin Municipal Health Bureau fund (2010K203).

**Address correspondence to:** Yuan Xue, Department of Orthopaedic Surgery, Tianjin Medical University General Hospital, 154 Anshan Road, Tianjin 300052, P.R. China. Tel: +86 22 60814688; E-mail: yuanx12@sina.com

**References**


DPPA reduces osteosarcoma growth


