

Punicalagin inhibited proliferation, invasion and angiogenesis of osteosarcoma through suppression of NF- κ B signaling

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Abstract. Osteosarcoma is the most common primary malignant bone tumor among children and young people and is associated with poor prognosis. Punicalagin is an antioxidant ellagitannin found in pomegranate juice with known antiproliferation and anti-angiogenesis properties. However, the antitumor effect of punicalagin on osteosarcoma requires further investigation. In the present study, the inhibitory effect of punicalagin on proliferation and invasion was evaluated in one human osteoblast cell line (hFOB1.19) and three human osteosarcoma cell lines (U2OS, MG63 and SaOS2). The cancer cell apoptosis ratio was determined using flow cytometry. NF- κ B signaling in these cells was also evaluated using western blotting analysis. A subcutaneous tumor xenograft model was initiated to study the efficacy of punicalagin on osteosarcoma development and angiogenesis *in vivo*. Punicalagin treatment significantly decreased osteosarcoma cell proliferation and increased apoptosis. In addition, the invasion potential of these cells in a transwell assay was also dramatically suppressed in osteosarcoma cells. Punicalagin not only induced the degradation of I κ B α but also the nuclear translocation of p65, suggesting the attenuation of NF- κ B signaling pathway following treatment. Moreover, punicalagin markedly downregulated interleukin (IL)-6 and IL-8 levels, which was consistent with the inhibition of NF- κ B signaling. An NF- κ B activator could reverse these effects. Using a tumor xenograft mouse model, it was demonstrated that punicalagin exposure inhibited osteosarcoma growth and angiogenesis *in vivo*. These observations confirmed the suppressive effect of punicalagin against osteosarcoma malignancies. The underlying molecular mechanisms may include inhibition of the NF- κ B signaling pathway.

Introduction

Osteosarcoma accounts <1% of malignancies overall, with an incidence of ~5 cases per million in individuals <19 years of age in the USA. However, it is the most widely diagnosed primary malignant bone tumor, particularly among children and young people. Males are prone to the onset of osteosarcoma, and the ratio of male to female incidence is ~3:2 (1). Osteosarcoma is thought to arise from mesenchymal primitive bone-forming cells, and is characterized by the sustainable production of malignant osteogenesis. In addition, the production of pro-angiogenic factors in the malignant development of osteosarcoma has also been suggested by a previous study, which concluded that osteosarcoma had a strong tendency to metastasize early and was associated with poor prognosis (2). Thus, the degree of osteosarcoma malignancy is extremely high, and increased tumor invasiveness and vascularity are associated with metastatic potential and poor prognosis.

The main current treatment strategy for patients with newly diagnosed osteosarcoma includes neoadjuvant chemotherapy, followed by surgical removal of the primary tumor and all metastatic lesions with clinical manifestations, as well as postoperative adjuvant chemotherapy (3). The three-drug chemotherapy regimen of cisplatin, doxorubicin and methotrexate constitutes the primary option for backbone treatment, and the overall 5-year survival rate in America for osteosarcoma has increased to 60-70% in patients receiving the three-drug regimen (4). Recently, biologic agents, such as muramyl tripeptide and IFN- α -2b, and additional cytotoxic chemotherapy, such as ifosfamide, have been introduced into clinical trials. However, these have failed to significantly improve the survival of young patients with osteosarcoma (5,6). Therefore, an improved understanding of the underlying mechanisms of tumor progression and angiogenesis in osteosarcoma is required in order to identify and develop more effective therapies.

Dysregulation in nuclear factor- κ B (NF- κ B) signaling is associated with excessive cellular proliferation and developmental signals during tumorigenesis. Indeed, this pathway has been reported to be involved in inflammatory proliferation and differentiation of osteosarcoma cells (7). NF- κ B can also regulate the generation of proinflammatory and proangiogenic cytokines around cancer cells (8). Thus, it

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Abbreviations: NF- κ B, nuclear factor- κ B; PMA, phorbol myristate acetate; mTOR, mammalian target of rapamycin; Ctrl, control

Key words: osteosarcomas, punicalagin, NF- κ B pathway, malignancy

was previously suggested that NF- κ B could serve a causative role in osteosarcoma progression (9). Punicalagin is an antioxidant ellagitannin found in pomegranate juice with known anti-proliferation or anti-angiogenesis properties against many cancer cell lines, including leukemia, glioma and prostate cancer cells (10). The present study aimed to examine the detailed function of the NF- κ B pathway in osteosarcoma, and to determine whether punicalagin can inhibit the NF- κ B pathway to suppress inflammation and osteosarcoma tumorigenicity. Combined treatment targeting the NF- κ B pathway may represent a novel and promising strategy to significantly enhance the therapeutic activity of routine anticancer drugs against osteosarcoma.

Materials and methods

Reagents and cell lines. A 50 mM stock solution consisting of 5 mg punicalagin (Sigma-Aldrich; Merck KGaA) in 1 ml DMSO were prepared. The stock was diluted to the desired concentrations with culture medium to give a water-soluble fraction, in which DMSO concentration did not exceed 0.2% in the highest punicalagin concentrations applied. The three human osteosarcoma cell lines (U2OS, MG63 and SaOS2) and one normal osteoblast cell line (hFOB1.19) were purchased from American Type Culture Collection and cultured according to the instructions. All of the cell lines were grown in Dulbecco's modified Eagle medium supplemented with 10% FBS (both from Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO₂ at 37°C. Following treatment, culture medium was prepared serum-free and collected in 24-h cultures. Phorbol myristate acetate (PMA; Sigma-Aldrich; Merck KGaA), an activator of the NF- κ B pathway, was added to cells together with punicalagin and incubated for 45 min in a humidified atmosphere with 5% CO₂ at 37°C.

Cell proliferation assay. Cell proliferation was examined using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). Cells were seeded at a density of 1×10^3 cells per well in 96-well plates, then treated with different concentrations of punicalagin or DMSO as control. Following 1-3 days of culture, 10 μ l CCK-8 reagent was added to each well and cells were cultured for 1 h. A microplate reader (Bio-Rad Laboratories, Inc.) was used to measure the absorbance at 450 nm.

Cell invasion assay. Transwell migration assays were performed with 8.0- μ m pore polycarbonate filter inserts (Corning, Inc.) coated with Matrigel™ (BD Biosciences) at room temperature for 1 h before use. Briefly, osteosarcoma cells in 100 μ M punicalagin contained or vehicle medium supplemented with 1% FBS were placed in the top chamber at a density of 1×10^4 cells/well. In the bottom chamber, complete medium with 10% FBS was used as a positive control. After 48 h of incubation, the migrated cells were fixed with 4% paraformaldehyde for 20 min and stained with 1% crystal violet for 10 min at room temperature. Images were captured with a light microscope at x400 magnification and the migrated cells were counted manually and averaged from 5 high-power fields.

Cell apoptosis analysis. To investigate early and late apoptotic cells, annexin V-FITC and propidium iodide (PI) double

staining was performed. The APOAF annexin V apoptosis kit (Sigma-Aldrich; Merck KGaA) was used for annexin V staining, according to the manufacturer's protocol. All samples were quantified using a Canto II flow cytometer (BD Biosciences) and analyzed with FlowJo version 7.6 software (TreeStar). Early apoptotic cells were defined as FITC^{high}PI^{low} cells and late apoptotic cells were defined as FITC^{high}PI^{high}. Additionally, FITC^{low}PI^{low} represented healthy cells and FITC^{low}PI^{high} accounted for cells debris that was eliminated.

Western blotting assay. The cells or tissues were homogenized in RIPA buffer (Beyotime Institute of Biotechnology). A BCA protein assay kit (Thermo Fisher Scientific, Inc.) was used to determine the protein concentration in lysates and conditioned medium. Equal amounts of protein (15 μ g) were loaded per lane and separated via SDS-PAGE (10% gel), then transferred to a PVDF membrane (Bio-Rad Laboratories, Inc.). The PVDF membrane was blocked with 5% skimmed milk in TBS + 0.1% Tween®-20 buffer on a shaker for 1 h at room temperature. The membrane was then incubated in 4°C with the following primary antibodies overnight: Anti-phosphorylated (phosphor)-inhibitor of κ B α (I κ B α ; Ser32; cat. no. 2859), anti-I κ B α (cat. no. 9242), anti-phospho-mammalian target of rapamycin (mTOR; Ser2448, cat. no. 2971), anti-mTOR (cat. no. 2983), anti-p65 (cat. no. 8242), anti-histone 2A family member X (H2AX, cat. no. 7631) (all 1:1,000; all from Cell Signaling Technology Biological Reagents Co., Ltd.), anti- β -actin (cat. no. sc-130656; 1:2,000), anti-interleukin (IL)-6 (cat. no. sc-130326; 1:500), and anti-IL-8 (cat. no. sc-8427; 1:500; all purchased from Santa Cruz Biotechnology, Inc.). After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (cat. no 7074; 1:1,000 Cell Signaling Technology Biological Reagents Co., Ltd.) for 1 h on the shaker at room temperature. The membrane was then incubated with chemiluminescence reagent (GE Healthcare Life) for 5 min at room temperature. The relative quantity of the protein was measured using ImageJ software v1.51 (National Institutes of Health).

Tumor xenografts. The *in vivo* experiment protocol was approved by the Institutional Animal Care and Use Committee at The Second Affiliated Hospital of Air Force Medical University and followed the Chinese national standards: Laboratory animal welfare ethics review guidelines for the humane and customary care and use of experimental animals. A total of 20 female 6-8-week-old, 18 g, Balb/c nude mice (n=5 per group) were purchased from Model Animal Research Center of Nanjing University. Mice were housed at 20-24°C with an average humidity of 40% and a 12-h light/dark cycle and received food and water *ad libitum*. Mice were then injected with osteosarcoma cells near the back of the neck at a density of 2×10^7 cells in 200 μ l PBS. Mice were anesthetized by inhalation using 2.0-2.5% sevoflurane during injection and measurement. After 1 week of tumor cell inoculation, 5 mg/kg punicalagin in saline or an equal volume (300 μ l) saline as vehicle (control) was injected intraperitoneally once a week for a total of 7 weeks, and the mouse health and behavior were monitored daily for 8 weeks. No death was observed prior to sacrifice. The tumor size was measured with a sliding caliper twice a week, and

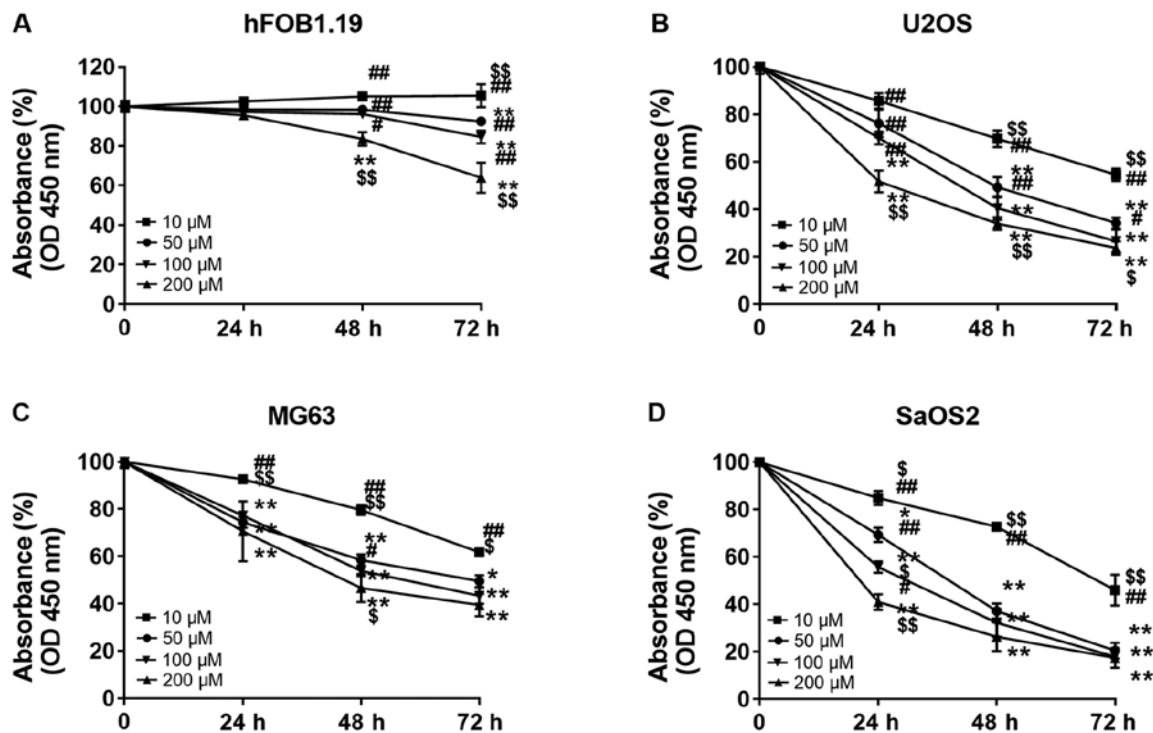


Figure 1. Effect of punicalagin on the viability of human osteoblast and osteosarcoma cell lines. Cells were treated with different concentrations of punicalagin for 24-72 h. Absorbance measured using a CCK-8 assay relative to the untreated subgroup in (A) hFOB1.19, (B) U2OS, (C) MG63, and (D) SaOS2. Data are presented as the mean \pm SEM of three independent experiments. * $P<0.05$ and ** $P<0.01$ vs. 10 μM punicalagin; \$ $P<0.05$ and \$\$ $P<0.01$, vs. 50 μM punicalagin; # $P<0.05$ and ## $P<0.01$, vs. 200 μM punicalagin. OD, optical density.

the tumor volume was calculated using the formula: $\text{Size, mm}^3 = [\text{tumor length} \times (\text{tumor width})^2] / 2$. When volume was $>500 \text{ mm}^3$, the experiment was stopped and the mice were sacrificed using CO_2 asphyxiation with a flow rate $\leq 50\%$ of the chamber volume per min, followed by cervical dislocation. Tumors were then harvested, weighed and snap-frozen in liquid nitrogen and stored at -80°C for subsequent use in immunohistochemistry assays.

Immunohistochemistry assay. Solid tumors were fixed with 10% formaldehyde for 48 h at room temperature and embedded in paraffin. Tissue slides were blocked with 1% BSA (Beyotime Institute of Biotechnology) in PBS for blocking for 1 h at room temperature. To identify infiltrating blood vessels, immunohistochemistry was carried out on 5- μm deparaffinized sections using an anti-CD31 antibody (cat. no. 77699; 1:150; Cell Signaling Technology Biological Reagents Co., Ltd.) at 4°C overnight, and then peroxide-conjugated secondary antibodies (cat. no. ab6721; 1:500; Abcam) for 1 h at room temperature with ABC Staining kits (Thermo Fisher Scientific, Inc.) were applied for generating chromogenic signals. Images were captured with a light microscope at magnification, $\times 100$.

Statistical analysis. Data are presented as the mean \pm standard error of the mean (SEM), unless otherwise stated. Analysis of two independent groups was performed using unpaired Student's *t*-test. One-way ANOVA followed by Bonferroni correction was used for multiple comparisons between groups. Statistical analysis was carried out using the GraphPad software v5.0 (GraphPad, Inc.). Each experiment was performed

in triplicate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Punicalagin treatment inhibits the proliferation of human osteosarcoma cell lines. In order to investigate whether punicalagin treatment could affect the viability malignant cells, hFOB1.19 osteoblast cells and U2OS, MG63 and SaOS2 osteosarcoma cells were treated with increasing concentrations of punicalagin (10, 50, 100 and 200 μM) for 24-72 h. Cell viability was then evaluated using CCK-8 assays (Fig. 1). Cell proliferation was suspended after 24 h but the viability of osteoblast cells did not significantly decrease at punicalagin concentrations $<100 \mu\text{M}$ or incubation time $<48 \text{ h}$. Overall, the viability of human osteosarcoma cell lines was decreased in a concentration- and time-dependent manner. In 2 of the 3 (MG2 and SaOS2) osteosarcoma cell lines, the decrease in cell viability was not further exacerbated following prolonged treatment $>48 \text{ h}$ and concentrations $>100 \mu\text{M}$. Thus, a concentration of 100 μM was selected for subsequent experimentation.

Punicalagin increases apoptosis of human osteosarcoma cell lines. In order to determine whether the decrease in cell viability following treatment with a moderate concentration of punicalagin was due to increased apoptosis, annexin V-FITC and PI double staining was used to assess the frequency of early ($\text{FITC}^{\text{high}}\text{PI}^{\text{low}}$) and late ($\text{FITC}^{\text{high}}\text{PI}^{\text{high}}$) apoptotic cells in U2OS, MG63 and SaOS2 cell lines. Treatment with 100 μM punicalagin for 48 h increased the cumulative percentage of early and late apoptotic tumor cells (Fig. 2).

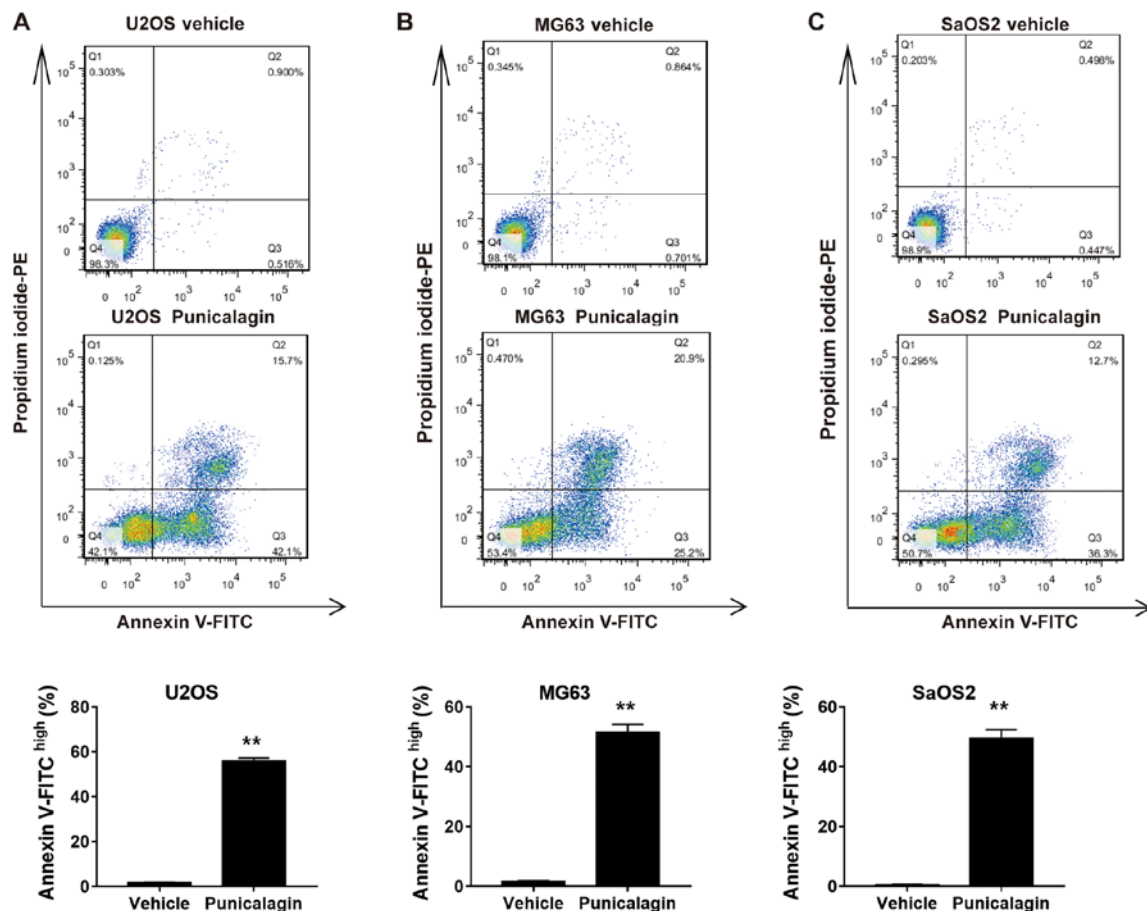


Figure 2. Effect of punicalagin on the apoptosis of human osteosarcoma cell lines. Cells were treated with 100 μ M punicalagin for 48 h, then analyzed using annexin V and propidium iodide double staining. Punicalagin treatment induced consistent changes seen as increased frequencies of both early (FITChighPIlow) and late (FITChighPIhigh) apoptotic cells in (A) U2OS, (B) MG63 and (C) SaOS2 cells. The representative diagrams of flow cytometry analyses were displayed. Data are presented as the mean \pm SEM of three independent experiments. ** P <0.01, vs. control group. PE, phycoerythrin; FITC, Fluorescein isothiocyanate; Ctrl, control.

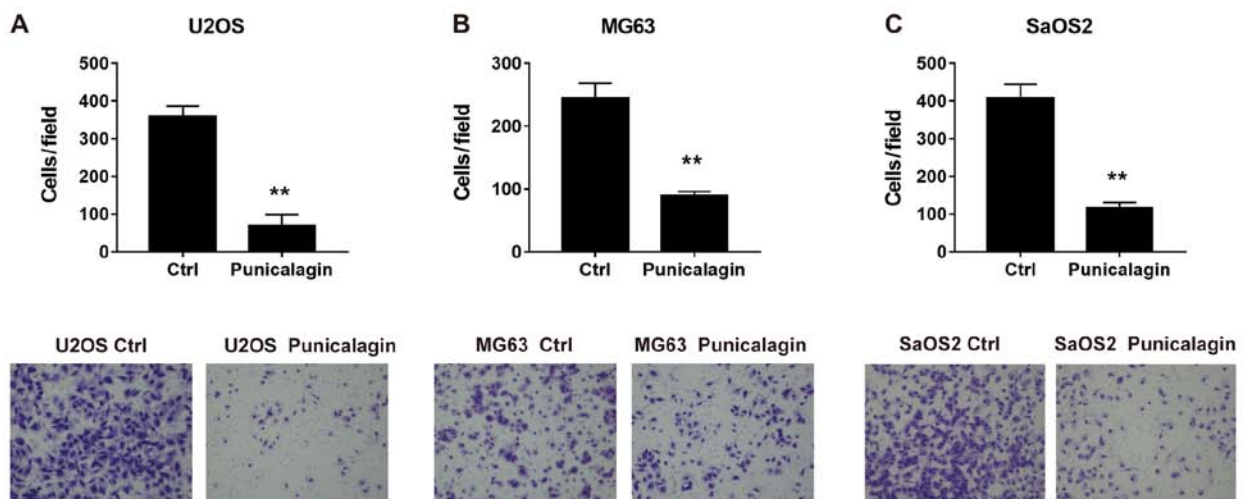


Figure 3. Effect of punicalagin on the invasion of human osteosarcoma cell lines (A) U2OS, (B) MG63 (B), and (C) SaOS2. Cells were treated with punicalagin for 24 h, then analyzed using a Transwell invasion assay. Data are expressed as the mean \pm SEM of three independent experiments. ** P <0.01, vs. control group. Ctrl, control.

Punicalagin suppresses the invasion of human osteosarcoma cells. To determine whether punicalagin could suppress the invasiveness of osteosarcoma cells, a Transwell Matrigel™ invasion assay was performed using U2OS, MG63 and SaOS2

cells in the presence or absence of punicalagin. Following treatment with punicalagin for 24 h, fewer migrated tumor cells were detected, suggesting that cell migration was inhibited in the presence of punicalagin (Fig. 3).

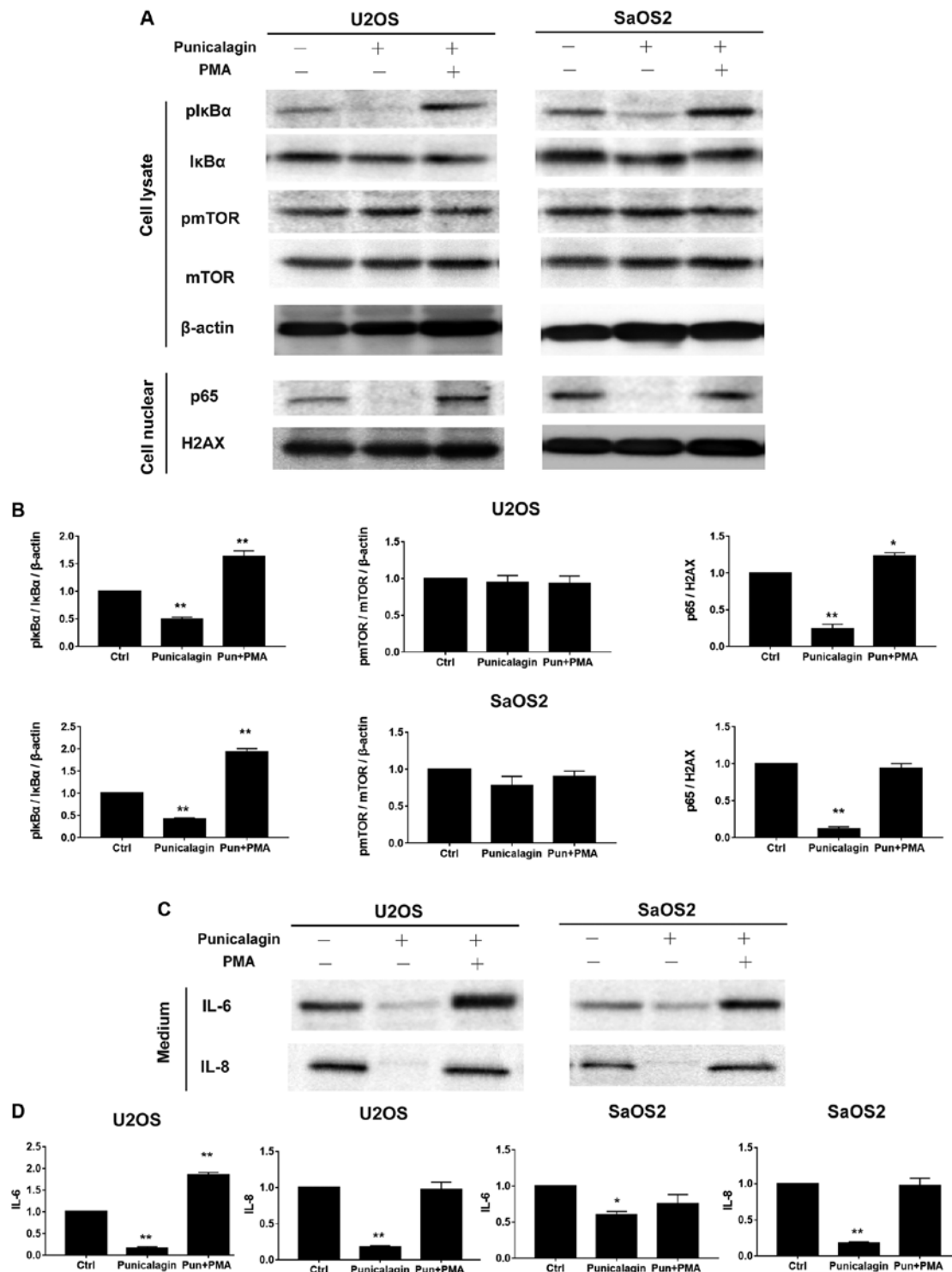


Figure 4. Punicalagin regulates the NF-κB pathway in human osteosarcoma cells. (A) Total cell extracts or nuclear fractions were collected and subjected to western blot analysis using anti-mTOR, pmTOR (Ser2448), IκBα, plkBα (Ser32), β-actin, nuclear p65 and H2AX. (B) Quantification of the results shown in (A). (C) U2OS and SaOS2 cells were also treated with punicalagin solution. The conditioned medium with or without NF-κB signaling activator, PMA and punicalagin treatment were used to determine the levels of IL-6 and IL-8. (D) Quantification of the results shown in (C). *P<0.05 and **P<0.01 vs. control group. Ctrl, control; p, phosphorylated; IL, interleukin; mTOR, mechanistic target of rapamycin kinase; NFκB; nuclear factor κB; IκBα, inhibitor of κBα, H2AX, histone 2A family member X; PMA, phorbol myristate acetate; Pun, punicalagin.

Punicalagin downregulates the NF-κB, but not mTOR signaling pathway in osteosarcoma cell lines. AKT signaling regulates cell survival. As one of the main downstream mediators, mTOR signaling is essential for cell proliferation, and suppressed mTOR signaling is associated

with apoptosis induction by various stimuli, delayed cell cycle progression and cell proliferation (11). The levels of phospho-mTOR/mTOR expression in osteosarcoma cells lysates were not altered by punicalagin treatment, but compared with those of the control group the levels of

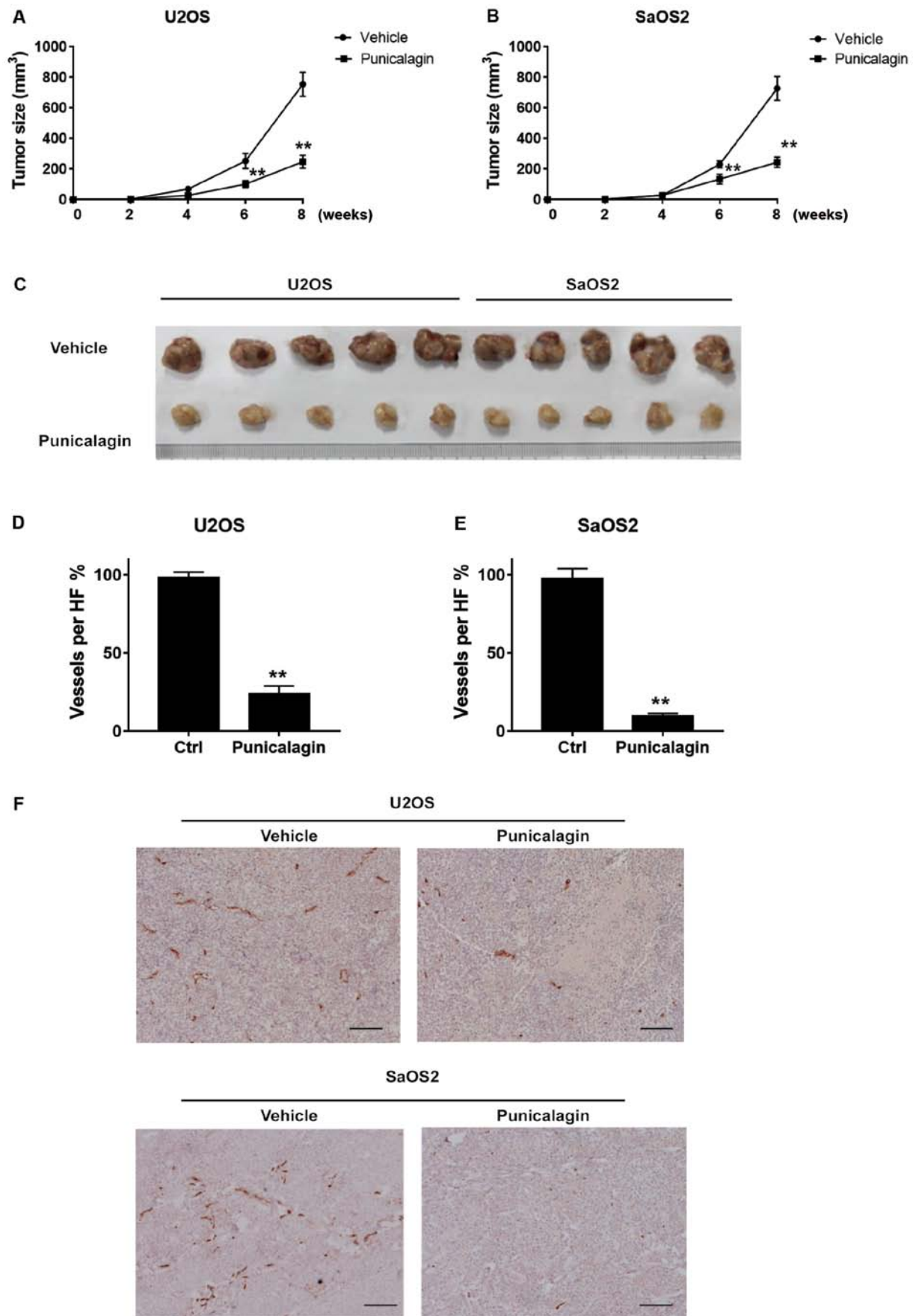


Figure 5. Punicalagin attenuates the malignant growth of osteosarcoma cells in mice. (A and B) Xenografted osteosarcoma growth curves of (A) U2OS and (B) SaOS2 were performed according to the xenograft tumor volume. (C) The representative osteosarcoma tissues after resection in mice xenograft models were captured. (D and E) Quantification of the angiogenesis results in xenografted osteosarcoma of (D) U2OS and (E) SaOS2 cells. (F) Punicalagin inhibits tumor angiogenesis represented by decreased blood vessel staining. ** $P < 0.01$ vs. vehicle group. Blood vessels are indicated by CD31 expression stained brown. Scale bar, 10 μ m. Ctrl, control.

pIkB α /IkB α , p65, IL-6, IL-8 were significantly reduced by punicalagin treatment (Fig. 4).

NF- κ B is a key regulator of inflammatory immune responses, including cytokine production (12). During tumorigenesis, these cytokine-associated chemotactic effects are required for the initiation of tumor-associated inflammation and neovascularization (13). Osteosarcoma cells were treated with saline (vehicle) or 100 μ M punicalagin alone for 48 h, following which, changes in the activated levels of NF- κ B represented by pIkB α /IkB α and p65, and its downstream inflammatory factors, including IL-6, IL-8 were examined (Fig. 4). Punicalagin affected the stable expression of NF- κ B in U2OS and SaOS2 cells. The expression levels of phosphor-IkB α , nuclear p65 and IL-6, IL-8 significantly decreased in U2OS and SaOS2 cells compared with untreated cells. To further evaluate the effect of punicalagin on the NF- κ B pathway, 200 nM PMA, an activator of the NF- κ B pathway, was added and cells were incubated for 45 min. Following the addition of PMA, the downregulation of IL-6 and IL-8 levels observed in punicalagin pre-treated osteosarcoma cells was reversed.

Punicalagin attenuates proliferation and angiogenesis of osteosarcoma cells in a murine tumor xenograft model. Proliferation and migration of osteosarcoma cells were significantly attenuated in a xenograft model in mice. Tumors typically had a length of 11-18 mm and a width of 8-12 mm in the vehicle group. By contrast, punicalagin treatment significantly decreased tumor growth, to a length of 7-11 mm and width of 6-8 mm (Fig. 5A-C). Thus, punicalagin injection resulted in slower malignant growth of human osteosarcoma cells in the mouse model *in vivo*. Staining of blood vessels with CD31 antibodies was then used to evaluate angiogenesis in malignant tissues of xenograft mice. CD31 staining represented the wall of the blood vessel of osteosarcoma core sections that were used in a rat subcutaneous model, as described in a previous study (14). The mean density of blood vessels in the punicalagin-treated was significantly reduced, compared with that in the vehicle group, which suggested that that punicalagin could inhibit tumor angiogenesis (Fig. 5D-F).

Discussion

The present study suggested that punicalagin treatment in osteosarcoma cells significantly decreased tumor cell viability and induced cell apoptosis. Punicalagin can inhibit proliferation and survival of osteosarcoma cells in a concentration- and time-dependent manner. These results were also replicated in a xenograft model, in which impaired angiogenesis was also observed following injection of punicalagin. The molecular mechanisms were further investigated using biological methods, which demonstrated that the therapeutic effects of punicalagin were associated with downregulation of NF- κ B but not mTOR signaling.

Osteosarcoma is the most commonly diagnosed primary solid bone malignancy. Metastasis is the main cause of death in patients with osteosarcoma, and treatment options remain unsatisfactory. The incidence of osteosarcoma in the general population is 2-3 per million per year. However, annual incidence is around 1.2-7.6 per million per year in people younger than 24 years of age worldwide (15). Although major efforts

have been made to establish the potential pathognomonic driver mutations in young patients, only sporadic mutations have detected in great majority of cases. Similar to the majority of common types of human cancer, osteosarcoma exhibits a high degree of mutational diversity. This diversity is driven by complex rearrangement processes, chromothripsis and chromothripsic amplification that predominate in osteosarcoma, although the underlying causes remain unknown (16). Currently, early detection, quick confirmation or targeted therapeutic strategies by molecular biology are not available in clinical practice. Therefore, the present study aimed to investigate the efficacy of a potential anti-cancer compound, punicalagin, in osteosarcoma cells.

Although they represent the main available treatment in osteosarcoma, chemotherapeutics are also toxic to normal tissue, and can lead to myelosuppression, opportunistic infection, heart damage and other adverse reactions, thereby decreasing the patient survival rate and quality of life (17). Therefore, new agents with fewer side effects and improved therapeutic advantages are required. Block *et al* (18) suggested that a phytochemical-rich diet, which includes compounds such as polyphenols, salicylates, phytosterols, saponins, glucosinolates, protease inhibitors, monoterpenes, terpenes, lectins, was associated with decreased risk of cancer. Punicalagin is one of the most abundant polyphenols in pomegranate. In addition, increasing evidence suggests that punicalagin inhibits tumor invasion and metastasis of various types of cancer, such as cervical (19), ovarian (20), colon (21) and lung cancer (22) as well as antioxidants in chronic inflammation (23). To the best of our knowledge, the present study was the first to identify that punicalagin could significantly inhibit osteosarcoma cell proliferation and invasion, induce apoptosis, and decrease angiogenesis. Thus, the results of the present study may provide insight into future therapeutic strategies against osteosarcoma.

However, as previously shown in both rats and humans, the poor bioavailability of punicalagin represents a considerable limitation to pharmaceutical research on its potential therapeutic effects *in vivo* (24). The low bioavailability of ellagic acid generated from punicalagin is due to its hydrophilic structure and large molecular weight, which limits its absorption by simple diffusion, including oral administration (25). In addition, extremely low lipid solubility further restricts its permeability through the lipophilic layer of the gastrointestinal tract (26). Furthermore, punicalagin can be metabolized into the bioavailable but relatively poor antioxidant, hydroxy-6H-dibenzopyran-6-one derivatives by the colonic microflora in healthy humans (27). Based on animal studies, the serum concentration after absorption of punicalagin in rodents was ~ 30 μ M (28), which is lower than the concentration used *in vitro* in the present study. Thus, the multifaceted therapeutic benefits of punicalagin observed in the present study may be difficult to fully replicate in patients. Furthermore, a relatively high concentration of punicalagin may result in non-specific effects due to the biological differences between osteoblasts and osteosarcoma cells. However, the development of novel punicalagin derivatives, compound preparation and administration methods may overcome these limitations in the future.

The precise mechanisms through which punicalagin inhibits osteosarcoma invasion and angiogenesis, as well

as its regulation, are not well understood. Several previous studies demonstrated that multiple signaling pathways, including the MAPK (29), β -catenin (19), TGF- β 1 (30), AKT, and JNK (31) pathways, were modulated by punicalagin administration. Furthermore, Adams *et al* (32) suggested that punicalagin decreased phosphorylation of the p65 subunit and binding of NF- κ B about 3.6-fold in colon cancer. In nerve cells, chronic neuroinflammation and oxidative stress were dramatically diminished by punicalagin via NF- κ B inhibition (33). Furthermore, vascular endothelial growth factor, an NF- κ B transcriptional target gene, was downregulated by punicalagin, thereby decreasing angiogenesis in the tumor environment (34,35). The *in vitro* and *in vivo* results of the present study were consistent with previous reports, and demonstrated the therapeutic potential of punicalagin against mesodermal illness like osteosarcoma, through modulation of NF- κ B activity.

In general, NF- κ B signaling controls many cellular processes, including immune responses, immune cell proliferation and viability, lymphogenesis and B cell maturation (36). The NF- κ B pathway is also involved in the regulation of skeletal muscle cell differentiation (37). Recently, activation of NF- κ B was demonstrated to increase glucose uptake and glycolytic flux in sarcoma cells, which suggested that NF- κ B played a crucial role in the development of osteosarcoma malignancies (38). Consistently, Gong *et al* (39) found at least 75% osteosarcoma tissues from patients showed positive stain of activated NF- κ B pathways and patients whose osteosarcoma with active NF- κ B had short median overall survival time as compared with patients whose osteosarcoma had inactive NF- κ B. Expression of metastasis-associated proteins, angiogenesis, cell invasion and metastasis have also been linked to NF- κ B activation in osteosarcoma (8). Liao *et al* (40) used short hairpin RNA to knockdown NF- κ B expression, which abolished cell invasion and metastasis in osteosarcoma. In another previous study, the NF- κ B inhibitor QNZ suppressed NF- κ B activation, which resulted in downregulation of proteins associated with metastasis, cell migration and cell invasion in osteosarcoma cells (41). The present study further demonstrated that NF- κ B is an important transcription factor during pathogenesis of osteosarcoma, and that punicalagin was involved in modulating the expression of molecules downstream of NF- κ B, such as IL-6, and IL-8.

A previous study demonstrated that IL-6 and IL-8 genes were directly regulated by the NF- κ B pathway (42) and that IL-6 and IL-8 levels increased with NF- κ B overexpression during chronic inflammation in bone and joint tissues (43). IL-6 and IL-8 activation promotes an inflammatory micro-environment during malignant progression (44) and these cytokine-associated chemotactic effects are required for the initiation of tumor-associated inflammation and neovascularization (45). The present study indicated that punicalagin decreased IL-6 as well as IL-8 production by osteosarcoma cells, which was consistent with angiogenesis inhibition in xenograft models. Thus, these findings further elucidate the mechanisms underlying the preventive and therapeutic potential of punicalagin against osteosarcoma.

Although a previous study suggested that pomegranate extract, including a large amount of active punicalagin, had a

strong anti-aging effect through the mTOR pathway (46), the present study failed to confirm this finding. Thus, the present results highlight the significance of punicalagin as a promising tumor suppressor in osteosarcoma by targeting NF- κ B, but not mTOR pathway. Further characterization of this compound will provide a new insight into punicalagin-mediated suppression of osteosarcoma genesis and development.

In conclusion, punicalagin treatment inhibited osteosarcoma growth, including proliferation, invasion and angiogenesis through NF- κ B suppression. Further in-depth and long-term studies are required in order to establish the therapeutic target of the NF- κ B signaling pathway in punicalagin-induced cell survival and inhibition of invasive abilities, as well as excessive angiogenesis. Nonetheless, the results of our present study provide preliminary evidence to support punicalagin, a phytochemical used in herbal medicine, as a novel and effective candidate for the systemic treatment and/or chemoenhancement of osteosarcoma.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HW designed and directed the study, and analyzed and interpreted the data. TH performed the experiments and wrote the manuscript. XZ performed the literature search, analyzed the data and designed the figures. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Animal Ethics Committee of The Second Affiliated Hospital of Air Force Medical University (approval no. 201904-11).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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