Wnt/β-catenin signaling modulates piperine-mediated antitumor effects on human osteosarcoma cells

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Abstract. The plant extract piperine is used as a traditional Chinese medicine due to its anti-inflammatory effects and efficacy against numerous types of cancer. The aim of the present study was to investigate the antitumor mechanism of piperine in human osteosarcoma U2OS and 143B cell lines. The effects of piperine on cell apoptosis and invasion of human osteosarcoma cells were assessed using flow cytometry and Transwell assays. Moreover, western blotting was used to measure the effects of piperine on the protein expression levels of the metastasis markers matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF). In addition, the involvement of the Wnt/β-catenin signaling pathway in modulating the effects of piperine was examined via western blot analysis. The results of MTT and Transwell invasion assays indicated that piperine treatment dose-dependently reduced U2OS and 143B cell viability and invasion. Furthermore, a significant reduction was identified in MMP-2, VEGF, glycogen synthase kinase-3β, and β-catenin protein expression levels, as well as the expression levels of their target proteins cyclooxygenase-2, cyclin D1, and c-myc, in U2OS cells after piperine treatment. In addition, similar results were observed in 143B cells. Therefore, the present study demonstrated the efficacy of piperine in osteosarcoma, and identified that the Wnt/β-catenin signaling pathway may modulate the antitumor effects of piperine on human U2OS and 143B cells.

Introduction

Osteosarcoma has an annual incidence rate of 5/1,000,000 individuals and a mortality rate of >50% in patients <20 years old worldwide (1-4). Surgery combined with radiotherapy and chemotherapy is the primary treatment method for osteosarcoma (5). However, ≤80% of patients have poor prognosis due to metastatic lesions that are already present at the time of diagnosis, and the 5-year survival rate is ~30% (6). Given this poor disease prognosis, the development of effective therapeutic strategies and pharmaceuticals to reduce osteosarcoma malignancy are important.

Piperine is an alkaloid found in Piper nigrum Linn and Piper longum Linn (Piper nigrum L., family piperaceae). Piperine is used as a food flavoring and as a traditional Chinese medicine due to its pharmacological benefits (7,8). Moreover, piperine is used to treat gastrointestinal disorders such as constipation and diarrhea (9). Furthermore, it has well-characterized anti-inflammatory (10) and antitumor effects in numerous types of cancer, including breast, lung and liver cancer, and lymphoma (11-14). Piperine has been reported to dose-dependently (15-20) regulate cell growth and differentiation via the Akt/JNK/MAPK pathway (21), and can increase cytokine production via the mTOR signaling pathway (22).

Tumor metastasis is a complex process involving tumor cell dissociation, extracellular matrix degradation, infiltration and adhesion to vascular endothelial cells (23). Notably, matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, and collagen type IV are significantly upregulated in osteosarcoma and metastases, and are indices of poor prognosis (24). Moreover, MMP-2 downregulation can inhibit osteosarcoma metastasis and infiltration (21,25). Vascular endothelial growth factor (VEGF) is known to promote angiogenesis, and its upregulation is correlated with poor osteosarcoma prognosis (26,27). Furthermore, VEGF downregulation has been shown to reduce vascular density and inhibit metastases in osteosarcoma (28).

While the antitumor effect of piperine on U2OS cells has been reported (21), its underlying molecular mechanisms of action are not fully understood. As the Wnt/β-catenin signaling pathway is known to regulate cell proliferation and differentiation (29,30), the present study hypothesized that it may be involved in modulating the antitumor effects of
piperine. Therefore, the aim of the present study was to test this hypothesis; the results may provide a novel insight into the antitumor mechanism of piperine.

Materials and methods

Chemical reagents. DMSO and MTT were purchased from Sigma-Aldrich (Merck KGaA). Piperine (molecular weight, 285.35 kDa; National Institutes for Food and Drug Control) was dissolved in DMSO at the concentration of 150 µM and stored at -20°C. An Annexin V-FITC/PI double staining cell apoptosis detection kit was obtained from Nanjing KeyGen Biotech Co., Ltd. NQBB FBS was obtained from Wuhan ChunjDuBio Co., Ltd. Anti-MMP-2 (cat. no. 10373-2-AP; 1:1,000) was purchased from ProteinTech Group, Inc. Anti-VEGF (cat. no. GB11034; 1:3,000), anti-c-Myc (cat. no. GB13076; 1:500), anti-cyclin D1 (cat. no. GB11079; 1:1,000), anti-cyclooxygenase-2 (COX2; cat. no. GB11072; 1:500), anti-β-catenin (cat. no. GB11015; 1:500) and anti-glycogen synthase kinase-3β (GSK-3β; cat. no. GB11099; 1:1,000) were purchased from Wuhan Servicebio Technology Co., Ltd.

Cell culture. Human osteosarcoma U2OS and 143B cells were provided by Cheeloo College of Medicine, Shandong University. 143B cells were identified by STR from Shanghai Cinoa Institute, and the results showed that the cells were not contaminated, had homology with HOS/Cinoasia Institute, and the results showed that the cells were not contaminated, had homology with HOS/KHOS-240s cells and were human osteosarcoma cells. The results showed that the cells were not contaminated, had homology with HOS/KHOS-240s cells and were human osteosarcoma cells. The results showed that the cells were not contaminated, had homology with HOS/KHOS-240s cells and were human osteosarcoma cells. The cells were cultured in McCoy's 5A medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS at 37°C in a 5% CO2 humidified incubator until cells reached the logarithmic growth phase; cells were then harvested for subsequent experiments.

MTT cell viability assay. U2OS cells (4x10³ cells/well) and 143B cells (1x10³ cells/well) were seeded in 96-well plates and incubated at 37°C in a 5% CO2 humidified incubator with different piperine concentrations (0, 50, 100 and 150 µM) for 24, 48 and 72 h. Subsequently, cell viability was determined using an MTT assay (Cell Titer 96AQ; Promega Corporation). The supernatant was aspirated and 0.05% DMSO (150 µl) was added to each well, and then shake at a low speed (3.2 g) for 10 min to fully dissolve the formazan. The optical density (OD) values of piperine-treated cells were measured at 490 nm using an ELISA microplate reader (Rt2100c; Rayto Life and Analytical Sciences Co., Ltd.). Inhibition rate %=(1-OD value of experimental group/OD value of 0 µM group) x 100%.

Flow cytometry. U2OS cells (5.0x10³ cells/well) and 143B cells (1.0x10³ cells/well) were seeded in 6-well plates and incubated at 37°C in 5% CO2 with different concentrations of piperine (0, 50, 100 and 150 µM) for 48 h. Cells were then harvested and 3 ml pre-chilled PBS was added at 4°C, which were centrifugated at 337 x g for 5 min at room temperature and 200 µl binding buffer was then used to suspend the supernatant. Next, double fluorescence staining was performed with 20 µg/ml PI and 5 µl Annexin V-FITC for 15 min at room temperature before analysis with a flow cytometer (Beckman CytoFLEX; Beckman Coulter, Inc.) FlowJo software version 10.0.7 (Stanford University). Annexin V-FITC (green) and PI (red) fluorescence intensities were detected using the FITC channel (FL1) and PI channel (FL2), respectively. Total apoptotic rate was calculated as follows: Total apoptotic rate=early apoptotic rate + late apoptotic rate.

Transwell invasion assay. A Matrigel Transwell 24 holes with an aperture of 3.0 µm (Corning, Inc.) assay was performed to assess cell invasion. Matrigel matrix (50 mg/l; BD Biosciences) was diluted in serum-free medium at a 1:3 ratio to reconstitute the basement membrane. U2OS and 143B cells (5x10^5 cells/ml) were separately added to the upper Transwell chamber, and 10% FBS-containing culture medium containing different concentrations (50-150 µM) of piperine was added to the lower chamber. After incubation for 48 h at 37°C in 5% CO2, the cells in the upper chamber were fixed with 75% ethanol at room temperature for 10 min, stained with 0.1% crystal violet for 5 min at 37°C and examined using light microscopy (Olympus Corporation) at x400 magnification. In total, five random visual fields were used to count the total number of migrating cells, and the images were analyzed using Image-Pro Plus 6.0 soft (Media Cybernetics, Inc.).

Western blot analysis. U2OS and 143B cells were treated with different concentrations of piperine (0, 50, 100 and 150 µM) for 48 h prior to protein extraction at 37°C. After the cells were harvested and washed with RIPA buffer (Wuhan Servicebio Technology Co., Ltd.; cat. no. G2002) total protein from cells was extracted using a protein extraction kit (cat. no. KGBSP002; Nanjing KeyGen Biotech Co., Ltd.). Protein concentration was measured by bichinchonic acid protein kit (Wuhan Servicebio Technology Co., Ltd.) Proteins (20 µg) were then separated by SDS-PAGE with 10% gels. The separated protein bands were transferred onto nitrocellulose membranes blocked with 5% skimmed milk/TBST(0.1% Tween-20) at 37°C for 60 min, which were probed with the respective primary antibodies [Anti-MMP-2 (1:1,000; ProteinTech Group, Inc.) Anti-VEGF (1:3,000), anti-c-Myc (1:500), anti-cyclin D1 (1:1,000), anti-cyclooxygenase-2 (COX2; 1:500), anti-β-catenin (1:500) and anti-glycogen synthase kinase-3β (GSK-3β; 1:1,000; Wuhan Servicebio Technology Co., Ltd.)] overnight at 4°C. Subsequently, the membranes were incubated with a corresponding horseradish peroxidase-labeled secondary antibody (1:3,000; cat. no. 23301; Wuhan Servicebio Technology Co., Ltd.) for 30 min at room temperature, before being developed with an enhanced chemiluminescence reagent (Wuhan Servicebio Technology Co., Ltd.). Protein bands were analyzed using AlphaEaseFC software (ver 4.0.0; Alpha Innotech).

Statistical analysis. Statistical analyses were performed using SPSS version 16.0 software (SPSS, Inc.). Data from ≤3 independent experiments are presented as the mean ± SD. A one-way ANOVA followed by Bonferroni post-hoc analysis was performed to compare groups. *P<0.05 was considered to indicate a statistically significant difference.

Results

Piperine inhibits cell proliferation and induces apoptosis. MTT assay results indicated that piperine significantly inhibited U2OS and 143B cell proliferation (P<0.05; Fig. 1). It was revealed that cell density was reduced in the presence...
of piperine compared with untreated cells, with the lowest OD$_{490}$ obtained for cells treated for 24 h with 150 µM piperine (P<0.01; Fig. 1A and C). Moreover, the strongest growth inhibition was observed in response to 150 µM piperine for 72 h (35% for U2OS and 35.7% for 143B; P<0.01; Fig. 1B and D). In U2OS cells, the apoptotic rate was positively associated with piperine concentration (Fig. 2). The apoptotic rate in the absence of piperine was 1.89%, whereas the apoptotic rates increased to 2.12, 4.63 and 19.1%, in the presence of 50, 100 and 150 µM piperine, respectively (P<0.05; Fig. 2A). For 143B cell the apoptotic rates was 10.29, 13.28, 24.85 and 36.7% in the presence of 0, 50, 100 and 150 µM piperine, respectively (P<0.05; Fig. 2B).

Piperine inhibits cell invasion by downregulating MMP-2 and VEGF protein expression. The present results suggested that osteosarcoma cells treated with 50, 100 and 150 µM piperine exhibited reduced cell(U2OS: Number of invading cells 266, 167 and 20 per field; 143B: 246, 128 and 45 per field, respectively; P<0.01; Fig. 3; Table I). Furthermore, western blotting results demonstrated a dose-dependent decrease in MMP-2 and VEGF protein expression levels in the presence of piperine, with the most significant reduction observed at 150 µM piperine for both proteins. However, with increasing concentration of piperine, U2OS had a more significant effect on MMP-2 and VEGF compared with 143B cells (Fig. 4).

Table I. Data from the Transwell assay

| Treatment group, µM | U2OS    | 143B   |
|---------------------|---------|--------|
| 0                   | 365.6±23.8 | 320.3±30.0 |
| 50                  | 266.2±39.7 | 246.9±48.3 |
| 100                 | 167.2±20.6 | 128.5±37.7 |
| 150                 | 20.4±4.8  | 45.8±10.2 |

Data are presented as the mean ± SD.
Figure 2. Piperine induces U2OS and 143B cells apoptosis. Flow cytometry profiles of Annexin V FITC/PI double stained (A) U2OS and (B) 143B cells after 48 h piperine treatment at 0µM, 50µM,100µM and 150 µM. C and D represent the apoptosis rate of U2OS and 143B cells after treatment with different concentrations of piperine for 48 h. **P<0.01 vs. Control (0 µM).

Figure 3. Transwell assay results showing piperine-mediated reduction in cell invasion. Images of (A-D) U2OS and (F-I) 143B cells treated with (A and F) 0, (B and G) 50, (C and H) 100 and (D and I) 150 µM piperine for 48 h. Magnification, x100. (E and J) Semi-quantitative profile of microscopy images of piperine-treated U2OS cells. *P<0.05, **P<0.01 vs. 0 µM.
Figure 4. Piperine downregulates VEGF and MMP-2 protein expression levels in U2OS and 143B cells. Western blot analysis of VEGF and MMP-2 protein expression levels in (A) U2OS and (C) 143B cells treated with 0, 50, 100 and 150 µM piperine. Actin was used as the loading control. (B and D) Semi-quantitative profiles of western blotting data of VEGF and MMP-2 at the four tested piperine concentrations. *P<0.05, **P<0.01 vs. 0 µM. MMP, matrix metalloproteinases; VEGF, vascular endothelial growth factor.

Figure 5. Effect of piperine treatment on the Wnt/β-catenin signaling pathway in U2OS and 143B cells. Western blot analysis of GSK-3β and β-catenin expression levels, and their target genes COX-2, cyclin D1 and c-myc after 48 h piperine treatment in (A) U2OS and (C) 143B cells. Actin was used as the loading control. (B and D) Semi-quantitative profile of the western blotting data. *P<0.05, **P<0.01 vs. 0 µM. COX-2, cyclooxygenase-2; GSK-3β, glycogen synthase kinase 3β.
the lowest protein expression levels were observed following treatment with 150 µM piperine (P<0.05; Fig. 5). In addition, reduced expression levels of the target proteins COX-2, cyclin D1 and c-myc were observed with increasing piperine concentrations (P<0.01; Fig. 5). In 143B cells, it was demonstrated that β-catenin protein expression was significantly reduced, and the expression levels of COX-2 and c-myc were decreased with increasing piperine concentrations.

**Discussion**

The present results suggested that piperine treatment increased apoptosis, and reduced cell invasion and proliferation of U2OS and 143B cells. Notably, cell proliferation was most significantly inhibited in response to treatment with the highest concentration of piperine (150 µM) for the longest treatment duration (72 h). Furthermore, the dose-dependent decrease observed in the protein expression levels of metastatic markers in the presence of piperine were in line with previous studies on the roles of these proteins in tumor metastasis and heterotopic angiogenesis (8,14,18). Therefore, the present results provided evidence on the role of piperine in inhibiting osteosarcoma cell proliferation and metastasis (21). In addition, the present western blotting results identified that the Wnt/β-catenin signaling pathway, which has a role in regulating osteosarcoma cell proliferation and apoptosis (31-34), may regulate piperine-mediated osteosarcoma apoptosis.

The core mediator of the Wnt/β-catenin signaling pathway, β-catenin, is regulated by phosphorylation, which is promoted by GSK-3β, and degradation via the ubiquitin/proteasome pathway (26). β-catenin mutations can cause abnormal activation of Wnt target genes, thus leading to cancer development (35). In addition, dysregulation of the Wnt signaling pathway can affect downstream gene expression (36). The present results suggested that piperine dose-dependently downregulated the protein expression levels of GSK-3β and β-catenin, as well as those of the downstream oncogenic proteins cyclin D1, c-Myc and COX-2. Therefore, piperine may effectively inhibit U2OS and 143B cell proliferation and metastasis, and ectopic angiogenesis via regulation of the Wnt/β-catenin signaling pathway. The PI3K/Akt pathway is frequently hyperactivated in osteosarcoma, and contributes to disease initiation and development (37). Moreover, inhibition of this pathway via small compounds is a potential therapeutic approach for osteosarcoma (38). Previous studies have shown that Wilms' tumor 1 knockdown in MG-63 cells results in deregulation of cell cycle proteins and downregulation of the PI3K/AKT pathway (39,40). Piperine has previously been reported to inhibit cell proliferation and metastasis of U2OS cells (21), and affect several signaling pathways, such as the Akt/MAPK and mTOR pathways (21,22,41), in several cancer cell types. However, to the best of our knowledge, the present study is the first to demonstrate that the Wnt/β-catenin signaling pathway may mediate the antitumor effects of piperine.

In conclusion, the present results suggested that the Wnt/β-catenin signaling pathway underlies the antitumor effects of piperine on U2OS and 143B cell proliferation, apoptosis and invasion. However, further research is needed to investigate the anti-osteosarcoma mechanism of piperine.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

LN contributed to the conception of the study. YBQ performed the experiments, data analysis and wrote the manuscript. WY and MS contributed to data interpretation and analyses. All authors have read and approved the manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.
