

Original Article

Icariin inhibits growth of human osteosarcoma cells by inducing apoptosis via downregulation of β -catenin signaling

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Abstract: Osteosarcoma is the most prevalent primary malignant bone tumor mainly endangering young adults. Icariin, derived from *Herba Epimedii*, has been shown to possess an anti-tumor effect in various cancers. However, whether icariin is effective for osteosarcoma has not been acknowledged. Our study aimed to investigate the anti-tumor effect of icariin and the potential mechanism on human osteosarcoma cell lines. In present study, icariin dose-dependently inhibited proliferation and promoted apoptosis in osteosarcoma cells, examined by MTT assay and Annexin V-FITC apoptosis detection. Moreover, we found icariin induced G0/G1 phase arrest and decreased the expression of cyclin D and p-RB, increased expression of Bax and attenuated expression of Bcl-2, and consequently augmented caspase-3 activity. Further, icariin dose-dependently inhibited β -catenin expression and activity, while overexpression of β -catenin signaling by adenoviruses system could abrogate the anti-tumor effect of icariin. Our finding indicated that Icariin could inhibit the proliferation by inhibiting the β -catenin signaling and induces apoptosis via upregulation the ratio of Bax/Bcl-2 in human osteosarcoma cells. Icariin is a promising agent candidate for osteosarcoma that deserves more attention.

Keywords: Osteosarcoma, icariin, β -catenin, apoptosis, proliferation

Introduction

Osteosarcoma is the most prevalent primary malignant bone tumor in young adult, with an incidence of 8.7 per million in children and adolescent [1]. Nowadays, the standard chemotherapeutic drugs, including doxorubicin, cisplatin, ifosfamide, etoposide and methotrexate, are reported to be tumor resistance [2] and associated with a variety of serious toxicities [3]. Moreover, it has suggested that the improvement of osteosarcoma treatment cannot be achieved simply by increasing the dose of chemotherapeutic drugs [4]. Therefore, based on the defect of current chemotherapeutic drugs, it is of great importance to develop new chemotherapeutic drugs.

Nature products derived from plant, such as vinblastine, camptothecin, paclitaxel, epipodo-

phyllotoxin, etc., play a non-substitutable role in the development for modern medicine. Icariin (C₃₃H₄₀O₁₅; molecular weight, 676.67, **Figure 1A**), a prenylated flavonol glycoside derived from *Herba Epimedii*, is considered to be the main active component contributed to the therapeutic effect of the herb. It has been revealed that icariin exhibited various biological effects, including anti-inflammatory [5, 6], cardiovascular protective [7], osteogenesis [8], estrogen-like activities [9] and immunomodulatory activities [10]. Additionally, increasing number of studies has demonstrated that icariin possesses anti-tumor effects. It has been confirmed that icariin is associated with significant antiproliferative effect on human tumor cell including gastric cancer, hepatocellular carcinoma, leukaemia cells, and gallbladder cancer [11-15]. However it has proliferative effect on

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MCF-7 breast cancer cell in vivo for its estrogen-like activities [16].

Icariin is effective on various cancers, though the anti-tumor effect of icariin on osteosarcoma remains unclear. Given the anti-tumor effect of icariin and dismal chemotherapeutic agents for osteosarcoma, we conduct the current study to evaluate the anti-tumor effect of icariin on osteosarcoma and characterize the underlying molecular mechanisms by using two human osteosarcoma cells.

Materials and methods

Chemical and reagents

Icariin (C₃₃H₄₀O₁₅, MW: 676.67, purity ≥ 98%) was purchased from Aladdin Co. Ltd. (Shanghai, China). Bicinchoninic acid (BCA) protein assay kit and was obtained from Pierce (Rockford, IL, USA). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (CA, USA). Monoclonal antibodies anti-β-actin, anti-Bax, anti-Bcl-2, anti-pRB, anti-cyclin D1, anti-cyclin E were purchased from Abcam (Cambridge, UK). Dimethyl sulfoxide (DMSO), propidium iodide (PI), and MTT were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents obtained from local area were of the highest analytical grade available.

Cell culture and treatment

Osteosarcoma cells 143B and MG63 were obtained from American Type Culture Collection (ATCC, Rockville, MD), and cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were routinely incubated in an atmosphere of 5% CO₂ at 37°C. All cell experiments were done using cells in exponential cell growth. Incubated for twenty-four hours after seeding, cells were treated with culture medium containing various concentrations of icariin (10⁻⁴, 10⁻⁵, and 10⁻⁶ M).

MTT assay for proliferation assay

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) Assay was performed to evaluate the proliferation of osteosarcoma cells. MTT assay is a rapid and sensitive procedure for assessing cellular toxicity of compounds *in-vitro*. Cells were seeded into 96-well plates at a concentration of 10⁵/ml and plates

were sealed and cultured for 24 hours before treatment of icariin. Cells were incubated for 48 hours after treatment. Following incubation, 20 μl of MTT was added to each well, and the cells were incubated for an additional four hours. Subsequently, media was carefully discarded and 100 μl of dimethyl sulfoxide were added to dissolve the formazan crystals, then the 96-well plates were put on a horizontal oscillator for ten minutes. The absorbance values were measured with the plate reader at a wavelength of 570 nm. Each experiment was conducted in triplicate, and the data are presented as mean values.

FACS assay of apoptosis

For apoptosis analysis, Annexin V-FITC/propidium iodide (PI) staining using an Annexin V-FITC apoptosis detection kit (KeyGEN Biotech, Nanjing, China) was performed by the flow cytometry according to the manufacturer's guidelines. Briefly, after 48 h of icariin treatment, the cells were washed with cold phosphate buffered saline (PBS) × 2, incubated with Annexin V-FITC/PI at room temperature for 5 min in the dark. The fluorescence of the cells was detected by the flow cytometry using a FITC signal detector (FL1) and a PI signal detector (FL2). According to the method, Annexin V-FITC (-)/PI (-) indicates survived cells, Annexin V-FITC (+)/PI (-) indicates cells of apoptosis in the early stage, and Annexin V-FITC (+)/PI (+) indicates cells of apoptosis or necrosis in the late stage. Each experiment was performed in triplicate and reproducible results were achieved.

Flow cytometry assay for cell cycle analysis

Cell cycle analysis was performed by detecting DNA content with propidium iodide (PI) staining. Briefly, the cells were incubated for 48 h before treating with different concentrations of icariin for 48 h. At the end of treatment, cells were harvested, washed twice with ice-cold PBS and fixed with 70% ethanol overnight at 4°C. Cells were washed twice with ice-cold PBS, resuspended in 1 ml PBS containing 50 μg propidium iodide, 200 μg RNase A and 0.1% Triton X-100, and incubated for 30 min at 37°C in the dark. The cell-cycle profiles were determined by flow cytometry and data were analyzed using Cell Quest Software (BD Biosciences, San Jose, CA).

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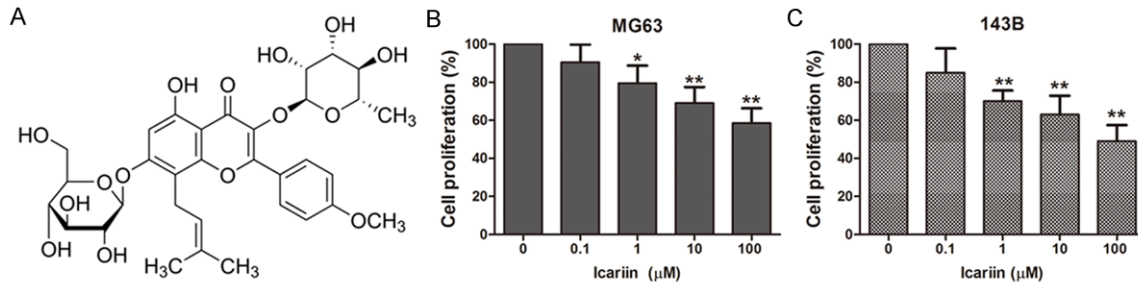


Figure 1. Effect of icariin on the proliferation in human osteosarcoma cells. A. The chemical structure of icariin. B and C. The human osteosarcoma cells MG63 and 143B were pretreated with different concentrations of icariin (0.1, 1, 10, or 100 μM) for 48 h. Cell viability was measured by MTT assay. Values are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$ compared with the control group.

Caspase-3 activity assay

The activity of caspase-3 was detected in vitro using a caspase-3 colorimetric assay kit (KeyGEN, Nanjing, China) according to the manufacturer's instructions. In short, following the treatment, the collected cells were lysed and centrifuged at 12000×g for 15 minutes at 4°C. The supernatant were collected and the protein concentrations were measured by the Bradford method. Then supernatant containing 50 μg of total protein were incubated with 5 l caspase substrate at 37°C for 4 h in the dark. The optical density (OD) of the caspase substrate was determined by a microplate reader at 405 nm, and the caspase 3 activity was calculated as a percentage of OD in icariin treatment cells relative to the control that were not treated with the icariin.

Quantitative Real Time PCR

Total RNA was extracted from the cells using Trizol (Invitrogen, USA) according to the manufacturer's protocol. The primer sequences were as follows: β-catenin (Forward: 5'-ATGGAGCCGGACAGAAAAGC-3'; Reverse: 5'-CTTGCCACTCAGGGAAGGA-3'), GAPDH (Forward: 5'-TGTTGCATCAATGACCCCTT-3'; Reverse: 5'-CTCCACGACGTA CTCAGCG-3'). Real-time PCR was carried out using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, USA) according to the manufacturer's instructions. The Real Time PCR System was employed for the thermal cycling reactions. After the normalizing with GAPDH, relative change in gene expression of β-catenin was determined by the comparative Ct method ($2^{-\Delta\Delta C_t}$ method).

Protein isolation and western blot

Cells were washed with PBS and lysed in cell lysis buffer. The lysate was centrifuged at 12000 g at 4°C for 10 min. The supernatant was collected and protein concentration was determined by BCA method. 40 μg total protein from each treated cell group was fractionated on 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel and then transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% (w/v) fat-free milk in Tris-buffered saline (TBS) containing 0.05% Tween-20, followed by incubation with a rabbit primary polyclonal anti-body at 4°C overnight. Then after washing with TBST for three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Antibody binding was visualized using enhanced ECL chemiluminescence system and short exposure of the membrane to X-ray films (Kodak, Japan).

Luciferase reporter assay

To investigate the activation of β-catenin signaling pathway modulated by icariin, TOPFlash (4 × TCF binding sites) luciferase reporter was used as reported previously. Luciferase activities were analyzed by the dual-luciferase reporter assay system (Promega, Madison, WI) with normalization to the control *Renilla*.

Adenovirus infection

Recombinant adenoviruses expressing β-catenin was used according to procedure described previous [17]. Additionally, the expression of GFP was as a marker for monitoring transfection.

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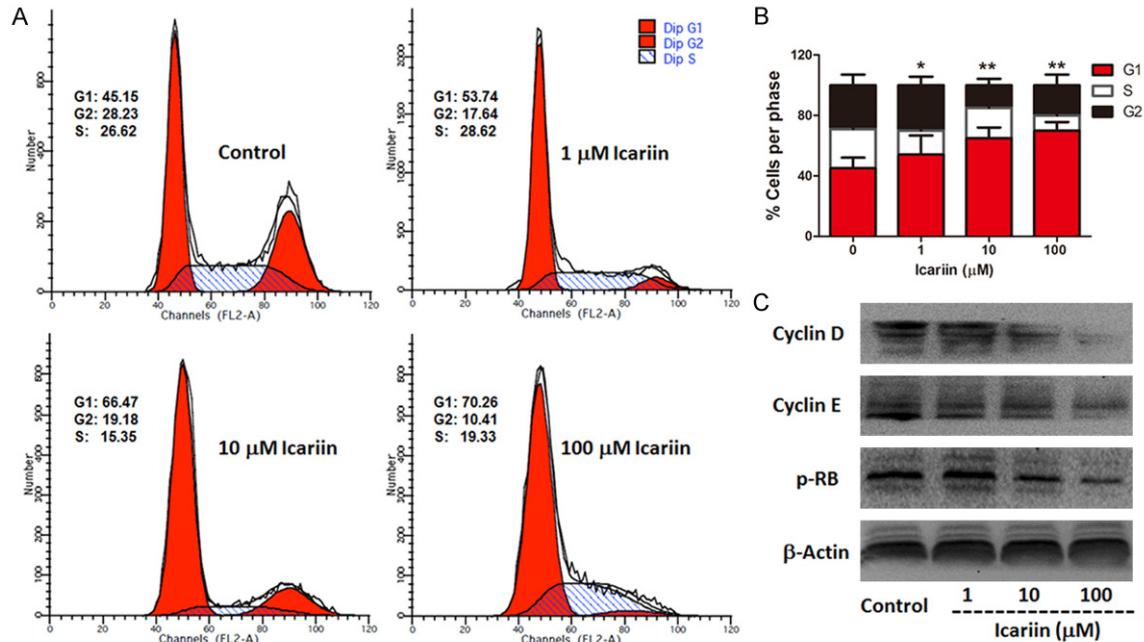


Figure 2. Effect of icariin on the cell cycle in human osteosarcoma cells. The human osteosarcoma MG63 cells were pretreated with different concentrations of icariin (1, 10, or 100 μM) for 24 h, the cell cycle distribution and cell cycle-related protein was examined by flow cytometry and western blotting, respectively. A. The representative images of cell cycle distribution. B. The percentage of cell cycle distribution. C. The expression of cell cycle-related proteins. Values are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$ compared with the control group.

tion efficiency. An analogous adenovirus expressing only GFP (Ad-GFP) was used as a control, and expression efficiency was determined by real-time PCR, western blotting, and functional assays of β -catenin signaling pathway.

Statistical analysis

The results were expressed as the means ± standard deviation (SD), either Student's t-test or one-way ANOVA by Prism GraphPad 4 software was used to achieve data analyses. A two-tailed P value of less than 0.05 was considered significant difference.

Results

Icariin inhibited the growth of osteosarcoma cells

To investigate the effect of icariin on the growth of osteosarcoma cells, two osteosarcoma cells lines, 143B and MG63, were incubated with a series of concentrations (10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} M) of icariin for 48 h, and next MTT assay was used to determine the cell viability. As shown in

Figure 1B and **1C**, icariin could significantly inhibit the growth of osteosarcoma cells in a dose-dependent manner, regardless of the cell types.

Icariin induced G1 phase arrest and down-regulated the expression of cyclin D, cyclin E and p-RB in osteosarcoma cells

To further illuminate the cell-growth suppressive effect of icariin, the cell cycle distribution was examined by flow cytometry after icariin treatment. Significant changes in cell cycle distribution were observed in MG63 cells (**Figure 2A** and **2B**). The rates of cells in G1 phase significantly increased while the rates of cell in S and G2 phase significantly decreased dose-dependently in icariin-treated cells, suggesting that icariin induces G1 phase arrest in human osteosarcoma cells.

Many cancer cells are characterized by overexpression of cyclins and mutation of RB, both of which could contribute to the continuous cycling of cancer cells [18, 19]. Therefore, we investigate the protein related to the G1 phase arrest, cyclin D, cyclin E and p-RB by using west-

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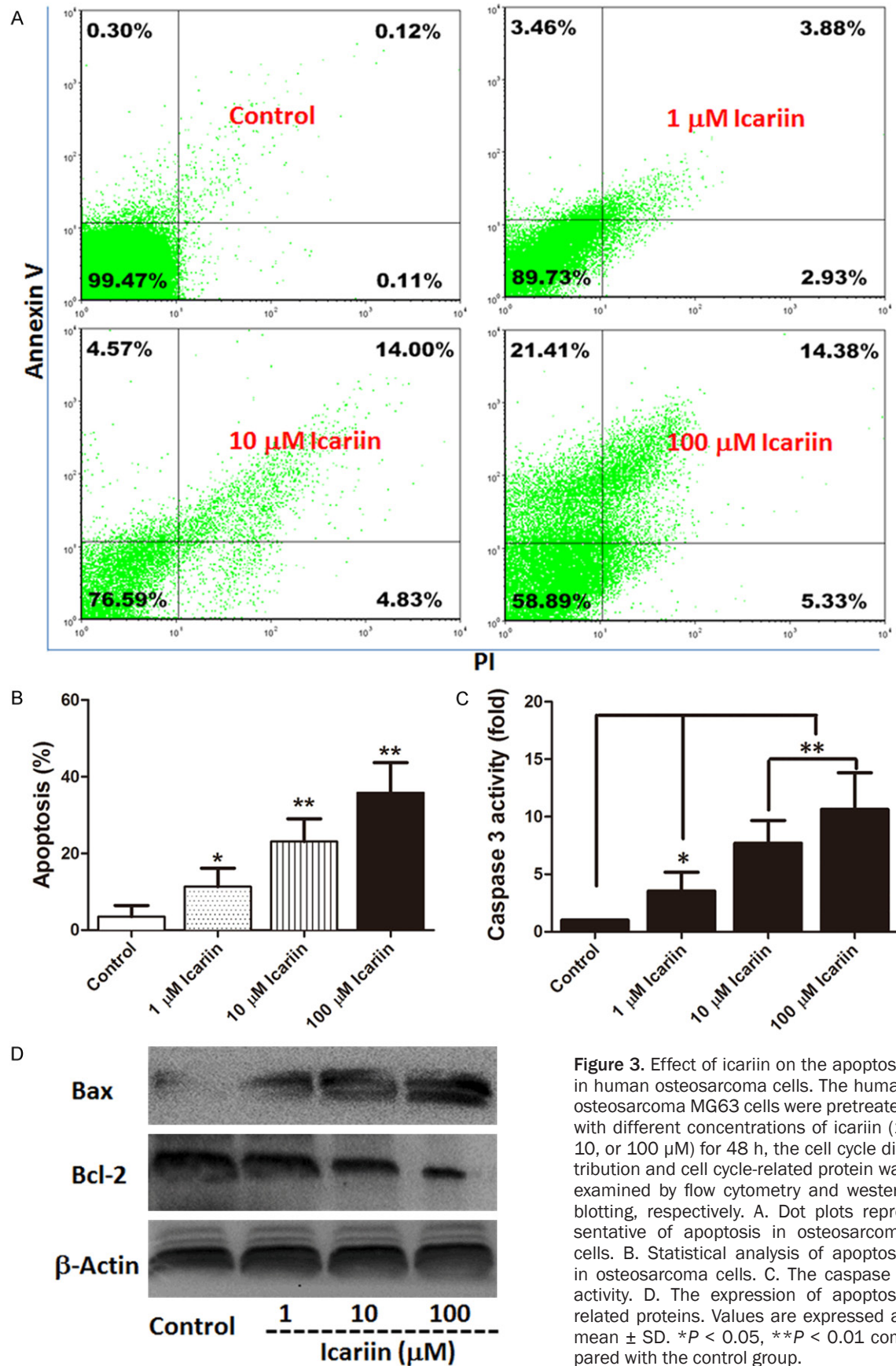


Figure 3. Effect of icariin on the apoptosis in human osteosarcoma cells. The human osteosarcoma MG63 cells were pretreated with different concentrations of icariin (1, 10, or 100 μM) for 48 h, the cell cycle distribution and cell cycle-related protein was examined by flow cytometry and western blotting, respectively. A. Dot plots representative of apoptosis in osteosarcoma cells. B. Statistical analysis of apoptosis in osteosarcoma cells. C. The caspase 3 activity. D. The expression of apoptosis related proteins. Values are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$ compared with the control group.

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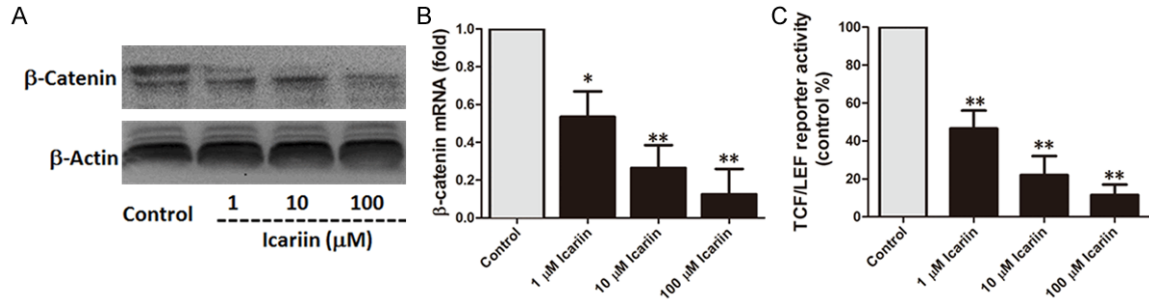


Figure 4. Effect of icariin on the expression and activity of β -catenin in human osteosarcoma cells. The human osteosarcoma MG63 cells were pretreated with different concentrations of icariin (1, 10, or 100 μ M) for 24 h, the mRNA and protein were extracted from the osteosarcoma. A. The protein of β -catenin was determined by western blotting. B. The mRNA of β -catenin was measured by qRT-PCR. C. The activity of β -catenin was assayed by the luciferase reporter assay. Values are expressed as mean \pm SD. * P < 0.05, ** P < 0.01 compared with the control group.

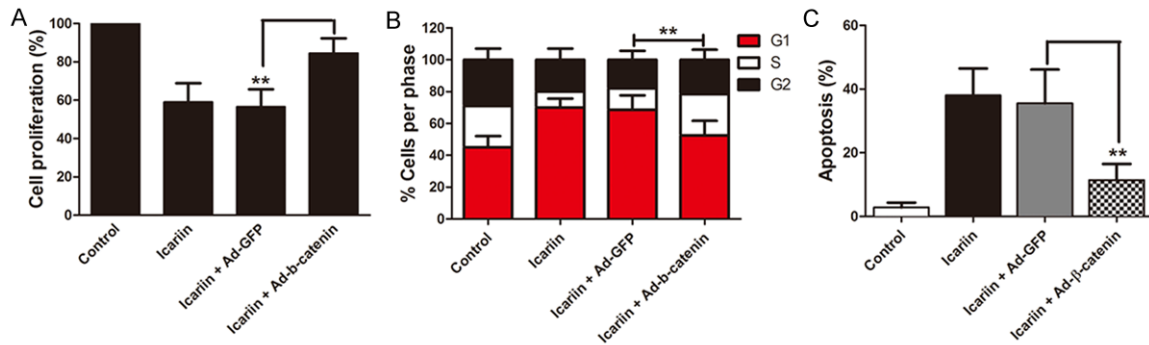


Figure 5. Overexpression of β -catenin reversed these beneficial effects of icariin in osteosarcoma cells. The human osteosarcoma MG63 cells were pretreated by 100 μ M icariin in the presence of Ad- β -catenin or Ad-GFP for 48 h, the proliferation and cell cycle and apoptosis were determined by MTT and flow cytometry, respectively. A. The proliferation of osteosarcoma cells. B. The cell cycle distribution of osteosarcoma cells. C. The apoptosis of osteosarcoma cells. Values are expressed as mean \pm SD. * P < 0.05, ** P < 0.01 compared with the control group.

ern blotting, it showed that icariin could induce dose-dependent downregulation of p-RB, cyclin E, and cyclin D in human osteosarcoma cells (**Figure 2C**). It indicated that icariin might induce G1 phase arrest through modulating cyclin D, cyclin E and p-RB.

Icariin regulated apoptosis and apoptosis-related proteins in osteosarcoma cells

To determine whether the cell-growth suppressive effect of icariin is due to pro-apoptosis, we examined the apoptosis of MG63 after exposure to icariin by flow cytometry using Annexin V-FITC/PI staining. Compared with control, the icariin treatment significantly increased the apoptosis rates, and a dose-dependent effect exists among the treatment groups (**Figure 3A** and **3B**). The caspase family is of importance to initiation, transduction, and amplification of

cell apoptosis. We used colorimetric assay to investigate the activation of caspase 3 in the apoptotic pathway. The results showed icariin could substantially and dose-dependently increased activation of caspase 3 in the osteosarcoma cells (**Figure 3C**).

It has been clearly illuminated that Bcl-2 family proteins are involved in the regulation of cell apoptosis [20]; among the family, the ratio of active pro- and anti-apoptotic protein dominates cell apoptosis. Accordingly, the effect of icariin on the expression of apoptosis-related proteins such as Bax and Bcl-2, was examined using immunostaining. As expected, compared with control, the expression of pro-apoptotic Bax was profoundly and dose-dependently increased while an inverse effect of icariin on the expression of anti-apoptotic Bcl-2 was observed (**Figure 3D**).

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Icariin suppressed β -catenin signal pathway

Previous study has reported that the aberrant wnt/ β -catenin signaling pathway is linked to human osteosarcoma [21]; we, therefore, wonder whether wnt/ β -catenin signaling was involved in anti-tumor effect of icariin on osteosarcoma. Quantitative Real-Time PCR and western blotting were used to detect the mRNA and protein expression of β -catenin after icariin treatment. As presented in **Figure 4A** and **4B**, the results showed that icariin profoundly decreased both β -catenin mRNA and β -catenin protein pattern, with a dose-dependent manner, in osteosarcoma cells. To further examine the activity of β -catenin, we performed a luciferase assay with the β -catenin reporter gene. The results of the luciferase assay manifested that icariin inhibited the activity of β -catenin in a dose-dependent manner (**Figure 4C**).

Overexpression of β -catenin reversed these beneficial effects of icariin in osteosarcoma cells

Over-expression of β -catenin by an adenovirus system reversed the anti-tumor effect of icariin, abrogating the inhibition of cell proliferation and promotion of cell cycle G1 arrest and cell apoptosis in human osteosarcoma cells (**Figure 5A** and **5C**).

Discussion

Osteosarcoma, though, presents a low incidence, it is fatal if untreated. Since 1980s, the 5-year survival rate in nonmetastatic osteosarcoma has increased remarkably from 20% to 65% owing to the use of multidrug chemotherapy and refined surgical techniques [22, 23]. However, despite the advance in surgery and systemic chemotherapy with current agents, the 5-year survival rate for localized osteosarcoma remains at 60%-70% and only 20% for metastatic disease. Developing new therapeutic agents is, therefore, of great importance to the improvement of osteosarcoma survival.

The results of present study showed that icariin, at various concentrations ranging from 10^{-6} to 10^{-4} M, significantly decreased the proliferation and increased apoptosis of osteosarcoma cells in a dose-dependent manner. We also observed icariin induced G1 phase arrest and down-regulated the expression of cyclin D,

cyclin E and p-RB in osteosarcoma cells. Additionally, after icariin treatment, Bcl-2 family protein Bax increased while Bcl-2 decreased, and the activation of caspase family member caspase-3 was significantly enhanced. Moreover, modulation of β -catenin using adenovirus expressing β -catenin revealed that β -catenin signaling might play an important role in anti-tumor effect of icariin, indicated by quantitative real-time PCR, western blotting, luciferase assay. Consistent with our results, several studies reported that icariin could increase expression of Bax and reduce Bcl-2 to contribute to apoptosis in gallbladder cancer [15], hepatocellular Carcinoma [11, 24], Leydig tumor [25]. Additionally, cell cycle arrest and down-regulation of cyclin D were also found to be involved in the anti-tumor effect of icariin [15, 25]. For another, Du et al. [26] found that icariin could enhance expression of Bax and decrease the expression of Bcl-2, leading to apoptosis of eosinophils and subsequently alleviating their infiltration in bronchial asthmatic mice. These findings demonstrate that icariin effectively inhibits the growth of various tumor cells, and it is also a promising anti-tumor candidate for osteosarcoma patients.

Two major pathways involved in apoptosis initiation. One is the cell-extrinsic pathway which is activated through tumor necrosis factor-related apoptosis-inducing ligand motivating cell-surface death receptors, and subsequently caspase-8 was activated. Another is cell-intrinsic pathway initiated by mitochondrial disruption, involving the release of cytochrome c and consequently activation of caspase-9 [20]. Both caspase-8 and caspase-9 activate caspase-3, a pivotal executive caspase in the cell apoptosis process [27]. In current study, we found that icariin could dose-dependently increase the activation of caspase-3 and this increasing tendency positively correlated apoptosis in the two osteosarcoma cells, indicating that icariin could enhance the activation of caspase-3 to promote apoptosis.

Cytochrome c release from mitochondria is a central event of the cell-intrinsic apoptosis pathway. It enters to the cytosol and binds to cytosolic Apaf-1 (apoptotic protease activating factor 1) and procaspase-9, resulting in activated caspase-9 [28]. Bcl-2 family protein play an indispensable role in the mitochondrial dis-

ruption and cytochrome c release, and the ratio of Bax and Bcl-2 (Bax/Bcl-2) could determine cell survival or apoptosis after an apoptotic stimulus [29]. Additionally, it has reported the balance of the Bax/Bcl-2 ratio could be affected by many chemotherapeutic agents [30-33]. In our study, icariin could increase the expression of Bax but decrease the expression of Bcl-2, causing the elevated Bax/Bcl-2 ratio. Moreover, activation of caspase-3 positively correlated the increased Bax while a negative correlation with bcl-2. Thus, our findings suggest the apoptosis induced by icariin in osteosarcoma cell could attributes to increasing Bax/Bcl-2 ratio.

Cyclin-dependent kinases (CDKs) are curial for cell cycle, and full CDK activity needs to combine with a specific cyclin to form CDK-cyclin complexes, which could phosphorylate proteins and consequently drive cell cycle progression [34]. Cyclin D could bind and activate CDK4 and CDK6, forming cyclin D-CDK4 and cyclin D-CDK6 complexes. These two complexes could phosphorylate retinoblastoma tumor suppressor protein, pRB, which result in release and activation of E2F transcription factors. Subsequently, E2F-induced gene expression is necessary for DNA replication and G1 to S cell cycle phase transition [35]. Amplification of Cyclin D and their proteins were documented in various human cancers, and an analysis of human cancer reported that cyclin D1 gene is the second frequently amplified locus in the human cancer genome [36]. Our results showed icariin induced G1 phase arrest in a dose-dependent manner, and decrease the expression of cyclin D1, cyclin E and pRB, suggesting icariin induce G1 phase arrest through downregulation of cyclin D, cyclin E and subsequent pRB.

It showed that aberrant over activation of Wnt- β -catenin pathway has been implicated in OS pathogenesis, and it contributes to osteosarcoma metastasis and chemotherapeutic resistance [37, 38]. Activation of canonical Wnt signaling inhibits GSK-3 β activity, causing β -catenin accumulation and translocation to the nucleus, where activated β -catenin can interact with the T cell factor/lymphoid enhancer factor (Tcf/Lef) family and activate the expression of proteins related to cell proliferation. Moreover, cyclin D1 is often shown to be upreg-

ulated by β -catenin signaling [39]. In our study, we found that icariin dose-dependently decreased the β -catenin mRNA and expression of protein pattern, both of which were in parallel with each other but was negatively associated with cell proliferation. Furthermore, luciferase reporter assay showed that activated β -catenin was decreased as the expression of β -catenin mRNA and protein pattern. More importantly, overexpression of β -catenin by adenovirus system could abrogate the anti-proliferation and pro-apoptosis effect of icariin. Taken together, our findings indicate that icariin inhibits cell proliferation through down regulation of cyclin D via inhibiting β -catenin.

In summary, our findings demonstrate that icariin inhibits the proliferation of osteosarcoma cells through downregulation of cyclin D and cyclin E by inhibiting the β -catenin signaling, and induces apoptosis through caspase-3 via upregulation the ratio of Bax/Bcl-2. These data indicate icariin might be a potential novel chemotherapeutic agent of osteosarcoma.

Disclosure of conflict of interest

None.

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