Ginsenoside CK induces apoptosis and suppresses proliferation and invasion of human osteosarcoma cells through the PI3K/mTOR/p70S6K1 pathway

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Abstract. Osteosarcoma is one of the most malignant bone tumors, and its major threats are aggressive invasion and early tumor metastasis, which result in a poor prognosis and high mortality. Accumulating evidence indicates that ginsenoside compound K (CK) has a significant antitumor effect, particularly on the inhibition of proliferation and invasion of numerous human tumors. In the present study, it was revealed that CK inhibited the viability and proliferation of osteosarcoma cells. Moreover, it was demonstrated that CK induced apoptosis and inhibited the migration and invasion of osteosarcoma cells via apoptotic staining, Annexin V/PI staining, and Transwell invasion assays. Furthermore, at the molecular level, the present results confirmed that apoptosis and invasion-related proteins were regulated by CK, which was possibly related to the blockade of the PI3K/mTOR/p70S6K1 signaling pathway. In summary, the present findings indicated that CK inhibited viability and proliferation, induced apoptosis, and inhibited the migration and invasion of osteosarcoma cells through the PI3K/mTOR/p70S6K1 signaling pathway.

Introduction

Osteosarcoma mainly occurs in teenagers and healthy young patients, and is one of the most common fatal primary malignant bone tumors (1,2), accounting for 5% of all pediatric tumors (3). The major threats of osteosarcoma are aggressive invasion and early multi-organ metastasis, especially pulmonary metastasis (4). Although survival has been greatly increased by surgical treatment combined with chemotherapy, the 5 year survival rate of patients with metastasis is still less than 35% (5). Therefore, the main strategy for the treatment of osteosarcoma is to block its invasiveness and metastasis to improve its prognosis. However, there are currently no effective drugs for osteosarcoma and the molecular mechanism remains obscure. Therefore, exploring targeted, highly effective treatments for osteosarcoma is urgently required.

The PI3K/mTOR/p70S6K1 pathway is an essential signaling pathway in cells, and has an extremely important biological function in cell growth, proliferation, apoptosis, angiogenesis and autophagy, as well as in other processes. Disorders of the pathway can cause a range of diseases, including cancer, neuropathy, and autoimmune diseases (6). The ribosomal 40S small subunit S6 protein kinase 1 (p70S6K1) is a direct substrate downstream of p-mTOR. P70S6K1 is phosphorylated by p-mTOR to promote the production of ribosomes, initiation factors, and elongation factors in cells, and to promote proliferation and invasion of tumor cells (7,8). Ginsenoside compound K (20-O-D-glucopyranosyl-20(S)-protopanaxadiol; CK; as shown in Fig. 1), is a major member of the original ginseng diol, saponins, and its study has been a popular research topic in the fields of anti-inflammatory and anti-tumor effects. Studies have revealed that ginsenoside CK is a compound with a variety of targets and pharmacological activities, which include anti-inflammatory (9), antitumor (10), anti-diabetic (11) and liver protection (12). However, to date, the antitumor mechanism of ginsenoside CK on osteosarcoma cells remains unclear. Therefore, in the present study, it was investigated whether CK exerted cellular antiproliferative effects and induced apoptosis in osteosarcoma cells. It was further explored whether the PI3K/mTOR/p70S6K1 signaling pathway played a decisive role in this process.

Materials and methods

Antibodies and reagents. Dulbecco's Modified Eagle's Medium (DMEM), high glucose medium, and fetal bovine serum (FBS) were obtained from Gibco; Thermo Fisher scientific, Inc. Human osteosarcoma cell lines, MG63 and U2-OS, were purchased from the Cell Bank of Shanghai Institutes for Biological Sciences. Ginsenoside CK (purity: >98%) was
after washing twice with PBS, 50 µl of TUNEL assay solution and 20 µl DAPI were added to each well of a 24-well plate and incubated at 37°C for 60 min in the dark. Then, the apoptotic nuclei were immediately observed using a fluorescence microscope, and the apoptotic rate was calculated.

**Annexin V/PI apoptosis detection.** Cell apoptosis was detected using an Annexin V/PI apoptosis detection kit according to the manufacturer's protocols. Briefly, after induction of apoptosis, the two cell types were harvested by trypsinization and washed twice in ice-cold PBS. The cells were centrifuged at 500 x g for 5 min and resuspended in 500 µl binding buffer, then incubated with 5 µl Annexin V-FITC and 10 µl propidium iodide (PI) in the dark at room temperature for 15 min. The samples were immediately analyzed using a FACSCalibur (BD Biosciences) flow cytometer and the data were analyzed using CellQuest software, version 5.1 (BD Biosciences).

**Cell migration and invasion detection.** The migration and invasive abilities of both groups of osteosarcoma cells were evaluated by Transwell migration and invasion systems. For the invasion assay, 20 µl Matrigel was applied to cover the bottom of the chamber after being washed with serum-free medium to create a Matrigel membrane by incubating at 37°C for 30 min. Cells (5x10⁴) with 200 µl of serum-free DMEM medium were seeded in the upper chamber of the Transwell system, and 500 µl of DMEM high glucose medium containing 10% FBS was added to the lower chamber as a chemoattractant. The Transwell system was incubated in a cell culture incubator for 24 h, and then the upper chamber was removed and the cells from the upper surface of the membrane were removed. Then, the cells invading to the lower surface of the membrane were stained with 0.1% crystal violet for 5 min at room temperature and observed using an inverted phase-contrast microscope. The procedure for the migration assay was the same as above, but a Matrigel membrane was not required.

**Western blot analysis.** Cells were lysed in a radioimmuno-precipitation assay (RIPA) lysis buffer (Solarbio Science & Technology) and the BCA method was used for protein determination. The protein samples from each group were resolved by SDS-PAGE and transferred to PVDF membranes (proteins were loaded by 50 µg/lane and the percentage of the gel was 10%). After transfer, the PVDF membranes were blocked with 5% (w/v) nonfat milk in TBST at room temperature for 2 h, and then incubated with the primary antibodies (1:2,000) in TBST buffer containing 1% (w/v) BSA at 4°C for 16 h. Following three washes in TBST, the membranes were incubated with the appropriate HRP-linked secondary antibody (goat anti-rabbit 1:1,000; cat. no. SA00001-2; ProteinTech Group) in TBST for 2 h at room temperature. Proteins were detected by an ECL system (GE Healthcare).
CK induces apoptosis of osteosarcoma cells detected by TUNEL assays. A TUNEL apoptosis kit was used to further detect the morphological changes of apoptotic nuclei. As revealed in Fig. 4, in the MG-63 group, the apoptosis rate of the NC group was only 1.52±0.63%, and the value increased to 25.33±2.68% after treatment with CK (20 µM). Similar results were also obtained in the U2-OS group [0.45±0.22% vs. 24.83±1.92% (NC group)]. These results indicated that CK induced apoptosis in both MG-63 and U2-OS cells.

CK induces apoptosis in osteosarcoma cells. The Annexin V/propidium iodide (PI) staining assay was employed to further confirm the apoptotic effect of CK. As revealed in Fig. 5A and B, after treatment with CK, the percentage of apoptosis in MG-63 and U2-OS cells in the experimental group was significantly higher than that in the control group (P<0.05). In the MG-63 group, both early apoptosis (5.42±1.90%) and late apoptosis (16.71±1.65%) were higher than that in the control groups (3.32±0.85 and 5.83±1.08%, respectively), and the total apoptosis was more pronounced (22.17±2.45 vs. 9.15±1.85%). U2-OS cells revealed a similar result (9.74±2.20 vs. 3.59±2.17% for total apoptosis). To validate the aforementioned conclusions at the cellular pathway level, the expression levels of apoptosis-related proteins were detected by western blot analyses. As revealed in Fig. 5, compared with the control group, the expression levels of the proapoptotic proteins cleaved caspase-3 and BAX were significantly upregulated in the CK group in MG-63 and U2-OS cells, while the expression of the inhibitory protein, Bcl-2, was downregulated (P<0.05). From the aforementioned results, it was concluded that CK significantly induced apoptosis in osteosarcoma cells.

CK inhibits the migration and invasion of osteosarcoma cells. In the previous results, it was demonstrated that CK inhibited proliferation and induced apoptosis of osteosarcoma cells. To further confirm the effect of CK on the migration and invasion of osteosarcoma cells, a Transwell assay was used. As revealed in Fig. 6A, the number of cells in the CK group passing through the Transwell chamber membrane were significantly lower in the MG-63 and U2-OS CK-treated groups than in the control group [invasion: MG63, 75.72±19.13 vs. 318.16±26.37 (control), P<0.05; U2-OS, 45.83±14.22 vs. 362.37±21.71 (control), P<0.05; migration: MG63, 123.15±12.39 vs. 398.63±31.40 (control), P<0.05; U2-OS, 132.94±29.91 vs. 453.33±34.17 (control), P<0.05], indicating that CK inhibited both the migration and invasion of MG-63 and U2-OS cells. To further characterize the relationship between CK and the mTOR signaling pathway, RAD001, a specific inhibitor of mTOR, was added. As revealed in Fig. 6B, the number of cells in the CK or RAD001 group passing through the Transwell chamber membrane was significantly lower than in the NC group, and this trend was more pronounced when cells were treated with both CK and RAD001. Therefore, it was hypothesized that CK played a similar role as an mTOR inhibitor, and that this effect was related to the mTOR signaling pathway. Furthermore, the expression of the invasion-related proteins,
MMP-2 and MMP-9 were detected, in each group by western blot analyses. As indicated in Fig. 6C, the results revealed that after treatment with CK, MMP-2 and MMP-9 expression levels were significantly downregulated in MG-63 and U2-OS cells (P<0.05), which was consistent with the aforementioned conclusions, and confirmed that CK inhibited the migration and invasion of osteosarcoma cells.

**CK is involved in the PI3K/mTOR/p70S6K1 signaling pathway in osteosarcoma cells.** To further investigate the antitumor mechanism of CK on MG-63 and U2-OS cells, the expression levels of related proteins in the PI3K/mTOR/p70S6K1 signaling pathway were investigated. The results of western blot analyses revealed that after treatment with CK, PTEN the major blocking protein of the PI3K/mTOR/p70S6K1 pathway was significantly upregulated in MG-63 and U2-OS cells (P<0.05; Fig. 7A). However, the core action proteins of the PI3K/mTOR/p70S6K1 pathway, including p-AKT and p-mTOR, were significantly downregulated in the CK group (P<0.05; Fig. 7A and B), indicating that the PI3K/mTOR/p70S6K1 pathway was blocked.
after treatment with CK. The downregulation of p70S6K1 expression in the CK group (P<0.05; Fig. 7B) further confirmed this conclusion. Furthermore, the expression levels of p-mTOR and p70S6K1 were downregulated when treated with RAD001.
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in U2‑OS cells, which was consistent with the effect of CK. When cells were treated with both CK and RAD001, they played a synergistic role and the downregulation of the expression levels of p‑mTOR and p70S6K1 were more pronounced (Fig. 7C). Overall, these conclusions confirmed that CK blocked the PI3K/mTOR/p70S6K1 signaling pathway of MG‑63 and U2‑OS osteosarcoma cells.

Discussion

Ginsenoside CK is one of the main metabolites of ginsenoside, which is a glycoside compound composed of sugars and aglycones, and belongs to a triterpenoid compound family. According to the structure of sapogenin, it can be divided into three categories: One is an oleanane‑type pentacyclic triterpenoid saponin, the sapogenin is oleanolic acid, and the other two are dammarane‑type tetracyclic triterpenoid saponins, which are the majority of ginsenosides and are the primary active ingredients of ginseng. According to different aglycons, the dammarane type tetracyclic triterpenoid saponin can be divided into the original ginseng diol saponin and the original ginseng triol saponin. Ginsenoside CK, a major member of the originally reported ginseng diol saponin, has been a topic of research in the fields of anti‑inflammatory and antitumor effects. Recent studies have revealed that ginsenoside CK has anti‑inflammatory, antioxidative, and neuroprotective effects (13‑16), and of particular interest are its antitumor effects. Studies have confirmed that CK has an inhibitory effect on various tumor cells, including liver, lung, breast and colon cancer as well as leukemia (12,17‑19). The antitumor effect of CK is mainly reflected in its ability to significantly attenuate the proliferation, invasion, and migration of tumor cells. Lee et al (20) revealed that CK significantly inhibited the proliferation and invasion of malignant glioma cells by blocking the PI3K/AKT/mTOR signaling pathway. Kang et al (21) reported that CK inhibited colon cancer cell proliferation and induced apoptosis by inhibiting histone deacetylase activity.
Osteosarcoma is one of the most malignant bone tumors, and its lethality is mainly reflected in the malignant, diffuse proliferative capacity, and early tumor metastasis. Therefore, it was speculated whether CK also had an inhibitory effect on the proliferation and invasion of osteosarcoma cells. To demonstrate the effect of CK on the in vitro viability and proliferation of osteosarcoma cells in vitro. Furthermore, it was revealed that the effect of CK on cell proliferation may be related to cell cycle distribution. Using PI staining cell cycle analyses in both MG-63 and U2-OS cells, it was determined that CK altered the distribution of cell cycle stages. After treatment with CK, the S-phase ratio of MG-63 cells decreased from 51.06±3.31 to 36.96±3.07%, compared to the control group, while the G2/M-phase ratio increased from 16.39±2.59 to 35.23±2.33%. Similarly, the proportion

![Figure 6](image-url)
of the S phase decreased from 38.00±3.57 to 28.73±4.08%, while the proportion of the G2/M phase increased from 36.25±2.77 to 45.96±3.41% in U2‑OS cells. Therefore, it was demonstrated that CK induced G2/M‑phase cell cycle arrest, and inhibited the viability and proliferation of MG‑63 and U2‑OS cells.

In order to analyze the apoptosis‑inducing effect of CK, apoptotic cell nuclear morphology assays, Annexin V/PI staining assays, and western blot analyses were performed. It was determined that the cell nuclei of the CK‑treated group exhibited typical apoptotic morphological changes. Similar results were detected by TUNEL assays, whether using MG‑63 or U2‑OS cells. The apoptotic nuclei were stained green, while the normal nuclei without apoptosis were stained blue by DAPI staining. The aforementioned results demonstrated that CK had an apoptosis‑inducing effect on both MG‑63 and U2‑OS cells using nuclear apoptotic morphological assays.

Therefore, using nuclear apoptotic morphological assays, it was confirmed that CK inhibited proliferation and induced apoptosis of MG‑63 and U2‑OS cells, and the results are consistent with previous studies which revealed that CK has antitumor effects on various tumor cells, including liver, lung, breast and colon cancer as well as leukemia (17‑21); however, another characteristic feature of osteosarcoma cells is their aggressive invasiveness and early metastasis. Since osteosarcoma is prone to early metastasis (most common in lung), most patients lose the opportunity for surgical treatment, and there is no effective
radiotherapy and chemotherapy for osteosarcoma in the clinic. Thus, patients usually have a poor prognosis and a high mortality rate (22,23).

To investigate the effects of CK on the migration and invasion of MG-63 and U2-OS cells, a Transwell assay was used and the expression levels of the invasion-related proteins, MMP-2 and MMP-9, were detected. The migration and invasion of MG-63 and U2-OS cells significantly decreased after treatment with CK, and downregulation of the expression of MMP-2 and MMP-9 also demonstrated that CK inhibited the migration and invasion of MG-63 and U2-OS cells. CK played a similar role to RAD001, a specific inhibitor of mTOR, and together they played a synergistic role in the effects of the migration and invasion. Chawla et al (24) reported that the mTOR inhibitor, Ridaforolimus, inhibited the phosphorylation of the mTOR effector protein, S6K, to block the PI3K/AKT pathway. Such inhibition also effectively inhibited the tumor characteristics of osteosarcoma, and achieved significant clinical effects. Moriceau et al (25) reported that the mTOR inhibitor, RAD001 (Everolimus), inhibited osteosarcoma cell proliferation in a dose- and time-dependent manner. Manara et al (26) reported that NVP-BEZ235, another mTOR inhibitor, significantly inhibited the proliferation and invasion of osteosarcoma cells, and was a possible novel potential targeted drug for the treatment of osteosarcoma. A number of previous studies have demonstrated that blocking the PI3K/mTOR/p70S6K1 signaling pathway by mTOR inhibitors inhibited osteosarcoma cell activity. Therefore, it was speculated that osteosarcoma cells may play a pathogenic role through the PI3K/mTOR/p70S6K1 pathway.

PI3K/mTOR/p70S6K1 studies have been a popular research topic in recent years. As an essential signaling pathway in cells, it plays an important biological function in cell growth, proliferation, apoptosis, angiogenesis, and autophagy. Disorders of the pathway can cause a range of diseases, including cancer, neuropathy, and autoimmune diseases (27). The phosphatidylinositol 3-kinase (PI3K) protein family is involved in the regulation of various cellular functions such as cell proliferation, differentiation, apoptosis, and glucose transport. Increases in PI3K activity are often associated with a variety of cancers (28). Cytokines such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), human growth factor (HGF), vascular protein I (AngI), and insulin activate PI3Ks, and the SH2 and SH3 domains of the p85 subunit of PI3Ks bind to the adaptor protein at a phosphorylation site. PI3K initiates phosphorylation of various PI intermediates after recruitment of activated receptors. Following this, PI3K converts PI3P to PI3P, a process that is particularly relevant to tumors (29). The result of PI3K activation is the generation of a second messenger, PIP3, on the plasma membrane. PIP3 binds to the PH domain-containing signaling proteins, AKT and phosphoinositide dependent kinase-1 (PDK1), which promotes PDK1 phosphorylation of AKT Ser308 to activate AKT (30,31). Phosphorylated AKT activates the mTOR complex (mTORC1), which activates the translation of proteins and enhances cell growth. AKT exerts anti-apoptotic effects by phosphorylating target proteins through various downstream pathways. ATK activates IκB kinase (IKKκ), which leads to the degradation of the NF-κB inhibitor, IκB, following which, NF-κB is released from the cytoplasm for nuclear translocation, and its target gene is activated to promote cell survival. AKT phosphorylates the Bcl-2 family member, BAD, which binds to 14-3-3 and prevents it from binding to Bcl-XL to initiate apoptosis (32,33).

PTEN is a PI3P-phosphatase that, in contrast to PI3K, converts PI3P to PI-4,5-P2 by dephosphorylation. PTEN reduces AKT activation and blocks all downstream signaling events regulated by AKT (34). Previous studies have confirmed that PTEN expression in osteosarcoma cells is significantly decreased compared to normal tissues (7,35), indicating that the pathogenicity of osteosarcoma is related to PTEN expression. In the present study, the expression of PTEN in MG-63 and U2-OS cells was significantly upregulated in CK-treated cells (P<0.05). In contrast, the expression levels of p-Akt and p-mTOR were downregulated compared with the control group (P<0.05). However, the expression of Akt and mTOR was not significantly altered in the two groups (P>0.05), indicating blockage of the PI3K/AKT pathway, as described previously. The phosphorylation of Akt was inhibited and the downstream mTOR complex could not be activated normally; thus, inhibiting cell viability and proliferation. Concurrently, the apoptotic proteins, caspase-3 and BAX were activated, which blocked apoptosis inhibition and triggered apoptosis.

p70S6K1 is a direct substrate downstream of p-mTOR. Phosphorylation of p70S6K1 by p-mTOR promotes the formation of ribosomes, initiation factors, and elongation factors in cells, and promotes the invasion and metastasis of tumor cells (8). The expression of p70S6K1 in MG-63 and U2-OS cells was significantly downregulated after CK treatment (P<0.05), indicating that the PI3K/mTOR/p70S6K1 signaling pathway was blocked, and the invasion and metastasis of tumor cells was inhibited. When RAD001 was added, the PI3K/mTOR/p70S6K1 signaling pathway was blocked, and the expression levels of p-mTOR and p70S6K1 were downregulated, and this result was consistent with the effect of CK. Furthermore, CK and RAD001 played a synergistic role in regulating the expression of proteins in the PI3K/mTOR/p70S6K1 signaling pathway.

In summary, the present study revealed that CK inhibited the viability and proliferation of osteosarcoma cells, which was related to G2/M-phase cell cycle arrest. CK induced apoptosis and inhibited the migration and invasion of osteosarcoma cells, and blocked the PI3K/mTOR/p70S6K1 signaling pathway of MG-63 and U2-OS osteosarcoma cells, which exhibited anti-tumor properties in osteosarcoma cells. However, this study also has some limitations. Cell viability assays on normal osteocyte cells as a healthy control group should be performed. Research on PARP activation status and the EGFR/RAS/MAPK pathways needs to be performed, to demonstrate the pro-oxidant or antioxidant effects of Ginsenoside CK in MG63 and U2-OS cells to further confirm our conclusion. In addition, apoptosis-related proteins, such as cleaved caspase-9 and cleavage of PARP-1 should also be investigated to research the specific mechanism of mitochondrial apoptosis induced by CK. The MAPK pathway is an important signaling pathway regulating osteosarcoma cells, and its relationship with CK and the PI3K/mTOR/p70S6K1 signaling pathway still requires further study.
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Availability of data and materials
The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions
KC and QR conceived and designed all the experiments. YW, ZM, ZL, and BS participated in the cell experiments. JJ, JX, TC and YH analyzed the results. YJ and LQ participated in the writing of the manuscript and revising it critically for important intellectual content. All the authors reviewed and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate
Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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