Cinobufacini Injection Suppresses the Proliferation of Human Osteosarcoma Cells by Inhibiting PI3K/Akt Signaling Pathway

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Research

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Abstract

Background: Cinobufacini injection (CI), an aqueous extraction from the *Cutis Bufonis*, is broadly used in clinical treatment of cancer in China. However, the underlying molecular mechanisms of CI in treating osteosarcoma (OS) remain unclear. Aberrant activation of PI3K-AKT signaling pathway is the cause of many types of cancer, including OS. Therefore, we investigated the effect of CI on proliferation, apoptosis and cell cycle of OS cells and elucidated the molecular mechanism of CI in inhibiting OS cells.

Methods: Cell proliferation of U2OS and MG63 cells after CI treatment was measured by CCK-8 assay, colony formation and morphological changes. Additionally, the cell cycle arrest and apoptosis induced by CI, were determined by FACS and Western blot analysis. The mechanisms of CI on OS were evaluated by RNA-seq and Western blot analysis.

Results: We founf that CI reduced the proliferation of U2OS and MG63 cells in a dose- and time-dependent manner. Furthermore, CI induced the U2OS cells cycle arrest in G0/G1 phase, but the MG63 cells cycle arrest in G2/M phase. Consequently, CI triggered the apoptosis in both OS cells, with enhanced caspase-3 activity and decreased expression of Bcl-2/Bax. In addition, RNA-seq data indicated that PI3K-Akt signaling pathway played an essential role in CI treatment. Moreover PI3K and phosphorylation of AKT (p-AKT) were significantly down-regulated by CI in both OS cells.

Conclusions: These results indicate that CI significantly inhibited the proliferation, induced the cell cycle arrest, as well as apoptosis in human OS cells, which is mediated by the inactivation of PI3K-Akt signaling pathway. These findings suggest that CI may have potential for the treatment of OS.

1. Introduction

Osteosarcoma (OS), as known as osteogenic sarcoma, is the most prevalent primary malignant bone tumor among child, adolescents and young adults [1] It is characterized by high aggression with rapid progression, high metastatic and lethal behaviour [2]. Although there are many options for OS therapy, including surgery, chemotherapy, hormone therapy and radiation therapy, the long-term survival rate has not been improved, especially for patients with multi-drug resistance, recurrence, or lung metastasis [3]. Therefore, elucidating the molecular mechanisms underlying OS and developing alternative therapies with lower toxicity are critical for achieving more favorable clinical outcomes and reducing mortality.

Normal cell proliferation depends on a complete and effective cell cycle under the regulation of several critical checkpoint kinases, such as cyclin-dependent kinases (CDKs) [4]. In malignance cells, cell cycle imbalance is an early step for tumor development. Many therapeutic agents were found to play an important part in the cell cycle arrest [5]. The checkpoint of G0/G1 or G2/M phase prevents entry into mitosis with DNA damage, causing cell cycle arrest as well as inducing apoptosis endpoints. Apoptosis is a type of programmed cell death with characterized morphology, such as cell shrinkage and DNA fragmentation [6]. The induced apoptosis contributes the prevention of tumor growth, and the related

mechanisms involve PI3K/Akt pathway [7, 8]. Hence, it is a promising therapeutic strategy to targeting PI3K/Akt for OS treatment.

Cinobufacini injection (CI), extracted from the skin of the Bufo bufo gargarizans Cantor, has the effects on clearing away heat and toxic material, inducing diuresis for removing edema. CI has been broadly used for clinical cancer treatment [9], including OS [10, 11], but the pharmacological mechanisms governing these processes remains unknown, which limits its clinical application and promotion.

In this study, we firstly employed the network pharmacological approach to explore the potential mechanism of CI in treating OS. The inhibitory role of CI in human OS cells was confirmed by CCK-8 assay, colony formation and morphological changes. Furthermore, we investigated the underlying molecular pathways by which CI induced cell cycle arrest and apoptosis, through the PI3K/Akt signaling cascade.

2. Materials And Methods

2.1. Cell Culture and Chemical Preparation

Human OS cell lines U2OS and MG63, were purchased from the American Type Culture Collection (Rockville, MD, USA). Both cell lines were cultured with Dulbecco's modified Eagle's medium (BI, Ashraf, Israel) supplemented with 10% FBS (Vian-Saga, Shanghai, China) at 37℃ in incubators with humidified air and 5% carbon dioxide. CI was from the Anhui Huarun Jinchan Pharmaceutical Co., Ltd (Anhui, China). CI was used at various concentrations to treat U2OS and MG63 cells.

2.2. Antibodies and reagents

The antibodies for PI3K (Cat. No: 4292S), p-AKT (Cat. No: 9271S), AKT (Cat. No: 9272S), Bax (Cat. No: 2772S), Bcl-2 (Cat. No: 4223S), cleaved-caspase 3 (Cat. No: 9661T), Caspase 3 (Cat. No: 9662), CDK4 (Cat. No: D963E), CDK1 (Cat. No: 77055S), cyclinD1 (Cat. No: 2922S) and cyclinB1 (Cat. No: 4138S) were obtained from Cell Signaling Technology (Boston, USA). The antibody for β-actin was from Beyotime Biotechnology (Shanghai, China).

2.3. Cell proliferation assay and morphological observation

The cell viability was measured by Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan). The U2OS and MG63 cells were cultured at a concentration of 2×10^3 cells/well in 100 μl in 96-well culture plates and incubated with various concentrations of CI for 24, 48, and 72 h. After that, the medium was removed, and the cells were washed twice with PBS. Medium (90 μL) and CCK-8 (10 μL) were subsequently added to each well and incubated for 2 h at 37°C. A microplate reader spectrophotometer was used to measure the optical density (OD) at 450 nm. At 24, 48 and 72 h after the exposure of the cells to various concentrations of CI, the cells in the plates were observed with a light microscope.

2.4. Colony formation assay

U2OS and MG63 cells were seeded into 6-well plates at a density of 2×10^3 cells/mL. After adhesion, the cells were treated with different concentrations of the CI and incubated for 2 weeks. Colonies were fixed with 4% paraformaldehyde and stained with crystal violet. All colonies were photographed under a scanner.

2.5. Cell Cycle Analysis

The cell cycle distribution was analyzed with the Cell Cycle Detection Kit (KeyGEN Biotech) following the manufacturer's instructions. U2OS and MG63 cells (2×10^5 cells/well) were seeded into 6-well plates overnight and then treated with the different concentrations of CI for 12 h. Cells were harvested, fixed with cold 75% ethanol, incubated with RNase A, stained with PI, and then detected on a LIFE Attune NxT flow cytometer.

2.6. Apoptosis Assay

Flow cytometry was conducted using the Annexin V-FITC/PI apoptosis detection kit (BD, United States, Cat. No: 556547) according to the manufacturer's instructions to assess cell apoptosis. U2OS and MG63 cells were seeded into 6-well plates at a density of 2×10^5 cells/mL (triplicate in each group), and incubated 24 h followed by the treatment with the different concentrations of CI For apoptosis assay, cells were harvested, washed with cold PBS for two times. 500 μL 1×binding buffer was then added to each tube and cells were resuspended. The supernatant cells were incubated with 5 μL Annexin V-FITC and 5 μL PI for 15 min at the room temperature in the dark. Finally, the apoptotic analyses were done by flow cytometry within one hour.

2.7. RNA-seq analysis

Total RNA was extracted with TRIzol reagent (Invitrogen, CA, USA) from U2OS and MG63 cells with or without CI treatment. Total RNA was then quality controlled and quantified using an Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations. After converting to cDNA and building the library, high-throughput sequencing was performed using the Illumina Hiseq platform in Novogene Genomics. Fold Change (FC) CI vs. control thresholds at 1 with an p-value inferior to 0.05 were used to consider differentially expressed genes (DEGs). KEGG pathway enrichment analysis of differentially expressed genes was performed by Novomagic cloud platform.

2.8. Western blot assay

U2OS and MG63 cells were seeded into 6-well plate with 2×10^5 cells/well and incubated 24 h followed by the treatment of the different concentrations of CI for another 24 h. After that, the cells were washed with PBS and lysed by RIPA lysis buffer with proteinase and phosphatase inhibitors. The protein concentration of each sample was measured with BCA protein Assay Kit (Solarbio, Beijing, China) and then adjusted to the same concentration with RIPA. Equal amounts of protein in each sample were loaded and separated by 10% SDS-PAGE and then transferred to PVDF membranes. Next, the membranes were incubated with different primary antibodies overnight at 4°C after blocking in 5% fat-free milk or 5% bovine serum

albumin at room temperature for 2 h, followed by 1 h incubation with the secondary antibody at room temperature. The membranes were scanned using a Amershan Imager 680. Finally, band blots were analyzed with Image J software.

2.9. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 5 software. All experiments were performed in triplicates and repeated at least three times to confirm the results. These data were expressed as means ± standard error of the mean (SEM) and analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. P -value < 0.05 was considered to be statistically significant.

3. Results

3.1. CI inhibited the growth of human OS cells in dose- and time-dependent manner

To investigate the effects of CI on the OS cell proliferation, U2OS and MG63 cells were directly treated with CI (1:800, 1:400, 1:200, 1:100, 1:50, 1:10) for 24 h, 48 h and 72 h. CI significantly reduced cell viability of both OS cells, in a dose- and time-dependent manner (Fig. 1A). Consistently, as evidenced by decreased clonogenicity (Fig. 1B), CI strongly inhibited cell proliferation in these two OS cells. Furthermore, observations under the light microscope indicated that the exposure to CI resulted in the apoptotic morphological changes of OS cells including decreased number of living cells, cell shrinkage, rounding and granulation. Morphological changes were seen with higher severity in dose- and time-dependent (Fig. 1C).

3.2. CI triggered cell cycle arrest in OS cells

To confirm the relation between the growth inhibition and cell cycle arrest, next we analyzed the function of CI on the progression of cell cycle. At given concentrations after 12 h, compared with untreated controls, G0/G1 accumulation and a downtrend in S peak was observed in CI treated U2OS cells. However in CI treated MG63 cells, G2/M accumulation and a downtrend in G0/G1 peak was observed (Fig. 2A). Furthermore, western blot results showed that cell cycle-related proteins CDK4 as well as CyclinD1 in U2OS, but CDK1 as well as CyclinB1 in MG63, were clearly down-regulated by CI treatment (Fig. 2B). Taken together, these findings suggest that CI encouraged U2OS cell cycle arrest at G0/G1 phase and MG63 cell cycle arrest at G2/M phase, by leading to regulation of the proteins related to cell cycle.

3.3. CI induced the apoptosis of OS cells

The network pharmacological analysis, morphological changes and cell cycle arrest indicated that apoptosis might be involved in the CI treatment on OS, which was detected through flow cytometric analysis. After 24 h treatment with 0 (control), 1:200 and 1:100 of CI in U2OS cells, and with 0 (control), 1:50 and 1:25 of CI in MG63 cells, Annexin V and PI staining assay showed that CI induced cell death in

dose-dependent manner (Fig. 3A). According to detect the apoptosis related protein expression, we found that CI increased significant cleaved-caspase 3 expression and decreased the ratio of Bcl-2 to Bax (Fig. 3B). Taken together, these results implied that CI induced apoptosis of both OS cells through the Bax, Bcl-2 and caspase-dependent pathway.

3.4. CI inhibits the PI3K/AKT pathway in OS cells

To investigate the expression of the genes affected by CI, RNA-seq assay of CI-treated U2OS and MG63 cells were conducted. In both OS cells, compared with the control at 24 h, CI treatment regulated 402 genes (Fig. 4A, B). The up-regulated genes were clustered to immunological pathways, but the down-regulated genes were involved in metabolic and energy process (Fig. 4C). Further KEGG pathway enrichment results indicated the regulation of PI3K-AKT signal pathway by CI (Fig. 4D). The protein expression of PI3K and the ratio of p-AKT to AKT were significantly decreased in a dose-dependent manner by Western blot (Fig. 4E). The results demonstrated that the effect of CI against OS via suppressing the PI3K/AKT signaling pathway.

4. Discussion

OS is the most common malignant bone tumor in children and adolescents. Despite great advances in the treatment of OS, the survival rate of patients with OS continues to be unsatisfactory in the metastatic and relapse setting [12]. Chemotherapy, a common treatment for cancer, improves dramatically the survival of the patient with OS [13]. However, the agents of chemotherapy have strong side effects, including alopecia, myelosuppression, mucositis, nausea, vomiting, cardiotoxicity, ototoxicity and nephrotoxcitiy [14]. Therefore, traditional Chinese medicine (TCM), with low toxicity and high efficiency, has become an appropriate alternative.

According to traditional Chinese theory, the main causes of OS are Yang-Qi deficiency, qi stagnation, blood stasis and cancer toxin stasis in the body. Based on the function of clearing away heat and toxic material, inducing diuresis for removing edema, *Cutis Bufonis* has been used in treatment of malignant tumor, sores and carbuncle. As the extract of *Cutis Bufonis*, CI has been widely used in clinical treatment of advanced tumors. Modern pharmacological studies have found that CI could inhibit the proliferation of OS, but the pharmacological mechanisms governing these processes have yet to be elucidated.

In view of the characteristics of multi-component and multi-target, the research on pharmacological mechanism of TCM has always full of difficulties and challenges. Transcriptomics plays an important role in the research of traditional Chinese medicine because of its holistic and systematic research characteristics at the whole genome transcription level. RNA sequencing technology, which consistent with the concept of TCM, has provide a new perspective for cooperating with a new realization of the mechanisms of drugs and has become an holistic approach that incorporating systems biology, bioinformatics, and polypharmacology to explore and comprehend the mechanisms of TCM [15].

The KEGG pathways of CI against OS include the PI3K-Akt signaling pathway, MAPK signaling pathway, Hippo signaling pathway and TNF signaling pathway. As one of the key pathways that we predicted, PI3K-Akt signaling pathway had higher degrees in the key network. It has been reported that PI3K-Akt signaling pathway, is closely related to many processes in amount of cancer, and participated in tumor growth and development [16]. PI3K-Akt-mTOR signaling pathway plays an important role in regulating cell cycle, proliferation, apoptosis and autophagy and involves in many cancer processes, including osteosarcoma [17, 18]. Therefore, PI3K-Akt signaling pathway was selected as pivotal research objects. Our experimental results further show that CI significantly inhibited the activation of the PI3K-Akt signaling pathway in OS cell, indicating that the PI3K-Akt signaling pathway was an important mechanism of CI for the treatment of OS. Although the published literature show that MAPK signaling pathway [19], Hippo signaling pathway [20] and TNF signaling pathway [21] are associated with the process of cancer, more *in vitro* and *in vivo* experiments and clinical trials are needed to further validate the pathways or mechanisms predicted in this KEGG analysis.

5. Conclusion

In this study, we systematically combine a method of RNA sequencing technology with bioinformatics to comprehensively predict the pharmacological mechanism of the actions of CI against OS at the systemic level and most of the predicted results are in agreement with the published literature. *In vitro* experiments, we confirmed the effect of CI against OS, which was associated with cell apoptosis and activation of the PI3K/Akt pathway. These results provided a reliable basis for the clinical application of CI.

Declaretions

Abbreviations

CI, cinobufacini injection; OS, osteosarcoma; CCK-8, Cell Counting Kit-8; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genome; TCM, traditional Chinese medicine.

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Declarations

Ethics approval and consent to participate

All procedures were performed in accordance with the guidelines of the Tianjin University of Traditional Chinese Medicine ethics committee.

Consent for publication

Not applicable.

Availability of data and materials

The data used to support the fndings of this study are available from the corresponding author upon request.

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Author contributions

CC and YZ performed the experiments. YC and YY analyzed the data and contributed the reagents/materials/analysis tools. SH and YW designed the research and wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

We have no conflicts of interest to disclose.

References

1. Saraf AJ, Fenger JM, Roberts RD. Osteosarcoma: Accelerating progress makes for a hopeful future. Front Oncol. 2018;8:4.
2. Otoukesh B, Boddouhi B, Moghtadaei M, Kaghazian P, Kaghazian M. Novel molecular insights and new therapeutic strategies in osteosarcoma. Cancer Cell Int. 2018;18:158.
3. Wang C, Ren M, Zhao X, Wang A, Wang J. Emerging roles of circular RNAs in osteosarcoma. Med Sci Monit. 2018;24:7043–50.
4. Osawa T, Davies D, Hartley JA. Mechanism of cell death resulting from DNA interstrand cross-linking in mammalian cells. Cell death disease. 2011;2:e187.
5. Wang H, Zhang T, Sun W, Wang Z, Zuo D, Zhou Z, Li S, Xu J, Yin F, Hua Y, Cai Z. Erianin induces G2/M-phase arrest, apoptosis, and autophagy via the ROS/JNK signaling pathway in human osteosarcoma cells in vitro and in vivo. Cell Death Dis. 2016;7(6):e2247.
6. Burgess DJ. Apoptosis: refined and lethal. Nat Rev Cancer. 2013;13(2):79.

7. Li Y, Liu Y, Shi F, Cheng L, She J. Knockdown of Rap1b enhances apoptosis and autophagy in gastric Cancer cells via the PI3K/Akt/mTOR pathway. Oncol Res. 2016;24(5):287–93.
8. Lu R, Zhao G, Yang Y, Jiang Z, Cai J, Hu H. Inhibition of CD133 overcomes Cisplatin resistance through inhibiting PI3K/AKT/mTOR signaling pathway and autophagy in CD133-positive gastric Cancer cells. Technol Cancer Res Treat. 2019;18:1533033819864311.
9. Yang T, Shi R, Chang L, Tang K, Chen K, Yu G, Tian Y, Guo Y, He W, Song X, Xu H, Ye Z. Huachansu suppresses human bladder cancer cell growth through the Fas/Fasl and TNF- alpha/TNFR1 pathway in vitro and in vivo. J Exp Clin Cancer Res. 2015;34(1):21.
10. Ma XC, Ding HQ, Shi JD, Hei L, Niu NK, Suo ZG, Shang YB, Lin S, Pu FF, Shao ZW. Cinobufacini from the skin of bufo bufo gargarizans induces apoptosis, possibly via activation of the wnt/β-catenin pathway, in human osteosarcoma cells. Nat Prod Commun. 2018;13(2):201–4.
11. Huang T, Gong WH, Li XC, Zou CP, Jiang GJ, Li XH, Qian H. Efficient Killing Effect of Osteosarcoma Cells by Cinobufacini and Cisplatin in Combination. Asian Pac J Cancer Prev. 2012;13(6):2847–51.
12. Lisa M, Rebecca JT, Sharon AS. Osteosarcoma Incidence and Survival Rates From 1973 to 2004: Data From the Surveillance, Epidemiology, and End Results Program. Cancer. 2009;115(7):1531–43.
13. Neyssa M, Mark G, Lisa T, Richard G. Biology and Therapeutic Advances for Pediatric Osteosarcoma. Oncologist. 2004;9(4):422–41.
14. Luetke A, Meyers PA, Lewis I, Juergens H. Osteosarcoma treatment - where do we stand? A state of the art review. Cancer Treat Rev. 2014;40(4):523–32.
15. Cao H, Xi S, He W, Ma X, Liu L, Xu J, Zhang K, Li Y, Jin L. The effects of Gentiana dahurica Fisch on alcoholic liver disease revealed by RNA sequencing. J Ethnopharmacol. 2020;29:113422.
16. Taskin E, Ozdogan K, Kunduz KE, Dursun N. The restoration of kidney mitochondria function by inhibition of angiotensin-II production in rats with acute adriamycin-induced nephrotoxicity. Ren Fail. 2014;36:606–12.
17. Wang B, Li J. Piceatannol Suppresses the Proliferation and Induced Apoptosis of Osteosarcoma Cells Through PI3K/AKT/mTOR Pathway. Cancer Manag Res. 2020;12:2631–40.
18. Meng CY, Zhao ZQ, Bai R, Zhao W, Wang YX, Xue HQ, Sun L, Sun C, Feng W, Guo SB. MicroRNA–22 mediates the cisplatin resistance of osteosarcoma cells by inhibiting autophagy via the PI3K/Akt/mTOR pathway. Oncol Rep. 2020;43(4):1169–86.
19. Fang JY, Richardson BC. The MAPK signalling pathways and colorectal cancer. Lancet Oncol. 2005;6(5):322–7.
20. Harvey KF, Zhang X, Thomas DM. The Hippo pathway and human cancer. Nat Rev Cancer. 2013;13(4):246–57.
21. Wolczyk D, Zaremba-Czogalla M, Hryniewicz-Jankowska A, Tabola R, Grabowski K, Sikorski AF, Augoff K. TNF-α promotes breast cancer cell migration and enhances the concentration of membrane-associated proteases in lipid rafts. Cell Oncol (Dordr). 2016;39(4):353–63.

Figures

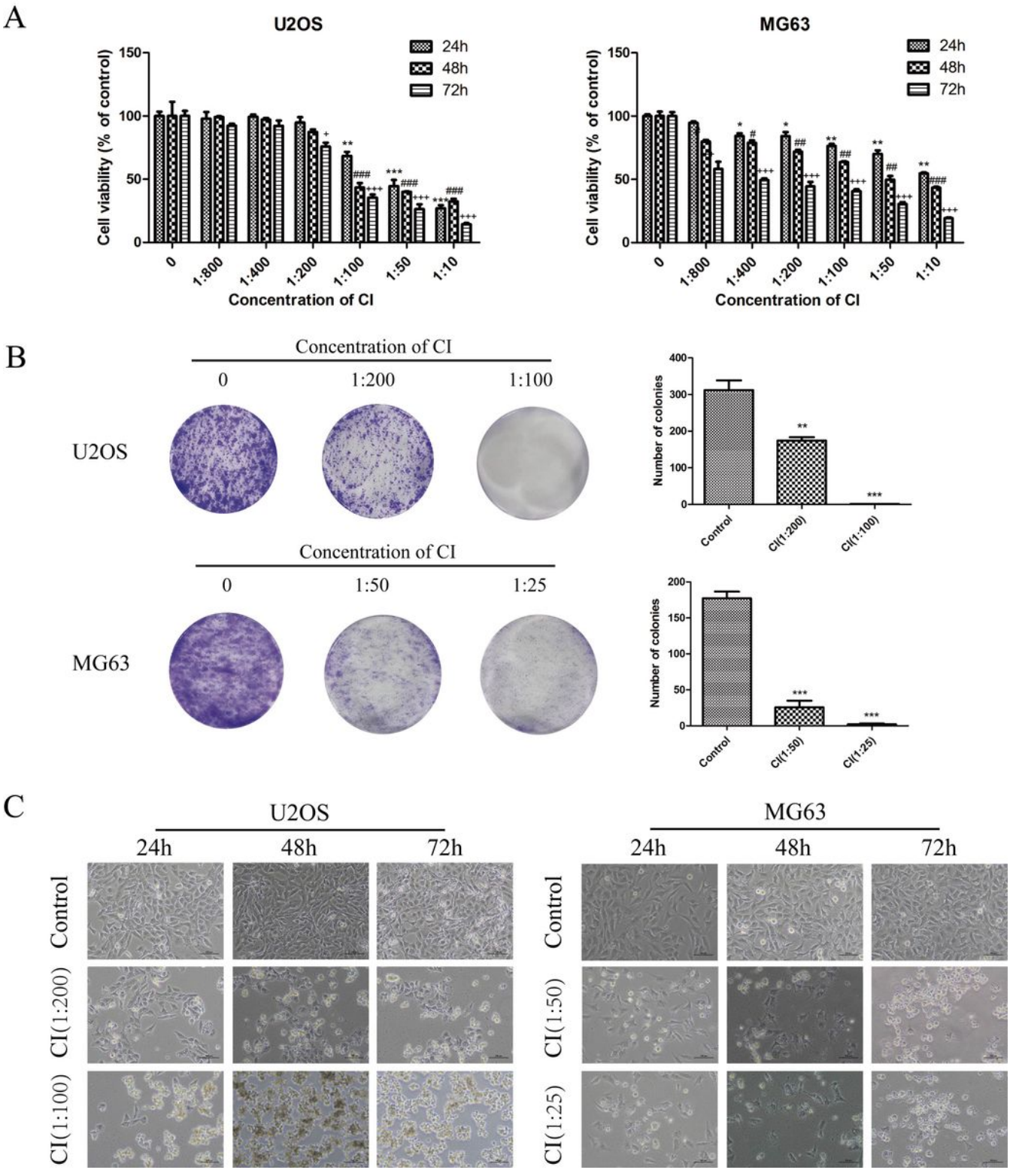

Figure 1

CI reduces cell viability of OS cells. A U2OS and MG63 cells were treated with CI (0, 1:800, 1:400, 1:200, 1:100, 1:50, 1:10) for 24, 48 and 72 h and cell viability was determined by CCK8 assay. B Colony formation assay of U2OS and MG63 cells exposed to CI (0, 1:200, 1:100 or 0, 1:50, 1:25) for 14 days. The

colony numbers (>50 cells/colony) were calculated manually. C Morphological changes of U2OS and MG63 cells were treated with CI (0, 1:200, 1:100 or 0, 1:50, 1:25) for 24, 48 and 72 h. *p < 0.05, **p < 0.01, ***p < 0.001 relative to the control group.

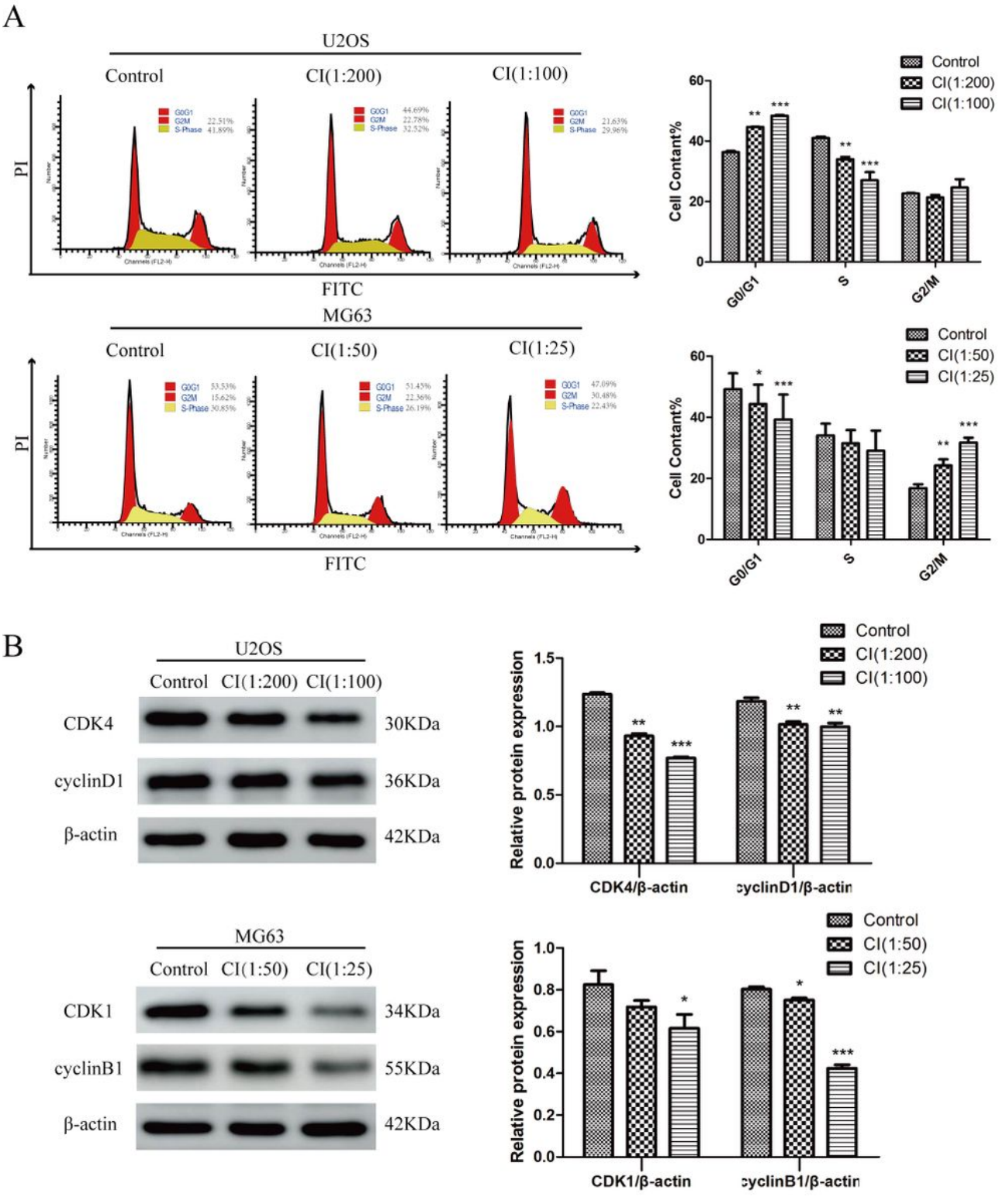

Figure 2

CI triggered cell cycle arrest in OS cells. A Cell cycle phases of U2OS and MG63 cells exposed to CI (0, 1:200, 1:100 or 0, 1:50, 1:25) for 12 h analyzed using flow cytometry. Cell cycle phase distribution

indicated as the mean±SD (n = 3). B Levels of expression of CDK4, cyclinD1, CDK1, cyclin B1 were determined using western blot assessment. *p < 0.05, **p < 0.01, ***p < 0.001 relative to the control group.

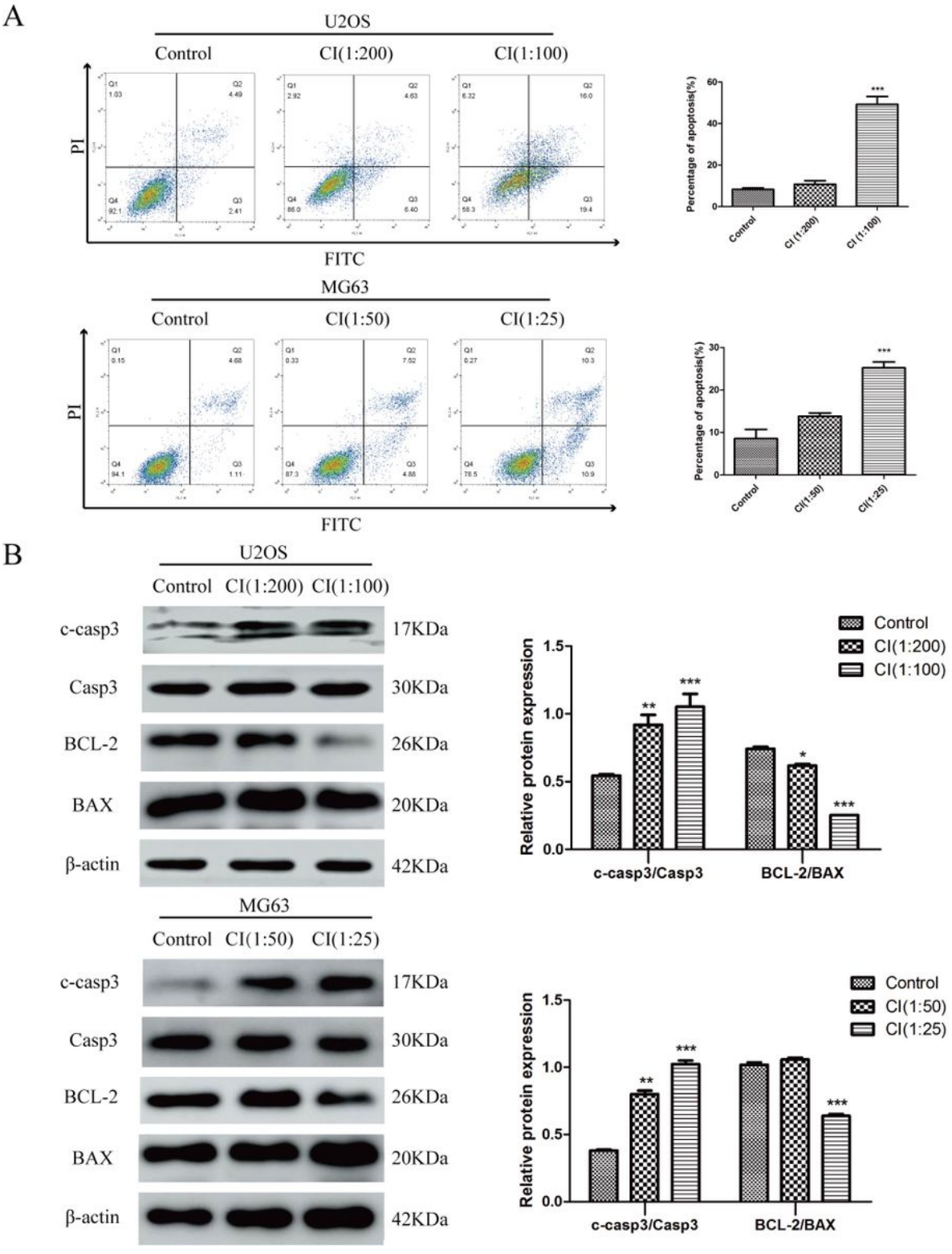

Figure 3

CI induces OS cell apoptosis. A The stained U2OS and MG63 cells exposed to CI (0, 1:200, 1:100 or 0, 1:50, 1:25) for 24 h. Apoptotic cell proportions are expressed as the mean±SD. B The expression and

statistic results of cleaved caspases 3, Caspases 3, BCL-2 and BAX in U2OS and MG63 after CI treatment. *p < 0.05, **p < 0.01, ***p < 0.001 relative to the control group.

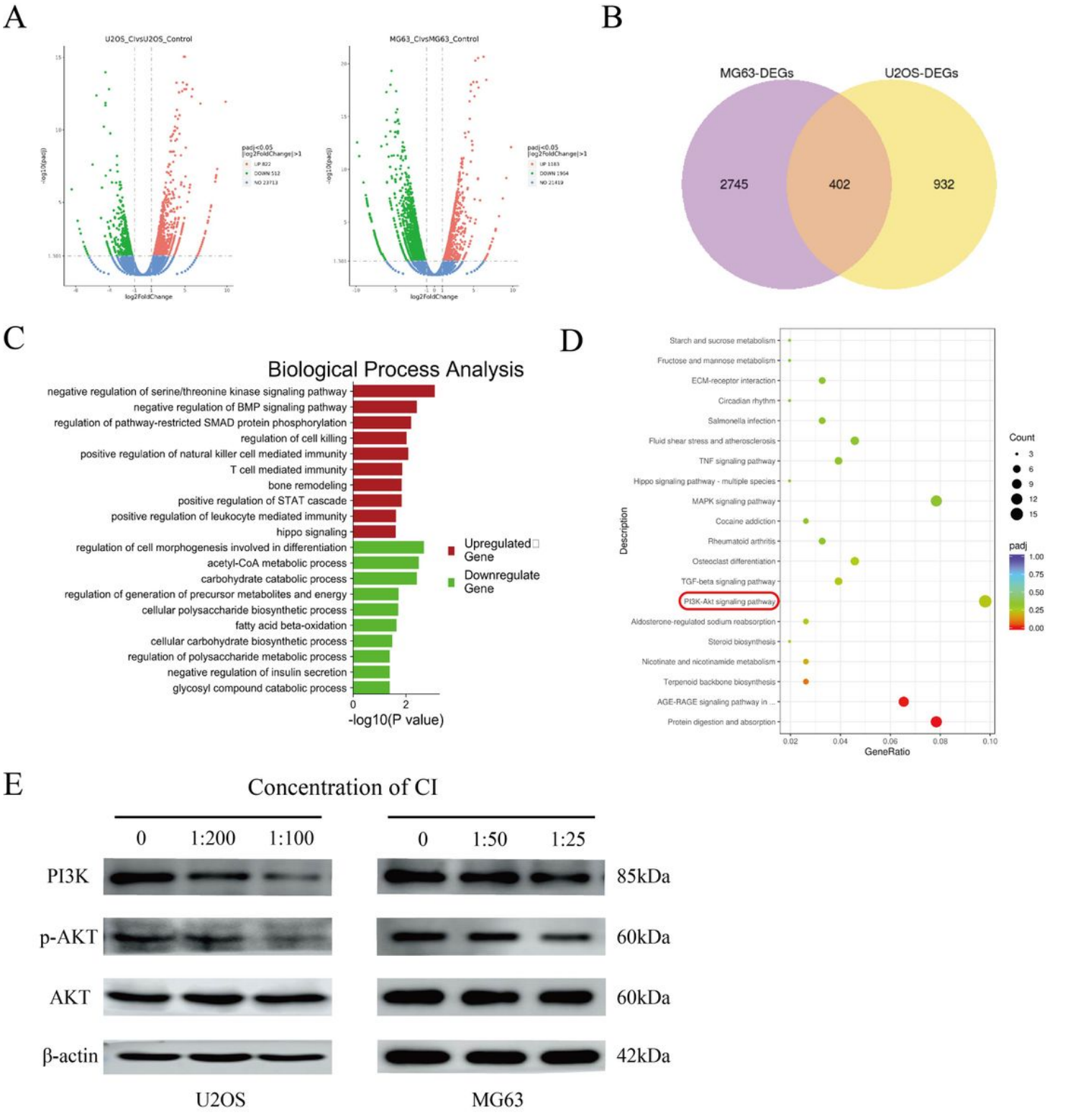

Figure 4

Analysis of CI-regulated gene expression in OS cells. A Volcano plot of the log2 Fold Change CI/control vs. the -log 10 p-value (y-axis) of the genes in U2OS and MG63 cells. B The intersection of differentially expressed genes between U2OS and MG63 cells were recognized as common differentially expressed

genes. C The cancer-related biological processes analysis of upregulated and downregulated genes in common differentially expressed genes. D The top 20 most significant KEGG pathways enriched by common differentially expressed genes. E The expression levels of PI3K, p-AKT and AKT were detected by western bolt.