

ICT1 Knockdown inhibits osteosarcoma cell proliferation by regulating cell apoptosis through targeting BCL-2

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Abstract

Background: Osteosarcoma (OS) which occurs mainly in the young people is a familiar malignant bone tumor. Apoptosis is a kind of programmed and managed cell death and promoting apoptosis of OS cells is an important treatment for OS. However, the pathways of controlling apoptosis in OS remain unclear. Immature colon carcinoma transcript-1 (ICT1) belongs to a family of four putative mitochondrial translation release factors. It controls the termination stage of translation.

Methods: In this study, the function of ICT1 was elucidated by a series of *in vivo* and *in vitro* assays. The potential downstream targets of ICT1 in the OS was confirmed by Quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting, while immunohistochemical analysis and rescue experiment were performed to confirm this result.

Results: ICT1 was significantly upregulated in osteosarcoma, and higher expression of ICT1 led to the worse survival rate. Furthermore, knockdown of ICT1 could inhibit the cell proliferation, but improved the apoptosis of cells *in vitro* and *in vivo*. In addition, our data demonstrated that BCL-2, apoptotic related-genes, is a potential target of ICT1 and the overexpression of BCL-2 partly reduced the negative influence of ICT1 knockdown on the pro-tumorigenic capabilities of OS cells *in vitro*.

Conclusion: The ICT1 protein was overexpressed in the OS and has identified a novel mechanism by which ICT1 inhibits osteosarcoma cell proliferation by regulating cell apoptosis through targeting BCL-2.

Background

Osteosarcoma is a common bone cancer in children and teenagers and originates in the interstitial cell line[1]. The heterogeneity, genomic instability and high risk of metastasis primarily to the lungs are the basic characteristics of osteosarcoma[2]. Currently, surgery, chemotherapy, immuno-therapy and gene therapy are the main treatments for osteosarcoma[3]. However, the strong proliferative and migratory capacity of osteosarcoma lead to the poor prognosis[4]. Therefore, it is important for us to investigate the novel oncogene which plays a crucial part in the beginning and progress of OS in order to develop better treatments for patients with this devastating disease.

ICT1 which is previously named DS-1 controls the termination stage of translation[5]. Besides, more further studies reported that ICT1 played a significant part in the development and progression of tumors. Several evidences have suggested that ICT1 was closely related to the proliferation in some human cancer cells, such as hepatocellular carcinoma, gastric cancer, non-small cell lung cancer and breast cancer[6-9]. However, the effect of ICT1 in OS is unclear.

Apoptosis belongs to a procedural cell death, which accounts for the vast majority of cell death in biological processes[10]. Proteins of the B-Cell CLL/Lymphoma 2 (BCL-2) family is a crucial part in the management of apoptosis and have pro-apoptotic and anti-apoptotic activities. Some proteins of this family, such as BCL-2, hinder cell death, while other members, including BAX, increase apoptosis. The

protein of BCL-2 , playing a crucial part in the control of prolonged cell survival and apoptosis, is a major anti-apoptotic protein[11]. Relevant studies showed that BCL-2 was also a protooncogene. In lung, prostate and laryngeal cancers, BCL-2 expression significantly associated with a poor prognosis[12-14]. Some recent studies reported the function of BCL-2 in OS. Apatinib promoted apoptosis via VEGFR2/STAT3/BCL-2 signaling in OS[15]. The expression of BCL-2 increased in recurrent pulmonary metastases compared with primary tumor in OS[16]. MiR-326 regulated cell survival and apoptosis by targeting BCL-2 in OS[17]. However, the correction between ICT1 and BCL-2 in OS is unclear.

In this present study, our data demonstrated that the ICT1 protein was observably increased in the OS and has identified a novel mechanism by which ICT1 inhibits osteosarcoma cell proliferation by regulating cell apoptosis through targeting BCL-2.

Materials And Methods

Cell culture and reagents

Two human osteosarcoma cell lines (MNNG-HOS,143B) and human osteoblast hFOB1.19 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The American Type Culture Collection (ATCC, Manassas, VA, USA) provided the human osteosarcoma U-2OS cell line. All cell lines were cultured under standard conditions. Human osteosarcoma cells were cultured at 37°C in a 5% CO₂ atmosphere. Meanwhile, the hFOB1.19 cells were cultured in a 5% CO₂ atmosphere at 34.5°C. Antibodies against ICT1 (AP20382b; Abgent, San Diego, CA, USA), Cleaved Caspase-3 (Immunoway, YT6161), Cleaved Caspase-9 (Immunoway, YP0598), BCL-2 (ab32124; Abcam, Cambridge, UK) and BAX (ab32503; Abcam, Cambridge, UK) were used.

Establishment of stable ICT1 knockdown cell lines and BCL-2 overexpression cell lines

The OBiO Technology (Shanghai, China) provided plasmids containing sh-ICT1 or BCL-2 and a negative control plasmid. The shRNA sequences for ICT1 are 5'-GCTGTTAATGCTTGTCTATAACTCGAGTTAGACAAGCATTACAGC-3' and 5'-GCAGAACATGAAACAAAGTGAACTCGAGTTCACTTGTTCACATTCTG C-3'. The overexpression RNA sequence for BCL-2 is : 5'-GTTGTAGCGGGACACCTACTGAAAGTTCTTCAGTAGGTGTCGGCTACAAAAAAACTTA-3'. HEK 293T cells provided by OBiO Technology (Shanghai, China) were used to package these plasmids into virus particles. Then, the titers of virus were measured. In order to constitute stable ICT1-knockdown cells and BCL-2 overexpression cells , the target cells were infected with 1×10⁸ lentivirus-transducing units with 6 mg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA). After 72 h, infected cells were screened with 2.5 mg/mL of puromycin. Finally, we used western blotting and qRT-PCR to measured the efficiency.

RNA isolation and qRT-PCR analysis

Total RNAs were extracted and reverse transcription were implemented as described before[18]. ICT1, BAX and BCL-2 transcripts were quantified with the SYBR Green qPCR Master Mix (Roche, Switzerland). The primer sequences used for measuring the expression levels of BCL2 , BAX and ICT1 are as follows: BCL-2: forward 5'-GCCCTGTGGATGACTGAGTA-3', reverse 5'-TTCAGAGACA GCCAGGAGAAA-3'; BAX: forward 5'-GCTGGACATTGGACTTCCTC-3', reverse 5'-GGCGTCCC AAGTAGGAGAG-3'; ICT1: forward 5'-CAGCCTGGACAAGCTC TACC-3', reverse: 5'-GGAACCTGACTTCTGCCTTG-3'. The reaction conditions were set up in accordance to the instructions. β -actin and 18S were used as internal controls.

Western blotting

Total cellular protein was extracted by making use of a protein extraction buffer (Beyotime, Shanghai, China). Proteins were split by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose (NC) membrane (Millipore, USA). After blocking, the membranes were incubated with the primary antibodies at 4 °C for one night, Then, the membrane were probed with the secondary antibodies. Finally, the bands were determined using a western electrochemiluminescence substrate (Share-bio, Shanghai, China).

Immunohistochemistry (IHC)

Alena Biotechnology Co., Ltd. (Xi'an, China) provided a microarray containing tissue from 40 OS patients. The IHC assay was carried out as previously described[19]. We employed the corresponding primary antibodies at 1:200 dilutions to detect the Ki67. We took photos of all the sections using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The final IHC score was counted by multiplying the intensity score with the quantity score as previously described[20]. These scores were judged independently by two veteran pathologists in a blinded manner.

Cell counting kit-8 (CCK-8) assay and Colony formation assay

The CCK-8 assay was performed according to the vendor's instructions (Dojindo Molecular Technologies, Japan). Briefly, 3×10^3 cells were seeded into 96-well plates. The absorbance at a wavelength of 450 nm was measured at 0, 24, 48, 72, 96, and 120h using a tablet reader (Thermo Fisher Scientific, Waltham, MA, USA).

The infected OS cells were cultured in a 6-well plate at an initial cell density of 1×10^3 cells/well. After two weeks, the ice-cold PBS was used to wash the colonies. Then, the cell pellet was fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Eventually, we took photos and counted cell numbers.

Cell apoptosis

In order to cell apoptosis analysis, the adherent cells were separated after culturing for 24 h in serum-free medium. Cells were stained with the Annexin V/FITC Kit (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. Finally, the flow cytometry was used to analyze results.

Xenograft Tumor in Nude Mice

The Balb/c nude mice (no sex limitation; 20-25 g) were fed and all animal experiments were taken place in the East China Normal University and were carried out according to the animal experimental protocols authorized by the Animal Care and Use Committee of the Animal Care and Use Committee of the Affiliated Hospital of Nanjing Medical University, Changzhou No.2 People's Hospital. The mice were assigned into several groups randomly. In brief, the Balb/c nude mice were anaesthetised with 1.5% pentobarbital sodium. Then, U-2OS cells (1.5×10^6) were subcutaneously inoculated into mice. The data of the tumor size was collected every 5 days. All of mice were euthanized by CO₂ After 20 days. All Tumors were removed and the weight and size data of which were recorded.

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay

The percentage of apoptotic cells in the xenograft tumors was quantified using the TUNEL kit (Roche, Basel, Switzerland). This assay was carried out as previously reported[20].

Statistical analyses

The Graphpad software was used for statistical analyses. The expression of all data was represented as mean \pm SD. The comparison between different groups was performed by Student's t-test. The $p < 0.05$ was considered to show a statistically remarkable difference.

Results

ICT1 expression increases in OS cell lines and high expression of ICT1 indicates poor survival rate of OS patients

First, the qRT-PCR analysis was used to detected the expression level of ICT1 in hFOB1.19 cells, a common osteoblast cell line, with that in human OS cell lines (MNNG-HOS, 143B and U-2OS). ICT1 was obviously upward in OS cell lines compared to that in hFOB1.19 cell line (Fig. 1A). Then, western blotting showed that ICT1 protein expression was remarkably upregulated in OS cell lines compared with hFOB1.19 cell line (Fig. 1B). Besides, the prognostic value of ICT1 in patients with OS was revealed by using a website (<http://hgserver1.amc.nl>) which is an online gene expression database. As shown in Fig. 1C, a KaplanMeier analysis showed that patients with ICT1 overexpression had a significantly poor prognosis ($p = 0.012$). In order to investigate the role of ICT1 in OS, The ICT1 stable knockdown cell lines (U-2OS and 143B cells) were built. The efficiency was validated by qRT-PCR and western blotting (Fig. 1D-F).

The knockdown of ICT1 inhibits proliferation and promotes apoptosis of OS cells *in vitro*

In order to further explore the biological role of the ICT1 in OS occurrence and development, we performed cell proliferation assay and cell apoptosis experiment. Following transfection of U-2OS and 143B cells with sh-NC, sh-1 and sh-2 vectors, we measured cell proliferation in OS cell lines. The consequences of

CCK-8 proliferation assay (Fig. 2A and B) showed that knockdown of ICT1 markedly inhibited the proliferation of OS cells. We found that the proliferation of OS cells obviously fall off when the expression of ICT1 was silenced through using colony formation assay (Fig. 2C and D). We used flow cytometry to measure the apoptosis and the consequences of cell apoptosis assay also indicated that apoptosis increased on account of the silencing of ICT1 in the OS cells (Fig. 2E and F).

BCL-2 is a potential target of ICT1

Since the knockdown of ICT1 promoted apoptosis of osteosarcoma cells, we measured the expression of BCL-2 and BAX which were apoptosis-regulating genes after the knockdown of ICT1. As is shown in Fig. 3A and B, in the U-2OS and 143B cell lines, the RNA expression of BCL-2 was markedly reduced while the RNA expression of BAX was remarkably increased after the silencing of ICT1. Then, by use of western blotting, we found that the protein expression of BCL-2 was markedly reduced and the protein expression of BAX was remarkably increased in the U-2OS and 143B cell lines after the silencing of ICT1 (Fig. 3C). Moreover, we found that Cleaved Capase-3 and Cleaved Capase-9 which are the other apoptotic genes were increased in the U-2OS and 143B cell lines (Fig. 3C). To further confirm this result, we used the tissue microarray through immunohistochemical analysis to determine the correlation between the expression of ICT1 and BCL-2. As shown in Figure 3D–3E, ICT1 protein expression levels in OS tissues have a positive correlation with the protein expression levels of BCL-2 ($r = 0.523, p < 0.001$). However, BAX and ICT1 expression have no obvious correlation. The above results indicated that BCL-2 is a potential target of ICT1.

The knockdown of ICT1 reduces cell growth of osteosarcoma cells *in vivo*

Moreover, we explored whether knockdown of ICT1 could inhibit proliferation and enhance apoptosis of U-2OS cell line *in vivo*. Firstly, The nude mice were injected subcutaneously with the stable ICT1 knockdown and control U-2OS cells. The data showed that the speed of the tumor growth was slower in the stable ICT1-knockdown group than in the control group (Fig. 4A-D). As is shown in Fig. 4E, the IHC staining and TUNEL assay indicated descending expression of Ki67 and upward proportion of apoptosis in the subcutaneous tumors of ICT1-knockdown group. Then, we confirmed that ICT1 protein level in xenografted tumor tissues had a positive correlation with the expression level of BCL-2 (Fig. 4F).

ICT1 improves proliferation and inhibits apoptosis by increasing BCL-2 expression in OS cells

The results showed that ICT1 positively regulates OS cell proliferation(Fig. 2) and we figured out whether the effects by ICT1 depend upon its target, BCL-2. First of all, we knockdowned ICT1 in wild-type and stable BCL-2 overexpression cell lines (U-2OS cells and 143B cells). The efficiency of overexpression was detected by western blotting (Fig. 5A). Then, the CCK8 assay and colony formation assay revealed that the overexpression of BCL-2 partly reversed the prohibitive functions of ICT1 knockdown on the pro-tumorigenic features of U-2OS cells and 143B cells *in vitro* (Fig. 5B-C). In a cell apoptosis assay, the effect of ICT1 knockdown on apoptosis was reversed partly by BCL-2 overexpression (Fig. 5D and E). The above data suggested the significant value of BCL-2 as a target gene of ICT1.

Discussion

Some reports have found the biological effects of ICT1 in the regulation of tumor growth and apoptosis. In the hepatocellular carcinoma, ICT1 promoted cell growth via improving cell cycle progression and resisting apoptosis[6]. In the non-small cell lung cancer, depletion of ICT1 inhibited proliferation and promote apoptosis[7]. In addition, ICT1 knockdown restrained cell growth through causing of cell cycle arrest and apoptosis in the breast cancer[9]. A recent study revealed that ICT1 was upregulated in gastric cancer and promoted its invasion and migration[8]. Whereas, the expression and biological effects of ICT1 in OS has not been studied. In our study, the data demonstrated that the levels of ICT1 expression were markedly higher in the OS cell lines by qRT-PCR and western blotting. Additional analysis showed that knockdown of ICT1 inhibited proliferation and promoted apoptosis of osteosarcoma cells *in vitro* and *in vivo*.

Apoptosis, a form of programmed and controlled cell death, is a noninflammatory or silent process[21]. Apoptosis make great contribution to cancer development and therapy and is regulated by many kinds of signaling pathways[22]. Therefore, targeting the apoptosis process has been confirmed to be an available approach for regulating tumor growth and raising anticancer medical treatment. The B-cell lymphoma 2 (BCL-2) family proteins, known as the apoptotic proteins, can be broadly divided into proapoptotic and antiapoptotic. BCL-2 which belongs to antiapoptotic is a typical class of oncogene[23]. Since Fukuhara and Rowley discovered BCL-2 protein, sufficient evidence consistently demonstrated that BCL-2 plays a huge part in cancer cell growth, metastasis, angiogenesis and apoptosis[24]. Furthermore, some recent studies confirmed that a few microRNA inhibited OS growth by effecting BCL-2 and some drugs promoted OS apoptosis through BCL-2[15, 25-27]. In our study, ICT1 can improve the cell growth, proliferation and resist the apoptosis of OS cells, which was in consistent with the previous reports. We proved that the knockdown of ICT1 can decrease the expression of BCL-2 in OS cell lines. Moreover, we confirmed that ICT1 protein levels had a positive correlation with the expression levels of BCL-2 in both xenografted tumor tissues and tissues of OS patients. The overexpression of BCL-2 partly reversed the inhibitory effects of ICT1 knockdown on proliferation of OS cell lines. Therefore, BCL-2 is very likely a target of ICT. However, the way of ICT1 regulating BCL-2 is unknown. The previous studies reported that BCL-2 is an anti-apototic molecule, which is phosphorylated at Ser70 by growth factor-activated protein kinases including PKC and the MAPKs (ERK1/2) can actively control the anti-apoptotic function of BCL-2[28-30]. It is unclear that wheather ICT1 leads to phosphorylation of BCL-2 to inhibit apoptosis. This question will be further explored in future studies.

Conclusions

In conclusion, the data of this study confirmed that ICT1 expression was increased in OS cells. ICT1 promoted OS cell growth both *in vitro* and *in vivo* by inhibiting apoptosis via increase of BCL-2 expression. This study may provided a biological perception into the evolution of OS and revealed that ICT1 can be thought as a new target for OS treatment in clinic.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

D.Z conceived and designed this study. J.X.T and Y.P.W wrote and executed the program. R.D analyzed the data. The article was written by Y.Q.J and X.H.P.

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Availability of data and materials

The data and results shown in our study are available from the corresponding author on reasonable request.

Consent for publications

Not applicable.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval The human study was approved by the Ethics Committee of the Affiliated Hospital of Nanjing Medical University, Changzhou No.2 People's Hospital. The animal experiments were approved by the Animal Care and Use Committee of the Affiliated Hospital of Nanjing Medical University, Changzhou No.2 People's Hospital.

Abbreviations

OS: osteosarcoma; ICT1: immature colon carcinoma transcript-1; DS-1: Digestion Subtraction 1; VEGFR2: Vascular Endothelial Growth Factor Receptor 2; STAT3: Signal Transducer And Activator Of Transcription 3; BCL-2: B-Cell CLL/Lymphoma 2; BAX: BCL2 Associated X; qRT-PCR: quantitative real-time polymerase chain reaction.

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Figures

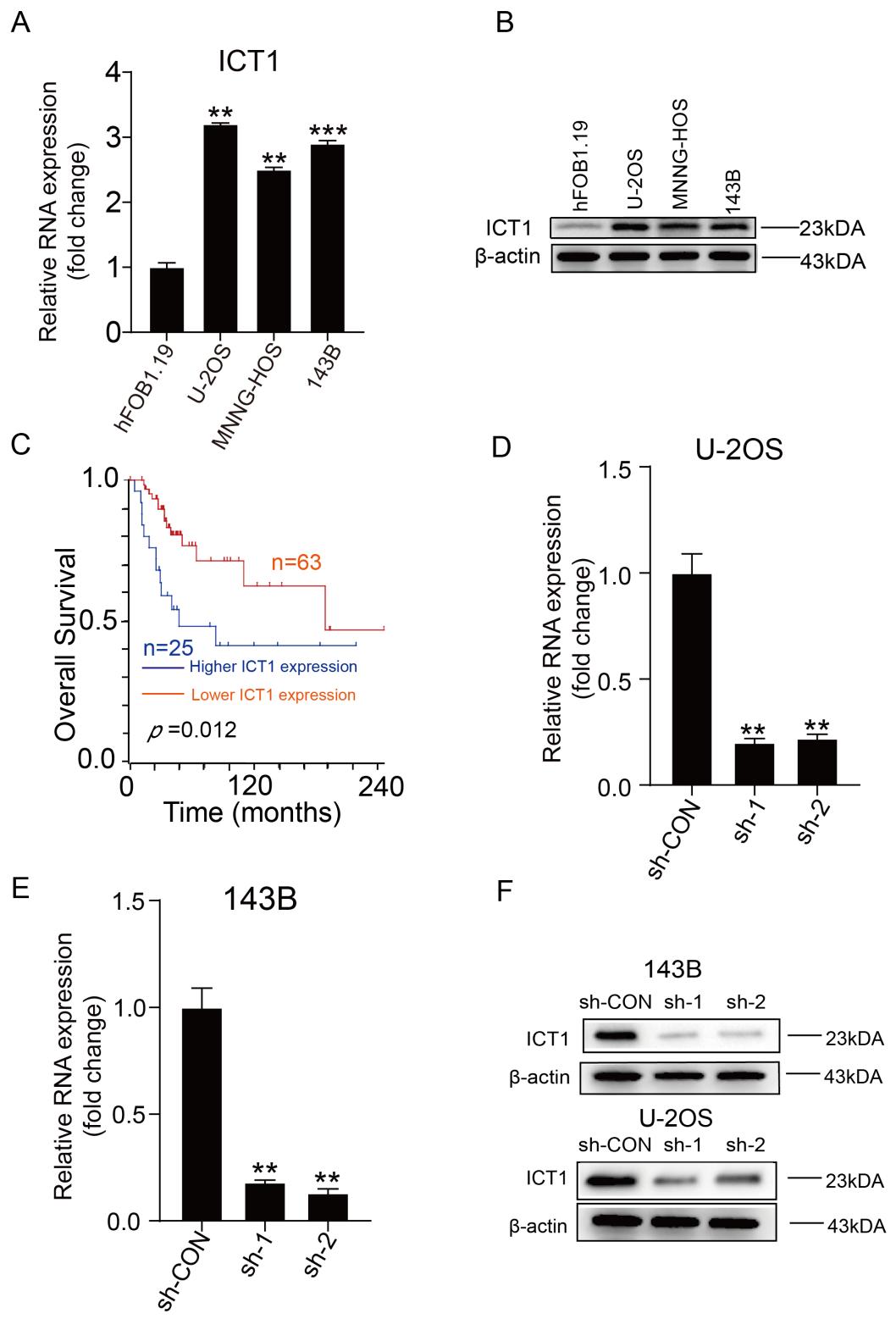


Figure 1

ICT1 expression level increases in osteosarcoma (OS) and the expression of ICT1 is closely related to the overall survival. A. The mRNA expression patterns of ICT1 in normal osteoblast cell line (hFOB1.19) and OS cell lines (U-2OS, MNNG-HOS and 143B) by qPCR. Values are means \pm SD, ** p < 0.01, *** p < 0.001 (Student's t-test). B. The protein expression patterns of ICT1 in normal osteoblast cell line (hFOB1.19) and OS cell lines (U-2OS, MNNG-HOS and 143B) by western blotting. C. Kaplan-Meier analysis of overall

survival rate related to the expression of ICT1 in 88 OS cases based on a human osteosarcoma gene expression database. (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>) D and E. Knockdown efficacy of ICT1 in wild OS cells (U-2OS and 143B) was determined by qPCR. Values are means \pm SD, ** $p < 0.01$ (Student's t-test). F. Knockdown efficacy of ICT1 in wild OS cells (U-2OS and 143B) was determined by western blotting.

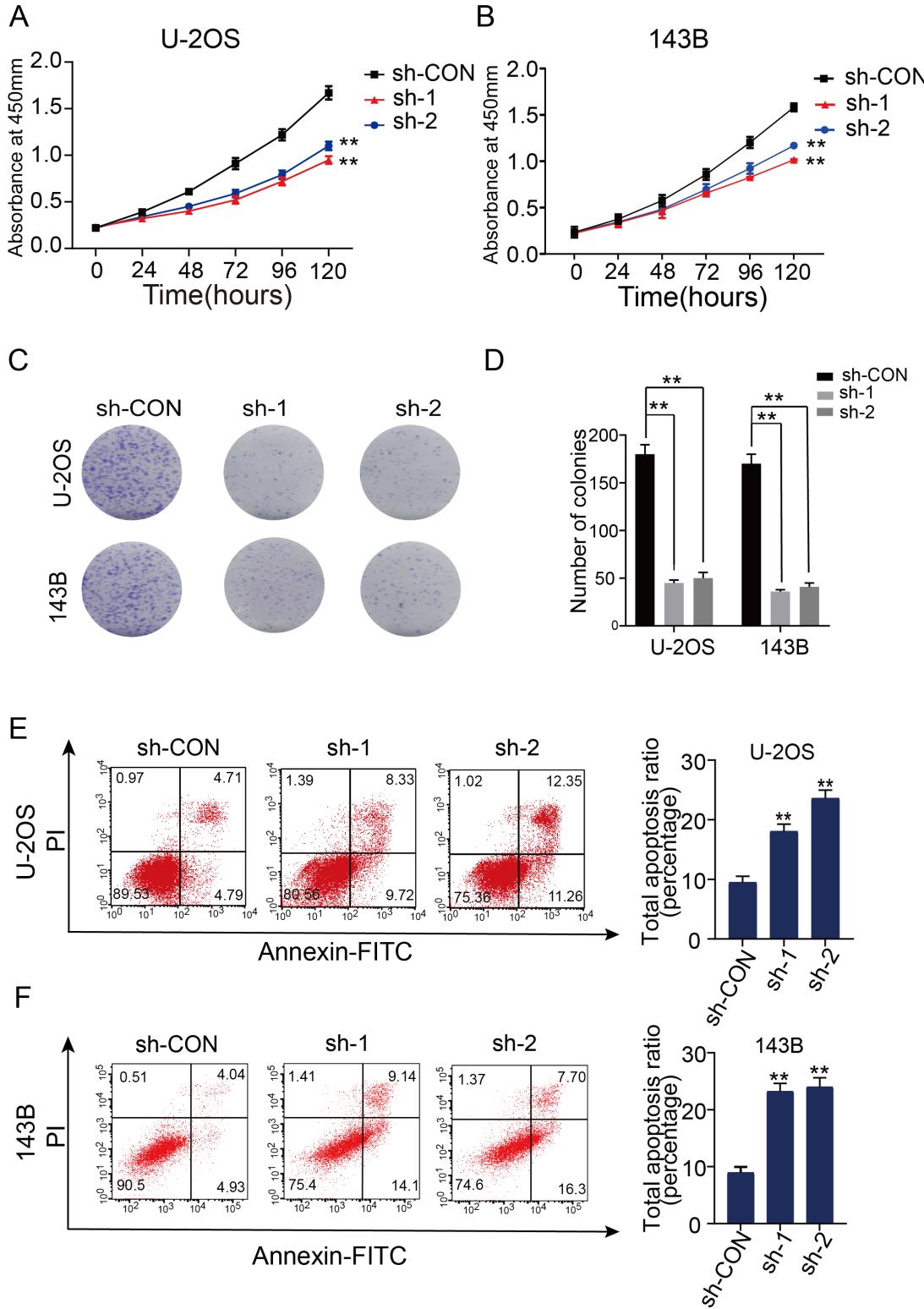
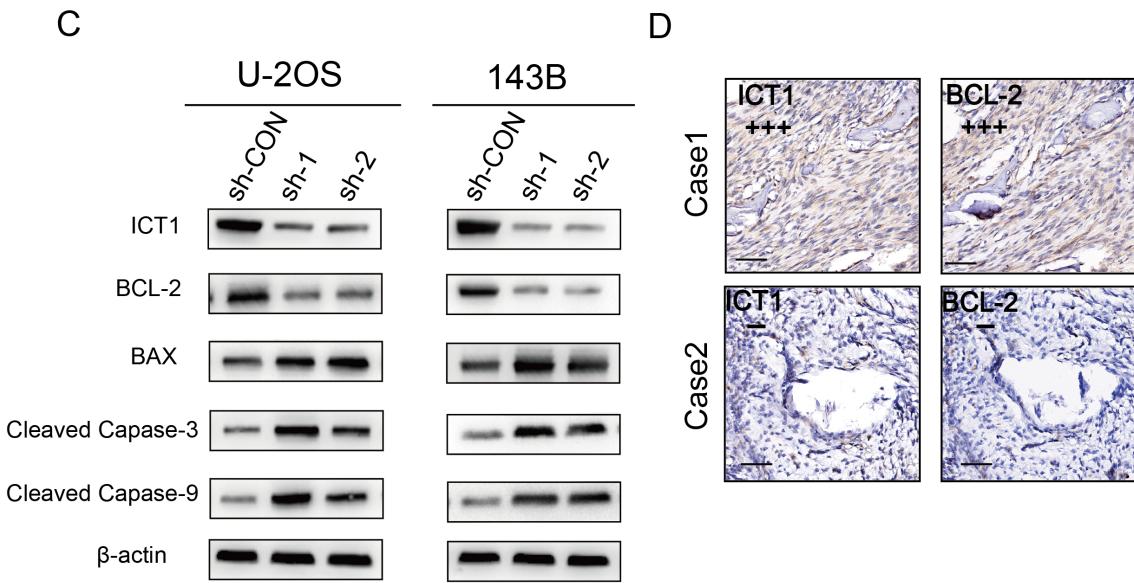
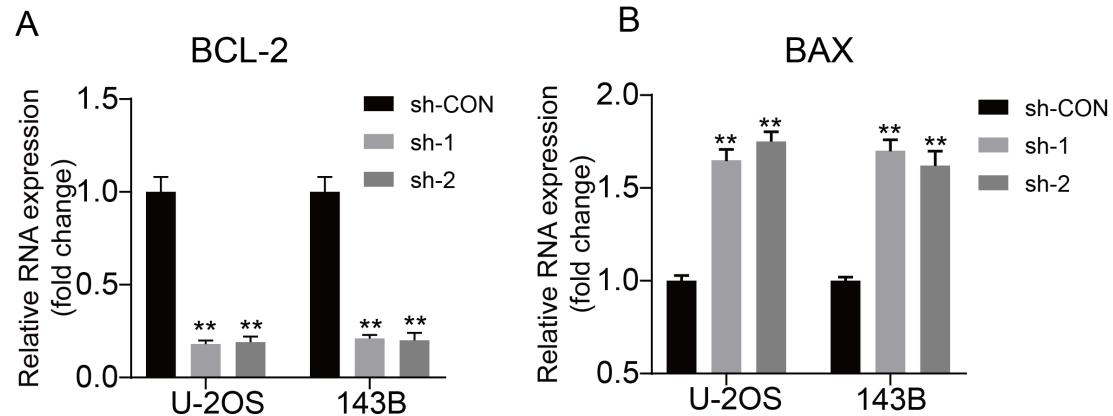


Figure 2

ICT1 promotes proliferation and inhibits apoptosis of OS cells in vitro. A and B. Knockdown of ICT1 inhibited U-2OS and 143B cells proliferation using the cell counting kit (CCK)-8 assay, Values are means ± SD, **p < 0.01 (Student's t-test). C and D. Knockdown of ICT1 suppressed OS cells (U-2OS and 143B) proliferation using colony formation assay. Representative photographs of the colony formation assay were shown in the left panel. Values are means ± SD, **p < 0.01 (Student's t-test). E and F. Knockdown of ICT1 significantly induced U-2OS and 143B cell apoptosis, Values are means ± SD, **p < 0.01 (Student's t-test).



E
Correlation between ICT1 and BCL-2

IHC grade	ICT1				
	— (n=6, %)	+(n=7, %)	++(n=23, %)	+++(n=4, %)	
BCL2	— (n=8)	4 (66.7)	2 (28.6)	2 (8.7)	0 (0.0)
	+	1 (16.7)	4 (57.1)	6 (26.1)	0 (0.0)
	++	1 (16.7)	1 (14.3)	13 (53.5)	1 (25.0)
	+++	0 (0.0)	0 (0.0)	2 (8.7)	3 (75.0)

Figure 3

BCL-2 is a target of ICT1 in OS cells. A and B. The mRNA expression levels of BCL-2 and BAX in sh-Control and sh-ICT1 OS cells (U-2OS and 143B), Values are means \pm SD, ** $p < 0.01$ (Student's t-test). C. The protein expression levels of BCL-2 and BAX was detected using western blotting in sh-Control and sh-ICT1 OS cells (U-2OS and 143B). D. IHC analysis showed representative positive (up) and negative (down) staining of ICT1 and BCL-2 in consecutive sections, Scale bar: 50 μ m. E. Statistical analysis of the correlation between ICT1 and BCL-2 expression in human osteosarcoma tissue microarrays. p value was calculated by the Spearman rank correlation test.

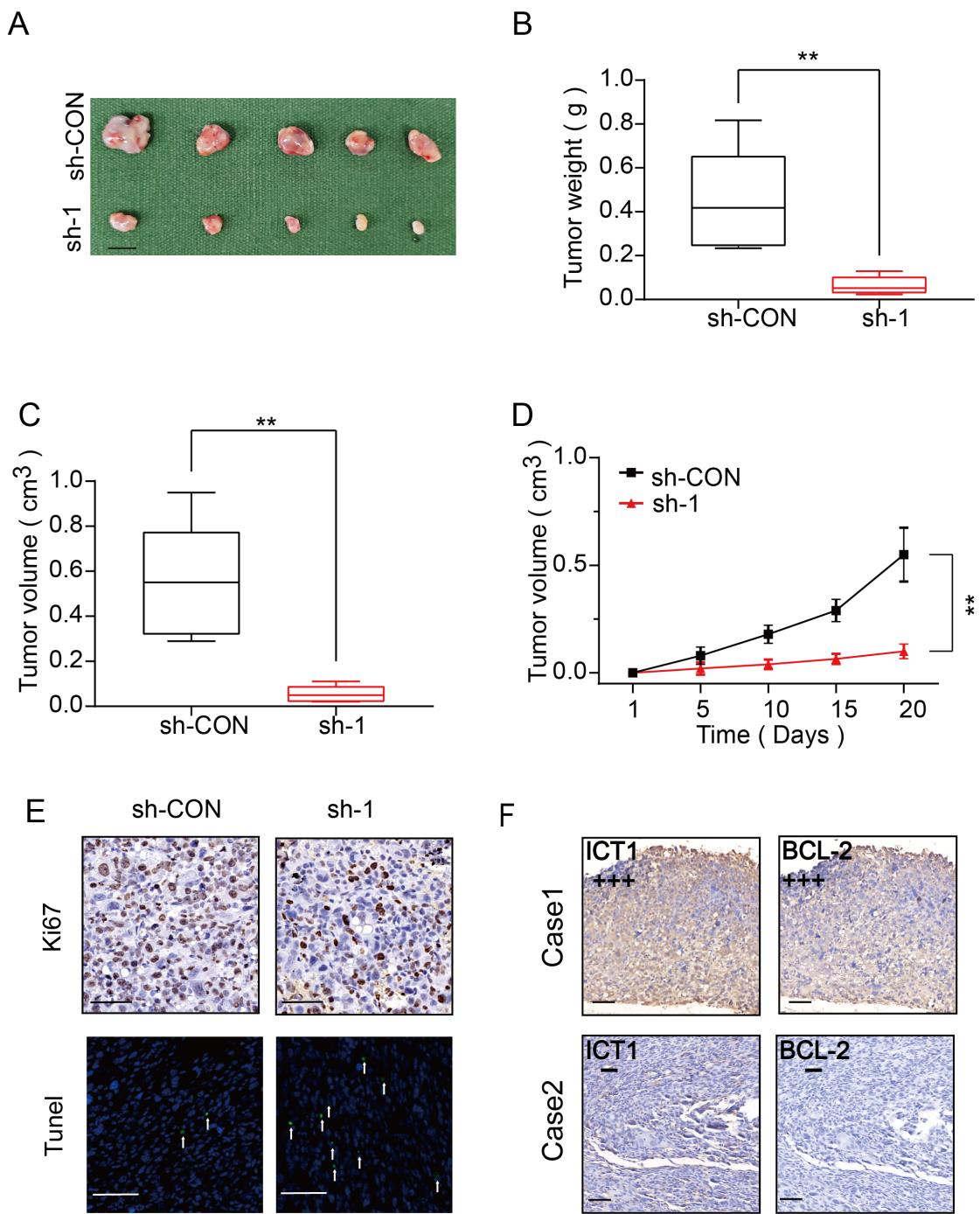


Figure 4

ICT1 promotes OS proliferation in vivo. A. Morphologic characteristics of excised tumors from nude mice in U-2OS/control group and U-2OS/ ICT1 knockdown group (n = 5). Scale bars= 1 cm. B. Tumor weight in sh-ICT1 group was reduced compared with sh-Control group (n = 5), Values are means \pm SD, **p < 0.01 (Student's t-test). C. Tumor volume in sh-ROBO1 group was reduced compared with sh-Control group (n = 5), Values are means \pm SD, **p < 0.01 (Student's t-test). D. Tumor volumes were measured with calipers

every 5 days. The growth rate in sh-ICT1 group was significantly slower than that in sh-Control group ($n = 5$), Values are means \pm SD, ** $p < 0.01$ (Student's t-test). E. Representative images of Ki67 and TUNEL staining in tissues from sh-ICT1 and sh-Control mice. A TUNEL-positive cell is indicated (arrow). F. IHC analysis showed representative positive (up) and negative (down) staining of ICT1 and BCL-2 in consecutive sections of xenografted tumor tissues, Scale bar: 50 μ m.

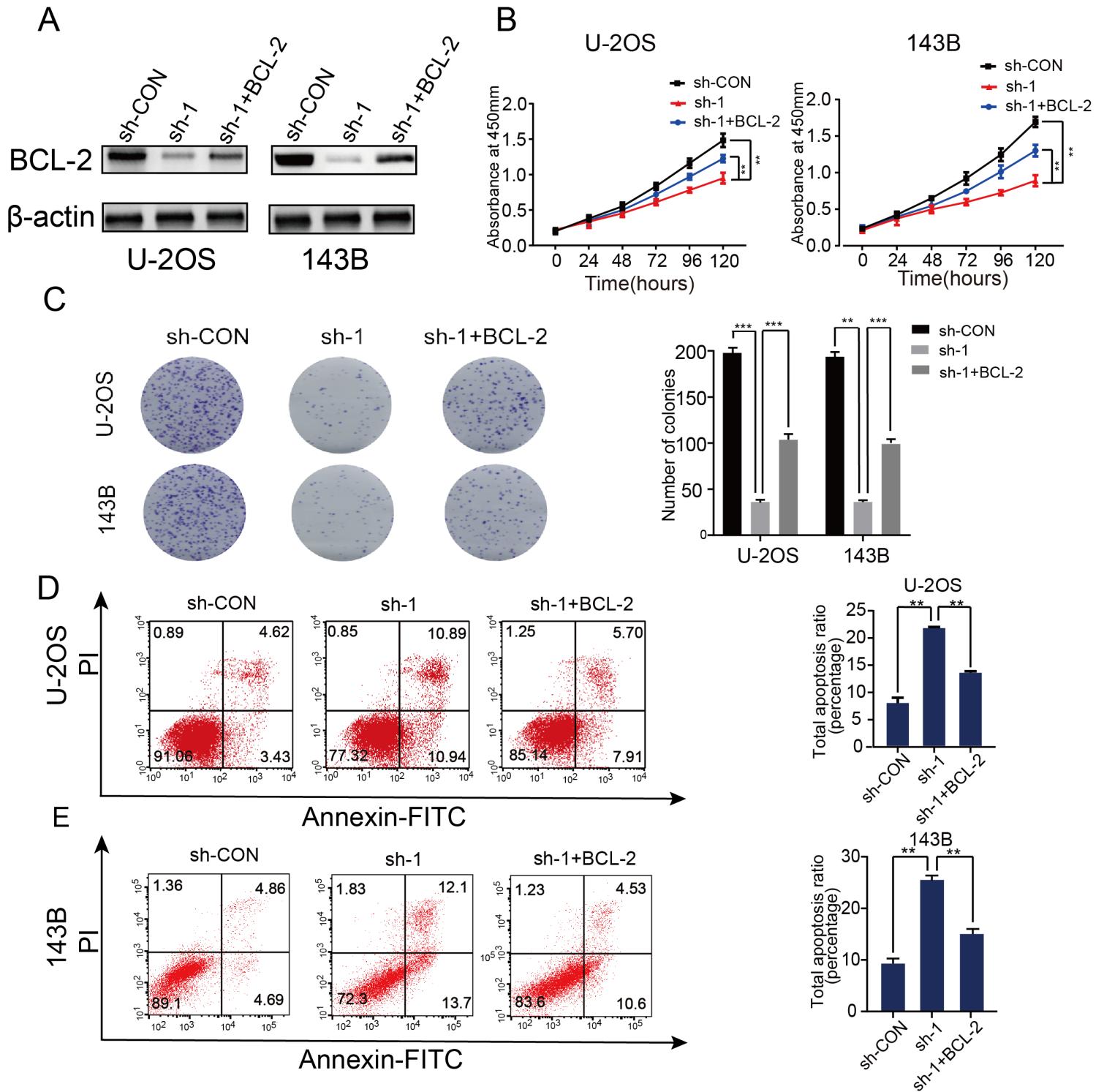


Figure 5

The effect of ICT1 knockdown on osteosarcoma cell proliferation was reversed partly by BCL-2 overexpression A. Overexpression efficacy of BCL-2 in sh-ICT1 OS cells (U-2OS and 143B) was detected by western blotting. B. Overexpression of BCL-2 partly reversed the suppressed effects of ICT1-knockdown on the CCK8 assay of U-2OS and 143B cells, values are means±SD, ***p <0.01 (Student's t-test). C. Overexpression of BCL-2 partly reversed the suppressed effects of ICT1-knockdown on the colony formation capability of U-2OS and 143B cells, values are means±SD, ***p <0.01 (Student's t-test). D and E. Overexpression of BCL-2 partly reversed the induce effect of ICT1-knockdown on the apoptosis of OS cells (U-2OS and 143B). Values are means±SD, ***p <0.01 (Student's t-test).