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 common malignant bone tumor. Apoptosis is a form of programmed and controlled 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) which is previously named DS-1 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 identified by qRT-PCR and western blotting, while immunohistochemical analysis and rescue experiment were performed to confirm this result.

Results

ICT1 was upregulated in OS, and higher expression of ICT1 was associated with worse overall survival rate. Functional studies suggested that knockdown of ICT1 could inhibit OS cell proliferation *in vitro* or *in vivo*. In addition, suppression of ICT1 could promote apoptosis of OS cells. Furthermore, we demonstrated that BCL-2, apoptotic related-genes, is a potential target of ICT1 and BCL-2 overexpression partly reversed the inhibitory effects of ICT1 knockdown on the pro-tumorigenic properties 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 primary bone malignancy in children and adolescents 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 role in the initiation and progression of OS in order to develop better treatments for patients with this devastating disease.

Immature colon carcinoma transcript-1 (ICT1) which is previously named DS-1 controls the termination stage of translation[5]. Besides, more further studies reported that ICT1 played a significant role in the occurrence and development of tumors. Several evidences have suggested that ICT1 was closely associated with proliferation in some human cancer cells, such as hepatocellular carcinoma, non-small cell lung cancer, gastric cancer and breast cancer[6–9]. However, the function of ICT1 in OS remains unclear.

Apoptosis is a procedural cell death and accounts for the vast majority of cell death in biological processes[10]. Proteins of the B-Cell CLL/Lymphoma 2 (BCL-2) family play an important role in the regulation of apoptosis and have pro-apoptotic and anti-apoptotic activities. Some members 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 role in the regulation 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 correlation between ICT1 and BCL-2 in OS is unknown.

In this study, we demonstrated that 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.

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 human osteosarcoma U-2OS cell line were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured under standard conditions. Human osteosarcoma cells were cultured at 37°C in a 5% CO₂ atmosphere. Meanwhile, the standard culture conditions were used for hFOB1.19 cells (34.5°C in a 5% CO₂ atmosphere). 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'-GCTGTTAATGCTTGTCTATAACTCGAGTTATAGACAAGCATTAAACAGC-3' and 5'-

GCAGAATGTGAACAAAGTGAAGTCGAGTTCACTTTGTTTACATTCTG C-3'. The overexpression RNA sequence for BCL-2 is : 5'-GTTGTAGCGGGACACCTACTGAAAGTTCTCTTCAGTAGGTGTCCCGCTACAAAAAACTTA-3'. HEK 293T cells were used to package these plasmids into virus particles and the viral titers were measured. In order to establish stable ICT1-knockdown cells and BCL-2 overexpression cells, the target cells were infected with 1×10^8 lentivirus-transducing units with 6 mg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA). Then, infected cells were screened with 2.5 mg/mL puromycin after 72 h. The efficiency was determined by western blotting and qRT-PCR.

RNA Isolation And qRT-PCR

Total RNAs were extracted and reverse transcription were performed as described before[18]. ICT1, BAX and BCL-2 transcripts were quantified with the SYBR® Premix Ex Taq™ kit (Takara Bio, Otsu, Japan). The primers used for detecting the messenger RNA (mRNA) 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'-GGCGTCCCAAAGTAGGAGAG-3'; ICT1: forward 5'-CAGCCTGGACAAGCTC TACC-3', reverse: 5'-GGAACCTGACTTCTGCCTTG-3'. The reaction conditions were: 95 °C for 5 min, 95 °C for 5 sec, 60 °C for 30 sec, and the fluorescence signal was detected from 40 cycles. β -actin and 18S were used as internal controls.

Western Blotting

Total cellular protein was extracted by using a protein extraction buffer (Beyotime, Shanghai, China). Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose (NC) membrane. After blocking, the membranes were probed with the following specific antibodies: anti-ICT1 (1:1000), anti-BCL-2 (1:1000), anti-BAX (1:1000), Cleaved Caspase-3 (1:10000), Cleaved Caspase-9 (1:1000) and anti- β -actin (1:2000), followed by species-specific secondary antibodies (1:10000). Finally, the bands were detected using a western electrochemiluminescence substrate (Share-bio, Shanghai, China).

Immunohistochemical Staining

A microarray containing tissue from 40 OS patients was obtained from Alena Biotechnology Co., Ltd. (Xi'an, China). The IHC assay was carried out as previously described[19]. Ki67 were detected employing the corresponding primary antibodies at 1:200 dilutions. We took photos of all the sections using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Scoring was measured according to the ratio of positive staining cells: 0–5% scored 0; 6–35% scored 1; 36–70% scored 2; more than 70% scored 3 and staining intensity: no staining scored 0, weakly staining scored 1, moderately staining scored 2 and strongly staining scored 3. The final score was determined using the percent of positive cell score $\times$

staining intensity score as follows: a score of 0–1 is “–”, a score of 2–3 is “+”, a score of 4–6 is “+ +” and a score of > 6 is “+ + +”; a total score < 4 was defined as low expression and a total score ≥ 4 was defined as high expression. These scores were judged independently by two senior pathologists in a blinded manner.

Cell Counting Kit-8 (CCK-8) Assay, Colony Formation Assay

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

The infected cells were seeded into a 6-well plate at an initial cell density of 1×10^3 cells/well. Colonies were washed with ice-cold PBS after two weeks. Then, the cell pellet was fixed with paraformaldehyde and stained with 0.5% (w/v) crystal violet. Finally, we took photographs and counted cell numbers.

Cell Apoptosis

For cell apoptosis analysis, the adherent cells were separated with 0.25% trypsin (without ethylenediaminetetraacetic acid) after culturing for 24 h in serum-free medium. Cells were stained with the Annexin V/FITC Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's explanations and analyzed by flow cytometry.

Xenograft Tumor In Nude Mice

The BALB/C nude mice (no sex limitation; 5-week-old) 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 approved by the East China Normal University Animal Care Commission. The mice were randomly assigned into several groups. 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. Tumor size was measured every 5 days. After 20 days, all mice were euthanized by CO₂. Tumors were removed, the weight and size of which were recorded.

Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-end Labeling (TUNEL) Assay

The proportion of apoptotic cells in the xenograft tumors was quantified using the TUNEL kit (Roche, Basel, Switzerland). This assay was performed as previously described[20].

Statistical Analyses

The Prism 5.0 software for Windows (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses. The expression of data was represented as mean $\pm$ SD. Student's t-test was used to perform the comparison between different groups. A p -value < 0.05 was considered statistically significant.

Results

ICT1 expression is upregulated in OS cell lines and ICT1 overexpression indicates poor prognosis of OS patients

First, the qRT-PCR analysis was used to compared the expression level of ICT1 in hFOB1.19 cells, a common osteoblast cell line, with that in human OS cell lines (MNNG-HOS, U-2OS, and 143B). ICT1 was markedly upregulated in OS cell lines compared to that in normal human osteoblast cell lines (Fig. 1A). Then, western blotting revealed that ICT1 protein expression was markedly increased in OS cell lines compared with normal human osteoblast cell lines (Fig. 1B). Furthermore, the prognosis of ICT1 in patients with OS was revealed by using a website (<http://hgserver1.amc.nl>) which is an online human OS gene expression database. As shown in Fig. 1C, a KaplanMeier analysis showed that patients with a higher expression of ICT1 had a significantly lower overall survival rate ($p = 0.012$). In order to investigate the function of ICT1 in OS, we established ICT1 stable knockdown cell lines (U-2OS and 143B cells). The knockdown efficiency was confirmed by qRT-PCR and western blotting (Fig. 1D-F).

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

In order to further explore the biological functions of the ICT1 in OS progression, 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. The results of CCK-8 proliferation assay (Fig. 2A and B) showed that knockdown of ICT1 significantly inhibited the proliferation of OS cells. Colony formation assay (Fig. 2C and D) revealed that the proliferation of OS cells obviously decreased when we reduced the expression of ICT1. We used flow cytometry to detect the apoptosis and the results 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 shown in Fig. 3A and B, the RNA expression of BCL-2 was markedly reduced and the RNA expression of BAX was remarkably increased in the U-2OS and 143B cell lines after the knockdown 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 knockdown 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 to determine the correlation between ICT1 and BCL-2 expression by immunohistochemical analysis. As shown in Fig. 3D–3E, ICT1 protein levels in OS tissues have a positive correlation with the 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*. The nude mice were injected subcutaneously with the stable ICT1 knockdown and control U-2OS cells. The results 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 shown in Fig. 4E, the IHC staining and TUNEL assay indicated descending expression of Ki67 and rising rate 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

We demonstrated that ICT1 promotes OS cell proliferation (Fig. 2); next, we tested whether these functions by ICT1 depend on its target, BCL-2. We first knockdowned ICT1 in wild-type and stable BCL-2 overexpression cell lines (U-2OS cells and 143B cells). Overexpression efficiency was confirmed by western blotting (Fig. 5A). As revealed by the CCK8 assay and colony formation assay, BCL-2 overexpression partly reversed the inhibitory effects of ICT1 knockdown on the pro-tumorigenic properties 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). These results validate the functional importance of BCL-2 as a target gene of ICT1.

Discussion

Some reports have discovered the important roles 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 inhibited cell growth via induction 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]. However, the expression and function of ICT1 in OS has not been studied. Here, we demonstrated that the levels of ICT1 were obviously higher in the OS cell lines compared to that in normal human osteoblast cell lines by qRT-PCR and western blotting. Cell proliferation assay and cell apoptosis assay also revealed 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 effective method for regulating tumor growth and enhancing anticancer therapies. 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 is important for 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 inhibit the apoptosis of OS cells, which was in consistent with the previous reports. We demonstrated 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. BCL-2 overexpression 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, whose phosphoylation at Ser70 by growth factor-activated protein kinases including PKC and the MAPKs (ERK1/2) can hibitpositively regulate the anti-apoptotic function of BCL-2[28–30]. It is unclear that wheather ICT1 leads to phosphoylation of BCL-2 to inhibit apoptosis. This question will be further explored in future studies.

Conclusions

In conclusion, we first demonstrated that ICT1 expression was upregulated in OS cells. ICT1 promoted OS cell proliferation both *in vitro* and *in vivo* by dampening apoptosis via increase of BCL-2 expression. Our findings offered perception into the biology of OS progression and revealed that ICT1 can be thought as a new molecular target for OS treatment.

Abbreviations

OS: osteosarcoma; ICT1: immature colon carcinoma transcript-1; DS-1: Digestion Substraction 1; BCL-2: B-Cell CLL/Lymphoma 2; BAX: BCL2 Associated X; qRT-PCR: quantitative real-time polymerase chain reaction.

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 All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figures

Figure 1

ICT1 expression level increases in osteosarcoma (OS) and the 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 $\pm$ 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 $\pm$ 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 $\pm$ 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, %)
BCL-2	— (n=8)	4 (66.7)	2 (28.6)	2 (8.7)	0 (0.0)
	+ (n=11)	1 (16.7)	4 (57.1)	6 (26.1)	0 (0.0)
	++ (n=16)	1 (16.7)	1 (14.3)	13 (53.5)	1 (25.0)
	+++ (n=5)	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-ROB01 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 $\pm$ 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 $\pm$ 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 $\pm$ SD, ***p <0.01 (Student's t-test).