

Original Article

Inhibition of beclin1 affects the chemotherapeutic sensitivity of osteosarcoma

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Abstract: This study was conducted to explore the role of autophagy in cisplatin-resistant osteosarcoma. Cisplatin-resistant osteosarcoma cell line (MG63/DDP) was obtained from parental MG63 by treating cisplatin with an intermittent stepwise selection protocol. The autophagy in MG63/DDP and MG63 was fully analyzed by immunofluorescence and western blot analysis. Meanwhile, the autophagy and the sensitivity to cisplatin for MG63/DDP and MG63 after inhibition of beclin1 were analyzed *in vitro* and *in vivo*. Increased autophagy was observed in cisplatin resistant MG63/DDP cells and in the cisplatin-treated MG63 and MG63/DDP cells. Meanwhile, inhibition the beclin1 significantly inhibited the formation of autophagosome and resulted in the increase in the sensitivity to cisplatin for both MG63 and MG63/DDP cells *in vitro* and *in vivo*. In conclusion, autophagy is implicated in the cisplatin resistant osteosarcoma, and inhibition of beclin1 could be a target for improving osteosarcoma therapy.

Keywords: Cisplatin, osteosarcoma, autophagy, beclin1, MG63

Introduction

Osteosarcoma (OS) is the most common primary bone tumor in humans, comprising 2.4% of all malignancies in pediatric patients and approximately 20% of all primary bonecancers [1, 2]. The chemotherapy with anticancer drugs, like cisplatin, doxorubicin, and methotrexate, has been accepted to be one of the most effective therapeutic approaches for the treatment of osteosarcoma [3-5]. However, the acquired drug resistance developed during the therapy has become important obstacle and reduced the survival rate of patients. To dates, many possible mechanisms responsible for resistance to anticancer drugs, especial for cisplatin, have been identified, including reduced cisplatin uptake, increased DNA repair, disruption of apoptosis, and detoxification of cisplatin [6-9]. Autophagy, a highly conserved survival response to growth limiting conditions, is associated with various anticancer drugs-resistant cancers [10-12]. However, the role of autophagy in cancer remains controversial. Constitutive autophagy may be a necessary way to protect against cancer through removing damaged

organelles and recycling macromolecules; while it may help cancer cells survive in response to cytotoxic drugs [13, 14]. Although we have reports indicating autophagy is implicated in the cisplatin resistant osteosarcoma [15-17], further investigation merits to be conducted to gain more evidence about the role of autophagy in cisplatin resistant osteosarcoma, especially evidence from *in vivo*. Based on the concept that the better understanding in molecular basis of such resistance, the more specific and effective strategy could be obtained. In this study, the autophagy was determined in cisplatin resistant osteosarcoma from cellular level, and its regulation was further validated *in vitro* and *in vivo*.

Materials and methods

Drugs and antibodies

Cisplatin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Paclitaxel, methotrexate and 5-fluorouracil were purchased from (Tianjin Jinyao Amino Acid Co., Ltd. Tianjing, China). Antibodies for beclin1, LC-I, and LC-II were

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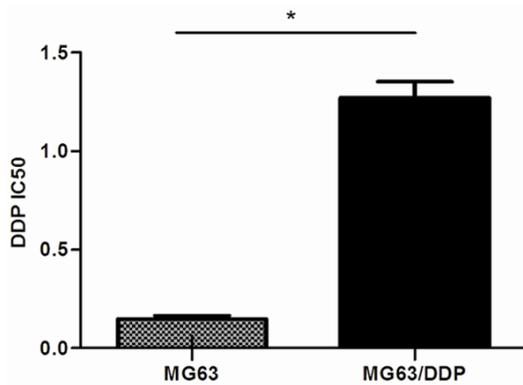


Figure 1. The 50% inhibitory concentrations (IC₅₀) of cisplatin in MG63 and MG63/DDP were calculated. MG63: Human osteosarcoma cell line; MG63/DDP is the cisplatin-resistant cell developed from the parental MG63 cells using an intermittent stepwise selection protocol. *means $P < 0.05$ compared to the controls with student's T-test ($n = 4$).

obtained from Santa Cruz Biotechnology, Inc. (Dallas, Texas U.S.A.).

Cell culture and treatment

Human osteosarcoma cell line (MG63) was purchased from the American Type Culture Collection (ATCC® CRL-1427™). All the cells were cultured in RPMI 1640 medium supplemented with 10% of fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Life Technologies/Gibco, Grand Island, NY, USA). The cells were grown at 37°C in a humidified incubator with 5% CO₂. The cisplatin-resistant MG63/DDP cells were developed from the parental MG63 cells using an intermittent stepwise selection protocol [18] over 6 months, ending with exposure to 1.2 g/L cisplatin. Before each experiment, MG63/DDP cells were cultured in drug-free RPMI 1640 medium for 2 weeks. MG63 and MG63/DDP were conducted to cytotoxicity assay [19, 20] and morphological evaluation by light microscopy and electron microscopy [11].

Immunofluorescence staining

Autophagosome formation in MG63 or MG63/DDP was examined by immunofluorescence with Lyso-ID® Green Detection Kit, which was purchased from Enzo Life Sciences, Inc., (Shanghai, China). The analysis was conducted according to the protocol of the kits.

Western blot analysis

Cell lysates were prepared with cell lysis buffer (20 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 1 mmol/L Na₂EDTA; 1 mmol/L EGTA; 1% Triton; 2.5 mmol/L sodium pyrophosphate; 1 mmol/L β-glycerophosphate; 1 mmol/L Na₃VO₄; 1 mg/mL leupeptin; 1 mmol/L phenylmethylsulfonyl-fluoride (PMSF); and 1 mmol/L PMSF). The lysates were cleared by centrifugation and total protein concentration was measured with the bicinchoninic acid assay Kit (Bio-Rad Laboratories). Proteins were resolved on a denaturing 10% SDS-PAGE gel and subsequently transferred to polyvinylidene fluoride membranes via semidry transfer. The membrane was then blocked in 5% dried milk or 3% bovine serum albumin in Tris-buffered saline and Tween 20 (10 mmol/L Tris, pH 7.5; 100 mmol/L NaCl; and 0.1% Tween 20), incubated with primary antibodies, and then with appropriate horseradish peroxidase-conjugated secondary antibodies. The signals were visualized by enhanced chemiluminescence (Pierce). Actin protein was used as a loading control.

Beclin1 inhibition by deoxyribozyme

Deoxyribozyme (DZ) sequences targeting human beclin1 (GenBank Accession NM_003766.3) and non-target sequences were constructed by Shenggon (Shanghai, China). The beclin1 DZ sequence (DZ) was *T*TG-ATGGAA GGCTAGCTACAACGA AGGAGCC*G*C, and that of non-target DZ (CON) was *T*TGATGGAA AAGAGGACCCGTCAT AGGAGCC*G*C. Transfections with DZ were performed using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Briefly, MG63 or MG63/DDP cells were placed into 6-well plates, and transfected the next day with 2 µM DZ or CON. Cells were harvested 2 days after transfection; whole cell lysates were isolated for western blots or real-time PCR. For cell viability assay, transfected cells were treated with cisplatin for 24 h, followed by cell viability assay.

Quantitative real-time PCR

cDNA from various cell samples were amplified by real-time quantitative PCR with specific primers for Beclin1 (upper CTCTCGCAGATT-CATCCCC, lower GACGTTGAGCTGAGTGTCCA)

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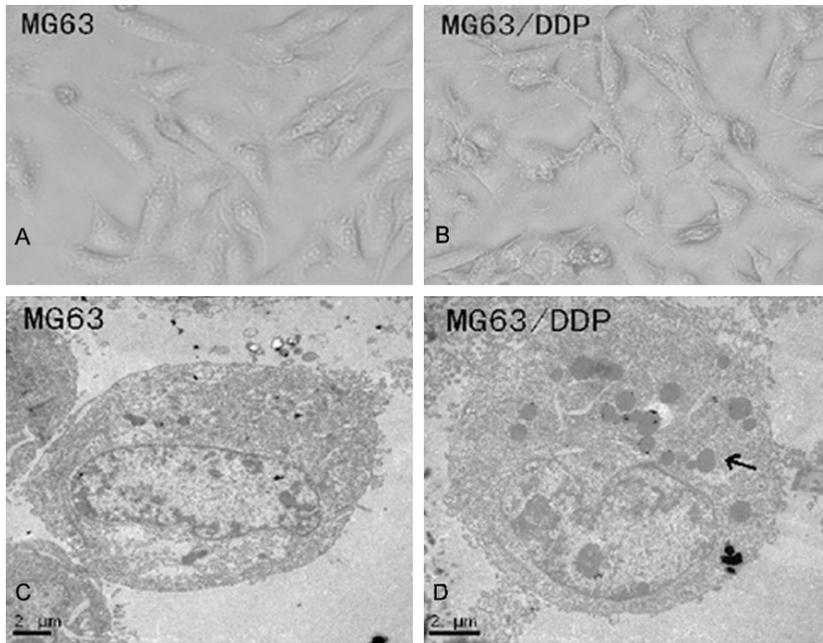


Figure 2. Morphological analysis of MG63/DDP and MG63 cells. A: Morphological analysis of MG63 cells under light microscopy. B: Morphological analysis of MG63/DDP cells under light microscopy. C: Morphological analysis of MG63 cells under electron microscopy. D: Morphological analysis of MG63/DDP cells under electron microscopy. MG63: Human osteosarcoma cell line; MG63/DDP is the cisplatin-resistant cell developed from the parental MG63 cells using an intermittent stepwise selection protocol.

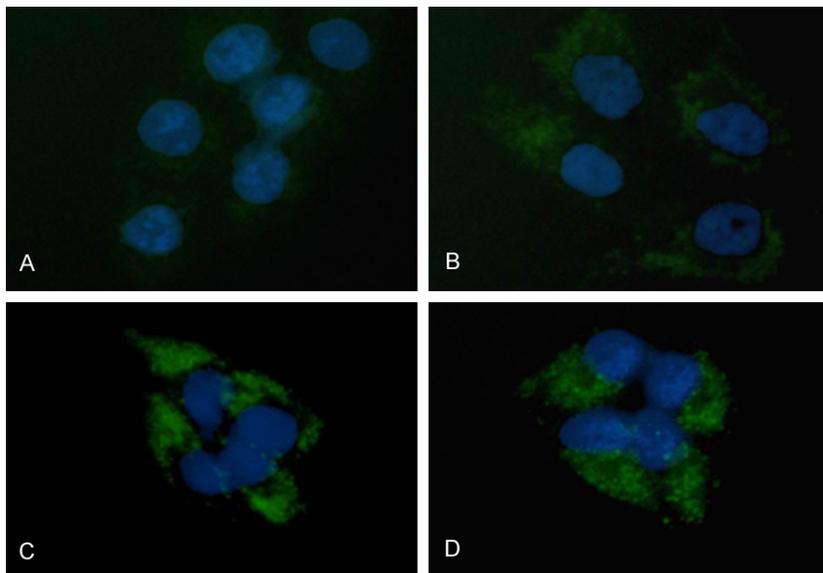


Figure 3. Autophagosome analysis of MG63/DDP and MG63 cells. A: MG63 cells were analyzed by immunofluorescence staining. B: MG63/DDP cells were analyzed by immunofluorescence staining. C: MG63 cells treated with DDP were analyzed by immunofluorescence staining. D: MG63/DDP cells treated with DDP were analyzed by immunofluorescence staining. MG63: Human osteosarcoma cell line; MG63/DDP is the cisplatin-resistant cell developed from the parental MG63 cells using an intermittent stepwise selection protocol; DDP: cisplatin.

and beta-actin (upper G G A C C T G A C T G A CTACCTC, lower TCATAC T T C C T G C T T G C - TG) with the iQ SYBR Green Supermix (Bio-Rad). Data were normalized to GAPDH expression. The control group was set as 1.

Mice xenograft models

To generate murine subcutaneous tumors, 1×10^7 MG63/DDP or MG63 cells were injected subcutaneously to the right of the dorsal midline in BALB/C mice (The Jackson Laboratory) as previously described [15, 21]. When the subcutaneous tumor size had reached the size of approximately 60-80 mm³, and the mice then assigned into four groups randomly for receiving the following treatment: 1) DZ; 2) CON; 3) DZ and cisplatin; 4) CON and cisplatin. DZ and CON were injected every other day for 7 times at dose of 34.5 mg/m¹ for 0.2 ml. Cisplatin was given through venous blood from the tails every other day for 7 times at dose of 34.5 mg/KG. Tumor volumes were calculated by the following formula: length* width *2 p/6 [15, 21]. At 18 days post treatment, the tumor was isolated for further analysis, like western blots. All animal experiments strictly followed the guidelines of the University Review Board. The study was approved by the Ethical Com-

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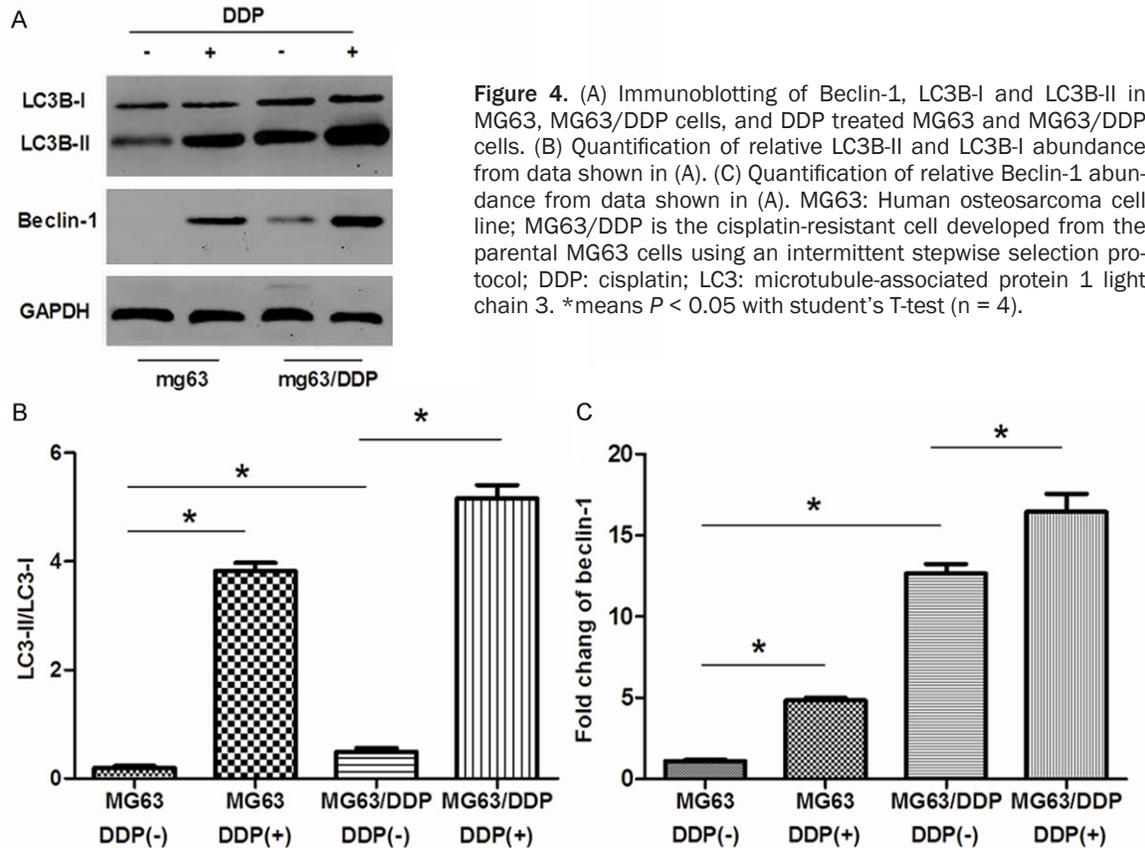


Figure 4. (A) Immunoblotting of Beclin-1, LC3B-I and LC3B-II in MG63, MG63/DDP cells, and DDP treated MG63 and MG63/DDP cells. (B) Quantification of relative LC3B-II and LC3B-I abundance from data shown in (A). (C) Quantification of relative Beclin-1 abundance from data shown in (A). MG63: Human osteosarcoma cell line; MG63/DDP is the cisplatin-resistant cell developed from the parental MG63 cells using an intermittent stepwise selection protocol; DDP: cisplatin; LC3: microtubule-associated protein 1 light chain 3. *means $P < 0.05$ with student's T-test ($n = 4$).

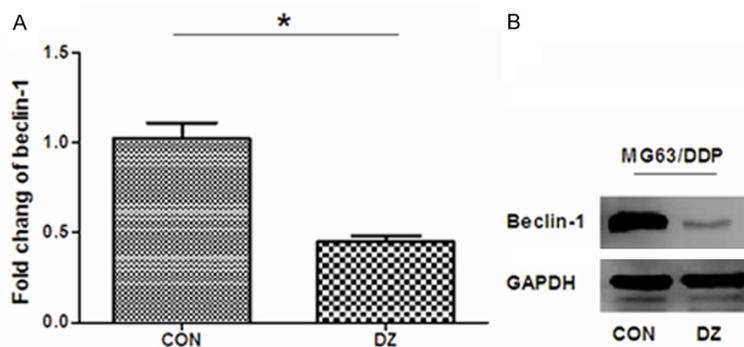


Figure 5. DZ affects the expression of beclin1. A: DZ inhibits the expression of beclin1 in mRNA levels in MG63/DDP cells. B: DZ inhibits the expression of beclin1 in protein level in MG63/DDP cells. MG63: Human osteosarcoma cell line; MG63/DDP is the cisplatin-resistant cell developed from the parental MG63 cells using an intermittent stepwise selection protocol; DDP: cisplatin; DZ: deoxyribozyme. *means $P < 0.05$ with student's T-test ($n = 4$).

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Statistical analysis

In all experiments, unless otherwise indicated, data are reported as mean \pm SEM in at least 3 replicates per group. Data were analyzed by

2-tailed Student t test or ANOVA least significant difference test, and $P < 0.05$ was considered significant.

Results

Cisplatin exposure affects MG63 morphology

Cisplatin resistant clone of MG63 was generated by continuous exposure to stepwise increasing concentrations of cisplatin starting from 0.2 g/L until reaching 1.2 g/L over a period of 6 months. After establishment of MG63/DDP cells, we characterized cell

cytotoxicity in response to cisplatin treatment in these cells comparing with those parental MG63 cells. MG63/DDP cells exhibited about 8.7 times more resistant than MG63 cells ($P < 0.001$) with the 50% inhibitory concentrations (IC50) of cisplatin in MG63 and MG63/DDP were about 0.15 ± 0.02 g/L and 1.3 ± 0.08 g/L, respectively (**Figure 1**). Noteworthy, MG63/

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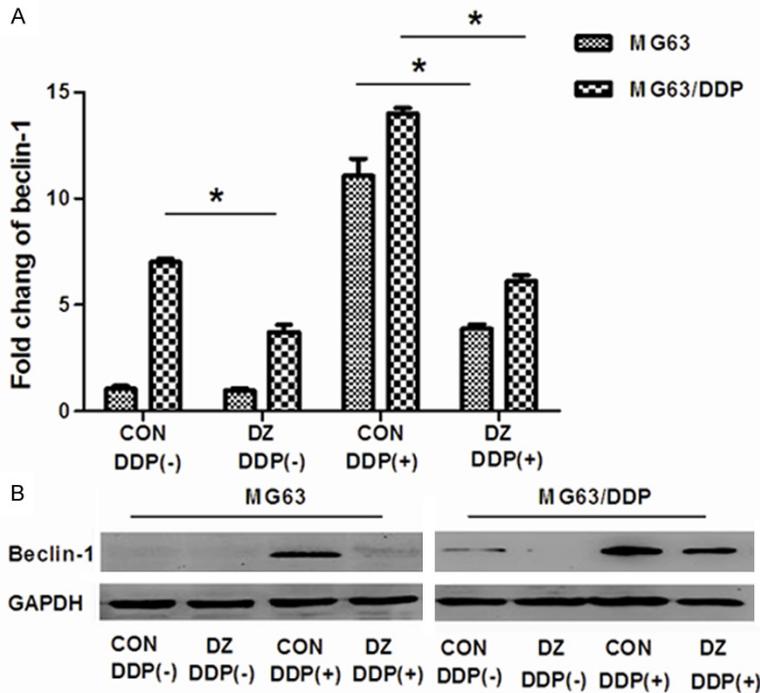


Figure 6. DZ affects the sensitivity to cisplatin. **A:** *In vitro*, DZ inhibits the expression of beclin1 in mRNA level in both MG63 and MG63/DDP cells treated with cisplatin. **B:** *In vitro*, DZ inhibits the expression of beclin1 in protein level in both MG63 and MG63/DDP cells treated with cisplatin.

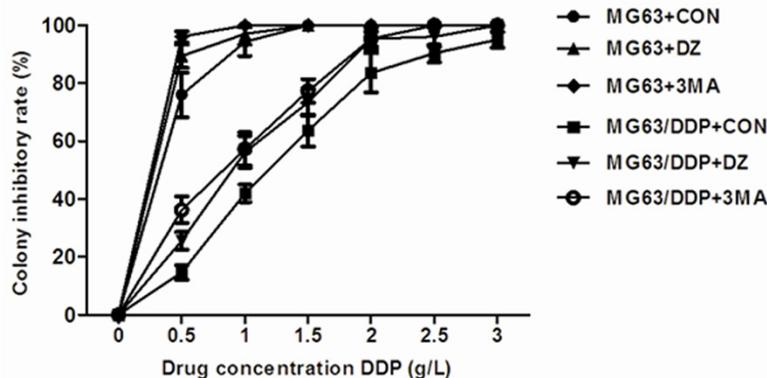


Figure 7. DZ increases the sensitivity to cisplatin for both MG63 and MG63/DDP cells. MG63: Human osteosarcoma cell line; MG63/DDP is the cisplatin-resistant cell developed from the parental MG63 cells using an intermittent stepwise selection protocol; DZ: deoxyribozyme. *means $P < 0.05$ with student's T-test ($n = 4$).

DDP also showed more ($P < 0.05$) resistant to other kinds of drugs, like paclitaxel, methotrexate and 5-fluorouracil (data not shown). Meanwhile, it was interesting to note that under light microscopy, MG63/DDP cells had an irregular shape and size, comparing to the parental

MG63 cells (**Figure 2A** and **2B**). Furthermore, more ($P < 0.05$) numbers of autophagosome were observed in MG63/DDP cells than those in the parental MG63 cells under electron microscopy (**Figure 2C** and **2D**).

Cisplatin exposure promoted autophagosome formation

As more autophagosome was observed in MG63/DDP cells under electron microscopy, thus we determined the autophagosome with immunofluorescence staining. As indicated in **Figure 3A** and **3B**, MG63/DDP cells exhibited more ($P < 0.05$) numbers of autophagosome than MG63 cells. Meanwhile, after treatment with cisplatin (0.5 g/L) for 12 h, both cells expressed large numbers of autophagosome (**Figure 3C** and **3D**). Beclin1 plays important role in the phagophore formation, which is the first step of autophagosome biogenesis [22]. The transformation of the native cytosolic form of microtubule-associated protein 1 light chain 3 (LC3-I) to membrane-bound lipidated (LC3-II) is recognized as an important process of autophagosome formation [23, 24], and the LC3-II is commonly used as a maker of autophagy [22]. Seminar to the observation from immunofluorescence staining, the abundance of beclin1 and

LC3-II were significant ($P < 0.05$) higher in MG63/DDP than those in MG63 cells (**Figure 4A-C**). Likewise, cisplatin treatment significantly ($P < 0.05$) increased the abundance of beclin1 and LC3-II in both MG63 and MG63/DDP cells, and it became more obvious in

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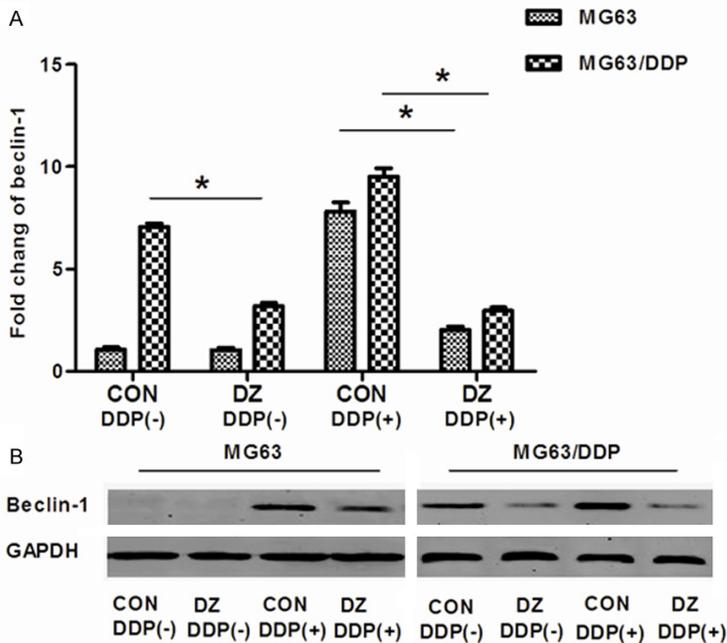


Figure 8. A: *In vivo*, DZ inhibits the expression of beclin1 in mRNA level in both MG63 and MG63/DDP cells after treatment with cisplatin. B: *In vivo*, DZ inhibits the expression of beclin1 in protein level in both MG63 and MG63/DDP cells after treatment with cisplatin. MG63: Human osteosarcoma cell line; MG63/DDP is the cisplatin-resistant cell developed from the parental MG63 cells using an intermittent stepwise selection protocol; DDP: cisplatin; DZ: deoxyribozyme. *means $P < 0.05$ with student's T-test ($n = 4$).

MG63/DDP cells (Figure 4A-C). Collectively, cisplatin resistance is associated with autophagosome formation.

Suppression of beclin1 increases sensitivity to cisplatin *in vitro* and *in vivo*

As autophagy is regarded to play important role in drug resistance, and increased expression of beclin1 has been observed in MG63/DDP cells, we used deoxyribozyme to knockdown the beclin1. As shown in Figure 5A and 5B, the designed DZ significantly ($P < 0.05$) inhibited the expression of beclin1 from mRNA and protein level in MG63/DDP cells. *In vitro*, DZ treatment significantly ($P < 0.05$) inhibited the expression of beclin1 from mRNA and protein level in both MG63 and MG63/DDP cells after treatment with cisplatin, resulting in the increase in the sensitivity to cisplatin for both MG63 and MG63/DDP cells (Figures 6, 7). As positive control, treatment with 3-methyladenine (Autophagy Inhibitor) also increased the sensitivity to cisplatin for both MG63 and MG63/DDP cells (Figure 7). Likewise, *in vivo*,

DZ treatment significantly ($P < 0.05$) inhibited the expression of beclin1 from mRNA and protein level in both MG63 and MG63/DDP tumors after treatment with cisplatin (Figure 8), resulting in the increase in the sensitivity to cisplatin for both MG63 (Figure 9A-D) and MG63/DDP tumors (Figure 9E-H). Together, beclin1 inhibition increases the cisplatin sensitivity.

Discussion

Autophagy has observed in cisplatin-based chemotherapy and has implicated in the acquired resistance of various cancers, like lung cancer [24], liver cancer [25] and ovarian cancer [26]. Likewise, increased autophagy is observed in cisplatin resistant MG63/DDP cells and in the cisplatin-treated MG63 and MG63/DDP cells with electron microscopy and immunofluorescence staining.

Noteworthy, similar change is observed about the beclin1 and LC-II in MG63/DDP cells and in the cisplatin-treated MG63 and MG63/DDP cells. Beclin1 and LC-II is the land maker for the initiation and maturation of autophagosome, respectively [22]. Indeed, previous investigations have reported that autophagy is implicated in the cisplatin resistant osteosarcoma [3, 16, 27]. In 2009, Zhang et al indicated that autophagy may be implicated in the carcinogenesis of osteosarcoma after analysis of specimens of osteosarcoma and normal bone tissues, and cisplatin may induce autophagy in the MG63 cells [16]. Subsequently, Huang et al found that cisplatin treatment will enhance the expression of high mobility group box 1 protein (HMGB1), which will increase drug resistance by inducing autophagy [3, 15]. Mechanistically, HMGB1 binds to Beclin1 and regulates the formation of the Beclin1-PI3KC3 complex to facilitate autophagic progression [15]. Thus, it is very interesting to analyze the expression of HMGB1 in MG63/DDP cells and in the cisplatin-treated MG63 and MG63/DDP cells.

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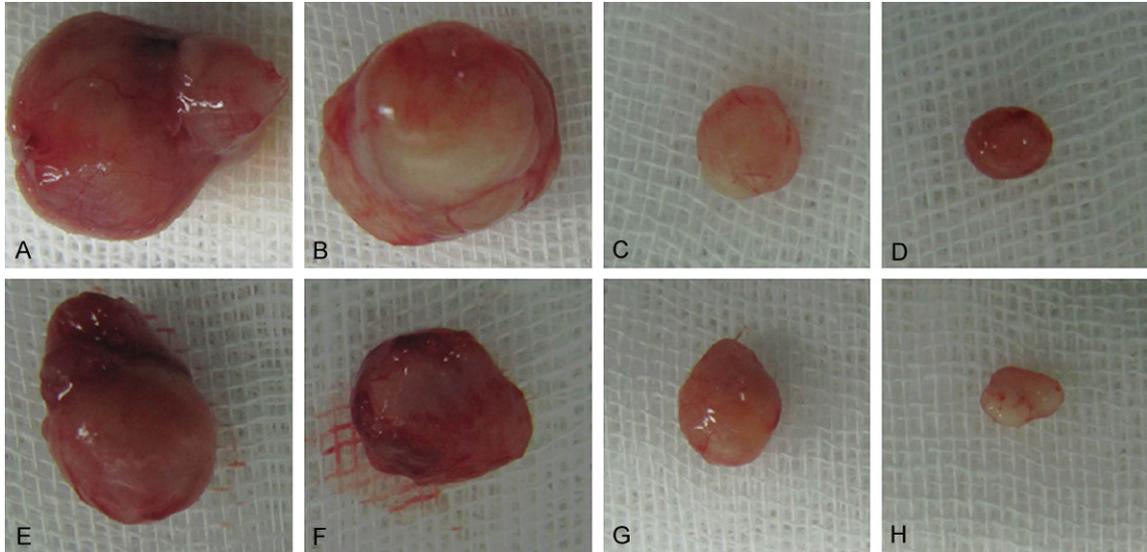


Figure 9. DZ affects sensitivity to cisplatin *in vivo*. A: MG63 cells without treatment. B: MG63 cells treated with DZ. C: MG63 cells treated with cisplatin. D: MG63 cells treated with DZ and cisplatin. E: MG63 cells without treatment. F: MG63 cells treated with DZ. G: MG63 cells treated with cisplatin. H: MG63 cells treated with DZ and cisplatin. MG63: Human osteosarcoma cell line; MG63/DDP is the cisplatin-resistant cell developed from the parental MG63 cells using an intermittent stepwise selection protocol; DDP: cisplatin; DZ: deoxyribozyme. Mice were injected with MG63 cells or MG63/DDP cells (n = 6).

Beclin1 help the class III phosphoinositide 3 kinase (PI3K) Vps34 to catalyze the formation of PI(3)P, which is an essential early step during phagophore formation [22, 28]. Thus, beclin1 as target to study the role of autophagy is widely used, and the knockdown of beclin1 will significantly affect the autophagy [11, 12]. Deoxyribozymes are DNA enzymes (DNAzymes) with the ability to bind to specific sequences of RNA, and to cleave the target site catalytically, thus they are widely used to inhibit targeted gene expression [17, 29]. Similarly, deoxyribozyme was used in this study to inhibit the beclin1, and the inhibition of beclin1 from mRNA level significantly inhibits the formation of autophagosome from the observation of the expression of LC-II. Intriguingly, the inhibition of autophagy results in the increase in the sensitivity to cisplatin for both MG63 and MG63/DDP cells *in vitro* and *in vivo*. Indeed, specific inhibition of early autophagy induction with siRNA targeted to Beclin1 significantly enhances the sensitivity to anticancer drugs in various kinds of cancers [11, 12]. Likewise, previous observation indicated that down-regulated autophagy by inhibitor (3-methyladenine) could increase chemotherapeutic sensitivity of cisplatin to osteosarcoma [16]. Similarly, the inhibition of autophagy by chloroquine, an inhibitor of lysosomal proteases, accelerates the cisplatin

induced cell death in Saos-2 cells [30]. Additionally, silencing of Beclin1-associated autophagy-related key regulator (Barkor/ATG14) significantly increases the sensitivity of Saos-2 cells to cisplatin by Barkorrelated endoplasmic reticulum - and mitochondrial-mediated apoptotic pathway [27]. Thus, the apoptosis after beclin1 inhibition in both MG63 and MG63/DDP tumors needs further investigations.

In conclusion, current study shows that autophagy has implicated in the cisplatin resistance in osteosarcoma, and inhibition of beclin1 increases the sensitivity to cisplatin for osteosarcoma.

Disclosure of conflict of interest

None.

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